NEURONAL SELF-DEFENSE: COMPENSATORY MECHANISMS IN NEURODEGENERATIVE DISORDERS

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NEURONAL SELF-DEFENSE: COMPENSATORY MECHANISMS IN NEURODEGENERATIVE DISORDERS

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At present, there are no effective cures for neurodegenerative diseases. Because unambiguous diagnosis is possible only after manifestation of symptoms, when a large proportion of neurons has been already lost, therapies are necessarily confined to alleviation of symptoms. Development of cures halting the disease course is hampered by our rudimentary understanding of the etiopathology.

Most neurodegenerative disorders are sporadic and age-related and - even for those of known genetic origin - the mechanisms influencing disease onset and progression have not been fully characterized. The different diseases, however, share important similarities in the mechanisms responsible for neuronal loss, which is caused by a combination of endogenous and exogenous challenges.

To counterbalance noxious stimuli cells deploy, at least during the initial pathogenic states, intrinsic neuroprotective responses. These are general compensatory mechanisms, common to several neurodegenerative conditions, which reprogram cellular physiology to overcome stress. Ineffective execution of these compensatory strategies severely threatens cellular homeostasis and favors onset of pathology. Therefore, a better understanding of these "buffering" mechanisms and of their interconnections may help to devise more effective therapeutic tools to prolong neuronal survival and activity, independently of the original genetic mutations and stress insults.

This Research Topic focuses on the initial compensatory responses protecting against failure of those mechanisms that sustain neuronal survival and activity. The collection intends to summarize the state-of-the-art in this field and to propose novel research contributes, with the ultimate goal of inspiring innovative studies aimed to contrast progression of neurodegenerative diseases.

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EDITORIAL
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Editorial: Neuronal Self-Defense: Compensatory Mechanisms in Neurodegenerative Disorders

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The Editorial on the Research Topic

Neuronal Self-Defense: Compensatory Mechanisms in Neurodegenerative Disorders

Neurodegenerative diseases (ND) are characterized by the progressive loss of specific neuronal populations with consequent deterioration of brain's function. While some ND have monogenic causes, the vast majority is sporadic. Accordingly, etiology varies greatly between these conditions; the pathogenesis, however, shares important common traits. Trophic deprivation, oxidative stress, accumulation of abnormal protein aggregates, and bioenergetics defects have been in fact described in most, if not all, ND. To counterbalance these noxious stimuli cells deploy intrinsic neuroprotective responses, at least during early pathogenesis. Adaptation includes strategies to optimize energetic resources, for instance reduction of rRNA synthesis to repress translation, suppression of transcription, and bioenergetics and metabolic redesign. Additional mechanisms include potentiation of antioxidant capacity, induction of endoplasmic reticulum (ER) stress, and activation of protein quality control systems and autophagy. Strategies to potentiate these naturally occurring processes might provide foundation to devise new experimental treatments.

This e-book contains a collection of reviews and original articles summarizing the state-of-the-art knowledge on protective responses sustaining neuronal survival and activity. The ultimate goal of this work is to inspire novel studies elucidating strategies to contrast these incurable disorders.

The first article reviews transcriptional deregulation in Huntington's disease (HD). Francelle and colleagues point out that while transcriptional changes are directly caused by mutant huntingtin, they may also represent a response to secondary stress conditions triggered by dysfunctional transcriptional machineries. They speculate that transient expression changes typical of HD may represent a self-defense mechanism because some of these altered genes activate pro-survival functions, which may be impaired during aging. Hence, the investigation of gene expression changes with aging in the striatum, the brain region mostly affected in HD, might help to develop strategies to modify mutant huntingtin toxicity (Francelle et al.). In line with this concept, Stilling and colleagues show how aging is associated with changes in gene expression and in RNA splicing, and with the upregulation of immune system functions in the hippocampus (Stilling et al.). Interestingly, affected genes are involved in synaptic function, therefore suggesting a potential pathogenic link between age-dependent transcriptional alterations and late onset of Alzheimer's disease (AD) (Stilling et al.).

A second original research article illustrates how two transcription factors important for the development of dopaminergic neurons also play a role in their maintenance in adulthood. Previous studies indicated that haploinsufficiency of the transcription factor Foxa 2 leads to abnormalities in motor behavior in old age and an associated progressive loss of dopaminergic neurons reminiscent of Parkinson's disease (PD) (Kittappa et al., 2007). Here Domanskyi and colleagues report that

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Foxa1 and Foxa2 co-evolved to compensate the lack of each other. By the generation and analysis of specific conditional double mutant mice they show the protective role of these transcription factors for the maintenance of dopaminergic neurons in adult stages (Domanskyi et al.).

A third original article addresses neuronal self defense mechanisms based on the regulation of rRNA synthesis. Downregulation of rRNA synthesis has been associated with neuronal loss in several ND, although disruption of nucleolar activity may trigger context-specific neuroprotective responses (Kiryk et al.; Kreiner et al., 2013). The study by Riancho and colleagues reveals that active nucleolar transcription may be a compensatory mechanism in a mouse model of amyotrophic lateral sclerosis (ALS) at both asymptomatic and symptomatic stages. High rate of rRNA transcription in ALS motor neurons could maintain protein synthesis when proteostasis is severely impaired (Riancho et al.).

Edwin Chan reviews aberrant RNA species—an emerging mechanism of toxicity in ND—particularly in polyQ diseases linked to nucleotide repeat expansion and in frontotemporal dementia/ALS (FTD/ALS). The piece discusses the impact of these mutant RNAs and proposes that toxicity may be ascribed to alternative splicing of RNAs, hairpin, and double-stranded CAG repeat RNAs, sequestration of cellular proteins, nucleolar stress, repeat associated non-ATG translation (RAN-translation) of CAG expansion transcripts (Chan).

The role of antisense long noncoding RNAs (lncRNAs) as regulators of neuronal gene expression is rapidly emerging (Ng et al., 2013). Recent studies indicate that they could contribute to ND. This mechanism has been for instance demonstrated in mouse, for the ubiquitin carboxy-terminal hydrolase L1 (Uchl1). Importantly in human UCHL1 (or PARK5) has been associated with familial PD. Carrieri and colleagues propose that increasing its expression might be beneficial and antisense (AS) Uchl 1 could be used to increase Uchl1 mRNA translation. They also show that AS Uchl 1 promoter region contains a binding site for Nurr1, a transcription factor required for differentiation of dopaminergic neurons. Moreover, AS Uchl 1 level is reduced in the prototypical MPTP model of PD, suggesting that this novel stress mechanism responding to stress might have a pathological relevance in PD pathophysiology (Carrieri et al.). In the context of this post-transcriptional regulation of protein synthesis, this group also proposes in a second original article the use of antisense long noncoding RNAs in neuronal cell lines to activate translation in a gene specific manner (Zucchelli et al.). Finally, Amadio and colleagues contributed with another original article on post-transcriptional regulation, proposing that ELAV (embryonic lethal abnormal vision) proteins to heme-oxygenase-1 mRNA—a gene activated to counteract oxidative stress—to increase its expression and elicit neuroprotection with beneficial effects (Amadio et al.).

After this initial series of articles focusing on transcriptional and post-transcriptional regulation, the collection continues with articles discussing compensatory mechanisms based on translation suppression.

The mammalian (or mechanistic) target of rapamycin (mTOR) acts as a central regulator of cell homeostasis, and its activity is tightly regulated (Laplante and Sabatini, 2012). mTOR plays an important role in neuronal homeostasis (Bateup et al.). In this context the minireview by Canal and colleagues focuses on the mTOR inhibitor RTP801/REDD1, a protein encoded by the stress responsive gene DNA damage inducible transcript 4. REDD1 expression is regulated during development and in response to DNA damage. In addition, REDD1 is induced by stressors, such as hypoxia and ER stress. Interestingly REDD1 is also found upregulated in PD. The authors propose a model in which REDD1 may have either a protective or toxic function in cell survival depending on its protein level that changes at different disease stages. At low level, REDD1 might be a protective compensatory response at early disease stage (Canal et al.).

The next group of articles deals with accumulation of protein aggregates, which constitutes another shared mechanism of neurodegeneration and represents a promising therapeutic target. The review by Angelika and Fabio Falsone focuses on different regulators of protein aggregates, including stress granules (SG) (Falsone and Falsone). SG are dynamic cellular compartments that contribute to the arrest of mRNA translation in response to stress insults, such as UV-irradiation, oxidative stress. SG are also linked to amyloidogenic proteins and irreversible protein aggregates perturb their normal dynamics. Alteration of SG dynamics however may be due to irreversible protein aggregates. The articles also explores the nexus between heat shock proteins and proteolytic pathways, and specifically focuses on the crosstalk between different protein degradation pathways such as ubiquitin-proteasome system, chaperone mediated autophagy and macroautophagy. The work reveals that these mechanism share a certain degree of redundancy and the function of the unaffected one can rescue malfunction of the other pathways (Falsone and Falsone).

Jaronen and coworkers focus in their review on the role of ER stress and unfolded protein response (UPR) on the protein disulfide isomerase (PDI), a chaperone regulator of misfolded protein degradation, in ALS (Jaronen et al.). PDI is upregulated in ALS and this upregulation is shown early in the disease progression in ALS rat models. PDI inactivation increases ER stress, defined as disruption of the ER linked to accumulation of misfolded proteins, while at early stages PDI could prevent aggregation of superoxide dismutase 1 (SOD1). However, at a later stage increased UPR can result in increased PDI activation that may result in neurotoxicity by increasing superoxide production or according to a second model by increasing hydroperoxyde (Jaronen et al.). A third mini-review on this topic, contributed by Ujval Anilkumar and Jochen Prehn, discusses non-cell death related functions of anti-apoptotic proteins such as the pro-survival BCL-2 in mitochondrial physiology and ER homeostasis (Anilkumar and Prehn). J proteins, which belong to the chaperone family, may exert neuroprotective effects by facilitating proteostasis, as also discussed in the mini-review by Koutras and Braun.

Sugaya and Nagano propose an alternative hypothesis to explain the variability in the onset of familial ALS (fALS) despite same SOD1 genetic mutation. Based on available data for SOD1-linked fALS and a risk-based modeling approach,

the authors suggest a SOD1 prion-like model of propagation. Spread of toxic aggregates through cell-to-cell transmission has been observed in several ND. However, the "protective aggregation hypothesis" according to which misfolded protein aggregates may represent a defensive response to protect cells from more toxic oligomeric species is hypothesized also for SOD1-linked fALS (Sugaya and Nakano). This article considers a "one-hit model," i.e., a mathematical model in which the risk of cell death remains constant. The next review article by Fiebich and colleagues, instead, formulates a two-hit hypothesis that includes also neuroinflammation (Fiebich et al.). Anti-neuroinflammatory approaches represent a promising strategy against neurodegeneration and this review discusses the role of P2 receptors, which can bind ATP released by injured cells. This represents a second detrimental event synergizing with aggregation and the possibility of decreasing neuroinflammation by targeting P2 receptors is accordingly discussed (Fiebich et al.).

In the context of neuroprotective strategies aiming at potentiating the cell antioxidant capacity, the inhibition of NMDA receptor and the calcium dependent enzyme nitric oxide synthase (nNOS) activities is discussed by Courtney et al. In particular the authors review the role of NOS1AP, an adaptor protein which is considered a nNOS inhibitor. As a modulator of the excessive nNOS, NOS1AP may play a role in the neuronal self-defense against the NMDAR mediated excitotoxicity dependent on nNOS signaling (Courtney et al.).

Ca^{2+} overload is a pathogenic feature of sporadic and fALS, characterized by degeneration of motor neurons (MNs). In this original article by Mühling and colleagues, pharmacological stimulation of Ca^{2+} transporters in this vulnerable neuronal population is presented as a potential neuroprotective strategy. The authors show that, in SOD1G93A mice, mitochondrial and plasma membrane Ca^{2+} transporters are expressed at higher levels in MNs dissected from the hypoglossal nucleus. These findings are in line with an activity dependent Ca^{2+} clearance deficit and could represent a compensatory response to the disease (Mühling et al.).

In the last review closing this e-book, Grzegorz Kreiner elaborates on the difficulties of modeling ND and arguments that, despite the unavoidable differences between human and rodents, compensatory mechanisms shown in mouse models could help developing new hypotheses to devise neuroprotective strategies (Kreiner).

We hope that the present e-book provides a better understanding of mechanisms and their interconnections regulating neuronal homeostasis, and how ineffective execution of compensatory strategies severely threatens cellular homeostasis favoring pathogenesis. We believe that this comprehensive collection represents an original reference and may inspire innovative studies that will devise more effective therapeutic tools to prolong neuronal survival and activity.

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Possible involvement of self-defense mechanisms in the preferential vulnerability of the striatum in Huntington's disease

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HD is caused by a mutation in the huntingtin gene that consists in a CAG repeat expansion translated into an abnormal poly-glutamine (polyQ) tract in the huntingtin (Htt) protein. The most striking neuropathological finding in HD is the atrophy of the striatum. The regional expression of mutant Htt (mHtt) is ubiquitous in the brain and cannot explain by itself the preferential vulnerability of the striatum in HD. mHtt has been shown to produce an early defect in transcription, through direct alteration of the function of key regulators of transcription and in addition, more indirectly, as a result of compensatory responses to cellular stress. In this review, we focus on gene products that are preferentially expressed in the striatum and have down- or up-regulated expression in HD and, as such, may play a crucial role in the susceptibility of the striatum to mHtt. Many of these striatal gene products are for a vast majority down-regulated and more rarely increased in HD. Recent research shows that some of these striatal markers have a pro-survival/neuroprotective role in neurons (e.g., MSK1, A2A, and CB1 receptors) whereas others enhance the susceptibility of striatal neurons to mHtt (e.g., Rhes, RGS2, D2 receptors). The down-regulation of these latter proteins may be considered as a potential self-defense mechanism to slow degeneration. For a majority of the striatal gene products that have been identified so far, their function in the striatum is unknown and their modifying effects on mHtt toxicity remain to be experimentally addressed. Focusing on these striatal markers may contribute to a better understanding of HD pathogenesis, and possibly the identification of novel therapeutic targets.

Keywords: striatum, Huntington, markers, cell death, excitotoxicity, signaling, gene products

INTRODUCTION

A SUMMARY OF WHAT IS HD

HD is a dominantly inherited disorder generally affecting young adults. Symptoms include involuntary abnormal movements (chorea, dyskinesia, dystonia), frontal cognitive deficits (e.g., perseveration) and psychiatric disturbances (Harper, 1991; Walker, 2007). The disease is fatal approximately 15 years after the onset of symptoms. There is no treatment available to slow the progression of this devastating disorder.

HD is caused by a mutation in the *HTT* gene encoding the protein huntingtin (Htt) that consists in a CAG triplet repeat expansion translated into an abnormal poly-glutamine (polyQ) tract within the N-terminal region of the protein (The-Huntington's-Disease-Collaborative-Research-Group, 1993). When considering cohorts of HD gene carriers, genetic studies showed that the longer is the CAG repeat expansion the earlier the disease onsets. However, there is a huge inter-individual variability in age of onset (and nature) of symptoms for gene carriers with similar CAG repeat numbers. Thus, apart from HD gene mutation, many genetic, epigenetic and environmental factors may affect the course of the disease (Sturrock and Leavitt, 2010). Deciphering these factors and the underlying mechanisms affecting the onset of this disease could constitute a real hope to find an efficacious treatment to slow the disease.

The mutant protein is cleaved by many proteases leading to the production of N-terminal fragments that form toxic oligomers (Roze et al., 2008b). Eventually mutant Htt (mHtt) forms intranuclear inclusions and somatodendritic aggregates that also contain ubiquitin and represent a histopathological hallmark of HD (Li and Li, 2004a).

Mechanisms of HD pathogenesis have been extensively studied in the past 20 years, since the gene has been identified and cloned. Thanks to many different genetic models (in cells, mice, rat, and even monkeys) a large spectrum of cellular defects has been identified and could contribute to neurodegeneration. For this reason the pathogenesis of HD is often considered multi-factorial. The

polyQ expansion in mutated Htt (mHtt) produces a gain-of-function that is toxic to neurons through several mechanisms. One major early event in HD is the alteration of transcription (Cha, 2007; Seredenina and Luthi-Carter, 2012). Importantly, reduced transcription of Brain Derived Neurotrophic Factor (BDNF), a major neurotrophic factor for striatal cells has been found (Zuccato and Cattaneo, 2007). Axonal transport alterations (Li and Li, 2004b; Roze et al., 2008b) leading to several cellular disturbance, including defects in BDNF secretion and transport (Gauthier et al., 2004) also contribute to neurodegeneration. Other alterations include intracellular signaling defects (Borrell-Pages et al., 2006), deregulated of the proteasome pathway (Finkbeiner and Mitra, 2008) and autophagy (Ravikumar and Rubinsztein, 2006), perturbation of calcium homeostasis leading to excitotoxicity (Cowan and Raymond, 2006; Raymond et al., 2011), mitochondrial defects and oxidative stress (Damiano et al., 2010).

In addition, the mutation in one allele is thought to produce a loss of function of wild type Htt (Cattaneo et al., 2005). Indeed, htt is involved in a large variety of physiological cellular processes. It regulates vesicle transport through regulation of molecular motors of the cytoskeleton, transcription of important pro-survival factors (such a BDNF) by interacting with key transcription factors and co-activators of transcription, cell division, intracellular signaling and ATP production (Zuccato and Cattaneo, 2014).

While wild type and mHtt protein are ubiquitously expressed in the brain, degeneration primarily affects the striatum. The contribution of striatal degeneration in motor and cognitive symptoms is not totally understood but neuropathological studies showed that striatal atrophy correlates with severity of symptoms (Myers et al., 1988). Recently, follow up of HD gene carriers cohort using Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) showed that even at presymptomatic stages, the atrophy of the striatum is detectable and may start even 10 years before onset of symptoms (Tabrizi et al., 2013). Other brain regions may also be damaged at early stages, such as the hypothalamus, and at later stages the cerebral cortex and other regions also degenerate (for a review, Brouillet et al., 1999; Petersen and Bjorkqvist, 2006). Thus, HD is not a selective striatal disease. Many innovative studies discovered extra-striatal and peripheral anomalies in HD animal models and for particular studies in HD patients (Martin et al., 2008; Obeso et al., 2014). However, the preferential striatal degeneration is an intriguing characteristic of this illness, and the underlying mechanisms may represent an important aspect of HD pathogenesis.

EXISTENCE OF POSSIBLE COMPENSATORY MECHANISMS IN HD

The existence of compensatory mechanisms in HD (as for other neurodegenerative diseases) is probable. Possibly, the best circumstantial evidence for this is that although mHtt is expressed in the brain of HD gene carriers since birth, degeneration and symptoms appear during adulthood (with the exception of long CAG repeat expansion carriers who develop the disease during childhood) (Harper, 1991; Walker, 2007). Similarly in genetic animal models, degeneration and symptoms occur in adult or aged animals (Menalled and Chesselet, 2002; Menalled, 2005). It has been shown that when mHtt is expressed in striatal neurons at similar levels for the same duration, its neurotoxic effects are significantly higher in aged animals, as compared to young animals (Diguet et al., 2009). The reason for this age-dependent phenomenon is unknown but it indicates that neurons possess the ability to partially counteract cellular stress induced by mHtt, a plasticity mechanism that may be progressively lost with aging. The aim of this review is not to cover all the possible compensatory mechanisms that may occur in the HD brain, but to focus on those that can be found in the striatum. However, a few examples of potential compensatory mechanisms that could be encountered in all cell types can be given.

There likely exist compensatory mechanisms at whole human brain level, to overcome cell dysfunction and/or neurodegeneration in the striatum of HD patients. For example, PET studies showed that effective learning performance on motor sequence learning tasks, normally associated with activation of the dorsolateral prefrontal cortex and the caudate nucleus, was not requiring the same brain regions in presymptomatic HD (pre-HD) patients and healthy volunteers (Feigin et al., 2006). In presymptomatic HD gene carriers, ventral prefrontal and orbitofrontal regions were used possibly via thalamic projections.

At cellular level, transient/reversible transcriptional and post-transcriptional mechanisms may intervene to compensate for cell suffering and degeneration pathways. For example, the loss of expression of the kinase PKCδ (Rue et al., 2014) is likely a compensatory mechanism. Indeed, the overexpression of PKCδ enhances mHtt toxicity *in vitro*. On the contrary, the knock down of PKCδ (using siRNA strategy or expression of a dominant negative form) significantly reduces mHtt effects. Interestingly the loss of PKCδ seems to occur through an increased degradation of the protein by neurons expressing mHtt (Rue et al., 2014).

Examples of potential compensatory mechanisms in HD can be found in studies related to defects energy metabolism that are thought to occur early in HD. Unexpectedly, recent experiments show that an early increase in the levels of high energy phosphate metabolites (ATP, phosphocreatine) can be found in the brain of HD mouse models (Mochel et al., 2012a; Tkac et al., 2012). Consistent with these observation in genetic models of HD, dynamic measurements of brain phosphocreatine levels during synaptic activation in HD patients using ^{31}P NMR spectroscopy also demonstrate abnormalities in the use of high energy phosphate metabolites (Mochel et al., 2012b). In R6/2 and Knock-in 111Q mouse models, early biochemical changes indicate that neurons tend to compensate by activating energy promoting cellular pathways (Mochel et al., 2012a). In particular, possible compensatory changes occur at the post-translational levels, leading to an increase in AMPK phosphorylation in HD mice, which could activate pathways leading to a more efficient metabolism.

Large scale analyses trying to broadly identify mRNA and/or protein expression changes provide a huge amount of information from which potential compensatory mechanisms in HD may be discovered. A well-controlled proteomic analysis of brain of R6/2 HD mice at different ages underlined that a number of proteins display transient /biphasic expression changes rather than an age-dependent progressive decline (Zabel et al., 2009). For instance, the absolute expression of the mitochondrial complex II subunit Ip (iron-sulfur), a key regulator of oxidative energy metabolism which is neuroprotective against mHtt (Benchoua

et al., 2006; Damiano et al., 2013), is early reduced in 2 week-old R6/2 mice, but is found to be increased in 8 weeks old of these mice and brings back at basal levels at 12 weeks old (Zabel et al., 2009).

Changes in the expression levels (decreases and more rarely increases) of mRNA in HD have been extensively explored in the last decade (Seredenina and Luthi-Carter, 2012). These changes may indicate two types of phenomena. On one hand, it indicates primary defects of transcription inherent to the presence of mHtt. In many cases, the direct interaction of mHtt with proteins that are part of macromolecular complexes involved in transcription regulation leads to a reduction of transcription and reduced levels of a large spectrum of gene products (Seredenina and Luthi-Carter, 2012). On the other hand, changes in mRNA levels (or protein) may not be directly linked to a primary effect of mHtt but could rather result from a physiological response engendered by the cellular stress induced by toxic gain of function of mHtt. Many expression changes identified in large scale analyses have been studied with the hypothesis that they were causal in HD pathogenesis. It is not always the case. Expression changes can represent self-defense mechanisms. To differentiate between the two above mentioned mechanisms, knock-down/knock-out or overexpression/neuro-rescue experiments in HD models are needed. It is beyond the scope of the present review to provide a detailed description of the gene products that have been experimentally tested. Here we will limit our review to gene products that have deregulated expression and that are preferentially expressed in the striatum. The review of the studies focused on "striatal gene products" illustrates that in some cases, expression changes may represent compensation or self-defense mechanisms while in others they directly contribute to degeneration of striatal neurons.

STUDYING THE PREFERENTIAL VULNERABILITY OF THE STRIATUM TO IDENTIFY POTENTIAL MODIFIERS

Working hypothesis

The particular vulnerability of the striatum in HD likely resides in its molecular complexity. Whether its particular vulnerability depends on only one or a subset of gene products, acting together, is unknown. Recent publications indicate that the experimental knock-down or overexpression of only one striatal gene product can significantly change the toxicity of muHtt in cell models and mouse models. In one instance, a single nucleotide polymorphism in a striatal gene, *ADORA2A* (adenosine receptor 2a) has been found to be associated with earlier onset of symptoms in large cohorts of HD patients (Dhaenens et al., 2009). Thus, striatal gene products can have a significant impact of HD. From a therapeutic point of view, this indicates that acting on one single target may be sufficient to alter the course of the disease. Therefore, trying to decipher the complex mechanisms underlying neurodegeneration in the striatum may help to more broadly highlight important factors of neuronal dysfunction and death, and to point potential therapeutic interventions for HD (Brouillet et al., 2005; Thomas, 2006; Brochier et al., 2008; Mazarei et al., 2010).

The study of these causal or compensatory changes in the striatum in HD may also help to better understand other neurological diseases where the striatum is functionally affected (e.g., Wilson, Parkinson, metabolic diseases, addiction, depression etc.).

The notion of striatal markers

The hypothesis that gene products preferentially expressed in the striatum (or more generally particularities of this brain region) could play an important role in the susceptibility of the MSN to mHtt toxicity has been studied for many years. Hypotheses related to particular properties of the MSN related to energy metabolism/oxidative stress, or glutamate –related excitotoxicity, and other types of neurotransmitter systems that could explain striatal atrophy in HD where proposed in the 80's and 90's (for a review, Brouillet et al., 1999). The most recent developments of transcriptomic analysis led to a broader "without *a priori*" approach of the working hypothesis that striatum vulnerability to mHtt could reside in the expression of one or a subset of striatal enriched gene products.

The notion of striatal marker stems on the contrast of expression between the striatum and other brain regions. Relatively old studies identified striatal markers based on studies using *in situ* hybridization, immunohistochemistry, and biochemistry (see references in Desplats et al., 2006, for a number of validated striatal markers). The identification of approximately 50 validated markers took approximately two decades. In-depth transcriptomic analyses using serial analysis of gene expression (SAGE) further characterized the molecular complexity of the striatum as compared with other brain regions in mice allowed for the identification of a large list of "striatal markers" in wild type mice (de Chaldee et al., 2003; Brochier et al., 2008; Mazarei et al., 2010). This approach, based on the collection of polyA-containing RNA, provided a ranking of the number of copies of the different RNA species in different regions in the mouse brain. Comparison between brain regions led to the identification of gene products whose expression shows high enrichment in the striatum. Known striatal markers were found, but many annotated gene products whose function in the striatum is unknown were also identified. Approximately, 100–150 striatal markers can be listed, many of which have been cross-validated in different studies (de Chaldee et al., 2003; Desplats et al., 2006; Brochier et al., 2008; Mazarei et al., 2010). Transcriptomic studies using oligonucleotide array or RT-PCR showed that the magnitude of transcriptional changes in the striatum of HD mouse models for these genes preferentially expressed in the striatum was higher than that of ubiquitously expressed genes (Desplats et al., 2006). In the SAGE studies by Brochier and collaborators (Brochier et al., 2008), a number of gene products of unknown neurobiological function showed reduced expression in the striatum of R6/2 HD mice. Transcriptomic DNA array data in HD models and HD brain show that amongst the RNAs whose expression is deregulated, those coding for striatal markers are proportionally more frequently altered (Hodges et al., 2006; Kuhn et al., 2007). Another study validated a number of these striatal markers and identified potentially new ones that were found to be deregulated in YAC128 HD mice (Mazarei et al., 2010). Supplemental Table 1 indicates the striatal markers that have been well validated based on the studies quoted above.

Thus, the notion of striatal marker has evolved with the progression of the analytical methods. The criteria to decide whether a gene product is "preferentially" expressed in the striatum remains debatable. In most cases, the currently available public databases (Allen Brain Atlas) providing gene products expression in the brain in mice and humans generally confirm that the "striatal markers" identified in the studies described above have preferential striatal expression. In general, the contrast of "striatal specificity" in comparison to the somatosensory and motor cerebral cortex is in the range of 3–10-fold enrichment. If we were to consider a lower contrast (a two-fold difference between cortex and striatum for example), the list of striatal markers would be much longer. In addition, it must be mentioned that some striatal gene products, although referenced as "striatal markers" can have stronger expression in other anatomically restricted brain regions such as the hippocampus or some thalamic nuclei.

This review aims at providing a concise overview of the striatal markers that have been experimentally assessed for their capacity to modify mHtt toxicity. These markers have a large spectrum of biological functions and the alteration of the expression levels in HD is not *a priori* indicative of their role in striatal vulnerability. The different striatal gene products that have been experimentally studied for their capacity to change mHtt toxicity can be classified as "protoxic," "neuroprotective," and "neutral." In some instances, the expression changes (up or down) suggest the existence of a compensatory "self-defense" mechanism. We will also point to the large list of the other striatal markers that remain to be fully investigated to determine their potential role in HD.

POTENTIAL PROTOXIC STRIATAL GENE PRODUCTS

D2-R (Dopamine type 2 receptor)

The hypothesis that dopamine, which is at high concentrations in the striatum compared to other brain areas, might play an important role in the preferential vulnerability of the striatum in HD has been suggested long time ago (Reynolds et al., 1998; Jakel and Maragos, 2000).

Anatomically, MSNs expressing D2-R (D2 MSN) receive preferentially inputs from the Pyramidal Track type (PT-type) cortical neurons whose projects ipsilaterally to the striatum. This preferential innervation is believed to release more glutamate which could contribute to make D2 MSNs more vulnerable to excitotoxicity (Reiner et al., 2003; Ballion et al., 2008). Many electrophysiological evidences suggest that D2 MSNs are more excitable than D1 MSNs (Cepeda et al., 2007; Kreitzer and Malenka, 2007) partly because they display fewer primary dendrites (Gertler et al., 2008). Electrophysiological recordings of D2 MSNs show a higher frequency of spontaneous excitatory post-synaptic currents (sEPSCs) than direct pathway. Moreover, D2 MSNs display large membrane depolarizations rarely seen in direct pathway MSNs (Cepeda et al., 2008) after the addition of $GABA_A$ receptor blockers inducing epileptic form activity in CPN (Galvan et al., 2012a). Taken together, these evidences support the idea that D2-MSN is a fertile ground to develop abnormal responses.

Studies performed in YAC128 HD mouse model conducted at a presymptomatic age (1.5 months) and at symptomatic age (12 months) revealed interesting findings concerning the indirect pathways. At presymptomatic age, no differences were observed in excitatory and inhibitory synaptic transmission compared to WT. When the animals are symptomatic and become resistant to excitotoxicity, the inhibitory transmission in YAC128 D2 MSNs is greatly increased (Andre et al., 2011). This may indicate that the indirect pathway is subject to compensatory mechanism in HD, resulting in turn to the slowdown of excitatory glutamatergic synapses in the striatum.

Whether these changes in D2 MSN are only related to D2-R signaling is not known. Direct support for a causal role for DA and D2-R in HD comes from the recent demonstration that the toxicity of the N-terminal fragments of mHtt is potentiated by dopamine in cells expressing mHtt exon 1 and transgenic HD mouse models (Charvin et al., 2005; Cyr et al., 2006; Stack et al., 2007; Benchoua et al., 2008). Dopamine modifies the formation of Htt-containing aggregates in primary striatal neurons transfected with exon 1 of Htt gene and exacerbates mHtt-induced cell death (Charvin et al., 2005). Of interest, this effect involves D2-R signaling, since dopamine effect is blocked by D2 antagonists (Charvin et al., 2005; Benchoua et al., 2006). Dopamine loses its detrimental effect when neurons are prepared from D2 receptor null mice (Charvin et al., 2005). Chronic blockade of the D2-R with a selective antagonist significantly reduces death of MSN in a lentiviral model of mHtt expression in rats (Charvin et al., 2008). Possibly, this "protoxic" effect of dopamine through D2-R stimulation may involve a reduction of the mitochondrial complex II, a key regulator of energy metabolism in neurons (Benchoua et al., 2008). D2-R stimulation increases mHtt toxicity in mouse striatal neurons via, among others, the activation of JNK pathway and activation of the Rho/ROCK-II pathway (Charvin et al., 2005; Deyts et al., 2009).

Thus, the presence of D2-R on MSN may render these neurons more susceptible to HD. However, expression of these receptors is down regulated early in HD as seen using biochemical experiments and PET scans in patients (Antonini et al., 1998; Glass et al., 2000). Whether this decrease is entirely caused by a direct regulation of D2-R transcription by mHtt is unknown. It is conceivable that this decrease is, at least in part, an attempt of MSN to reduce cellular stress generated by mHtt.

D1-R (Dopamine type 1 receptor)

In line with a role of D2-R, D1-R may also be involved in the vulnerability of the striatum. Stimulation of D1-R promotes the aggregation of N-terminal fragments of mHtt and cell death in cell line in culture (Robinson et al., 2008). The mechanisms are unknown but a protoxic role for D1-R has been suggested to be mediated by regulation of glutamatergic synapse and facilitation of excitotoxicity (Tang et al., 2007). Supporting this view, experiments in cells immortalized from knock-in HD mice (111Q) showed that activation of D1-R exacerbates mHtt–induced cell death (Paoletti et al., 2008). D1-R activation facilitates glutamate receptor-mediated activation of the Ca^{2+}-dependent protease calpain that in turn cleaves Cyclin dependent kinase 5 (Cdk5). Cleavage of Cdk5 activator p35 into p25 would be neurotoxic to striatal neurons (Paoletti et al., 2008). As for D2-R, D1-R expression being reduced in HD patients and HD models, this may also be seen as a self-defense mechanism to reduce mHtt toxicity.

CalDAG-GEFI (a.k.a. RASGRP2, calcium and DAG-regulated guanine nucleotide exchange factor I)

CalDAG-GEFI is a guanine-nucleotide exchange factors (GEFs) activated by diacylglycerol (DAG) and Ca^{2+}. CalDAG-GEFI has substrate specificity for Rap1A, and was found to be enriched in the basal ganglia (Kawasaki et al., 1998). This striatal gene product has been rarely studied, and its neurobiological function is not totally understood.

A pioneering study showed that expression of this gene product may render striatal cells more vulnerable to mHtt (Crittenden et al., 2010). Interesting, it was shown that striatal neurons of R6/2 mice with the highest level of mHtt-containing aggregates had the lowest levels of CalDAG-GEF. Since macroscopic aggregates are thought to be neuroprotective since they sequester mHtt toxic soluble oligomeric species, these results indicated that the presence of high levels of CalDAG-GEF may lead to increased levels of toxic species of mHtt in transgenic mice. Supporting this view, knock-down of CalDAG-GEF in a brain slice model of HD is neuroprotective against mHtt-induced neurodegeneration. The mechanisms underlying its "pro-toxic" properties are not determined. One possibility is that it may inhibit Ras-dependent activation of the Erk/MAP kinase cascade in striatal neurons. Thus, its diminished expression in HD may allow "re-activation" of the pro-survival Erk/MAP kinase pathway to block mHtt toxicity (Crittenden et al., 2010).

RGS2 (Regulator of G-protein signaling 2)

The RGS2 protein is a member of the RGS family of proteins that binds Gα subunits of heterotrimeric G proteins. RGS2 interfere with Gαq and Gαi to reduce their rate of hydrolysis of GTP to GDP and thus inhibits the signal transduction from GPCRs. RGS2 play a key role in synaptic plasticity (Kehrl and Sinnarajah, 2002). RGS2 directly interacts with adenylyl cyclases to inhibit the production of cAMP. RGS2 may also regulate GPCR-mediated Akt signaling (Anger et al., 2007). RGS2 expression is reduced in the HD brain and HD mouse models. Seredinina and collaborators studied whether the loss of RGS2 could exacerbate or reduce neurodegeneration induced by overexpression of mHtt in striatal neurons using lentiviral vectors (Seredenina et al., 2011). Results showed that increased expression of RGS2 further aggravates mHtt-induced neurodegeneration. Underlying mechanisms of RGS2 protoxic effects are not fully deciphered but the authors provided preliminary data indicating that they may implicate regulation of Erk/MAP kinase signaling.

Rhes (a.k.a. RASD2, Ras homolog enriched in striatum)

Rhes is a small G-protein that displays striking enrichment in the striatum and can regulate signaling through G-protein coupled receptors (Falk et al., 1999; Vargiu et al., 2004; Mealer et al., 2014). It has been described as a mediator of mHtt cytotoxicity (Subramaniam et al., 2009), acting as a regulator of SUMOylation. The presence of Rhes in MSN would favor the accumulation of toxic oligomeric species of mHtt in the cytoplasm. More recently, the deletion of Rhes has been found neuroprotective in HD R6/1 mice (Baiamonte et al., 2013).

Rhes binds Beclin-1 and activates autophagy, a lysosomal degradation pathway critical in aging and neurodegeneration (Mealer et al., 2014). Activation of autophagy has been shown to be neuroprotective in HD models (Ravikumar and Rubinsztein, 2006). Rhes-induced autophagy is inhibited by mHtt. The restricted expression of Rhes and its effect on autophagy may explain the selective striatal pathology and delayed onset of HD.

DGK (Diacylglycerol kinase)

The expression of DGK is increased in the striatum of R6/2 HD mice. Zhang and collaborators deciphered the potential role that this increase may have in striatal degeneration/dysfunction after having identified this kinase as a potential therapeutic target based on a screening of kinase inhibitors in a cellular models expressing mHtt (Zhang et al., 2012). The inhibitor of DGK (R59949) blocked induction of cell death pathways triggered by serum withdrawal in knock-in (111Q/111Q) HD striatal cells. Knockdown of all isoforms of DGK using siRNA strategy demonstrated that selective inhibition of DGKε was responsible for the neuroprotective effect of the inhibitor. Zhang and collaborators found that knocking down DGK gene in a fly model of HD was neuroprotective. Altogether these data indicate that increased DGK in the striatum could contribute to striatal degeneration. DGK increase could be considered as a protoxic event in HD pathogenesis.

Calcineurin (or protein phosphatase 3, formerly known as protein phosphatase 2B)

Since 1986, calcineurin has been identified by Goto as a marker of neuronal degeneration in the striatum of HD patients (Goto et al., 1986). Calcineurin has preferential expression in the striatum and is downregulated in HD patients and mouse models of HD (Xifro et al., 2009). Calcineurin dephosphorylates Htt at serine 421, inhibition of calcineurin restores axonal transport and transport of BDNF vesicles (Pineda et al., 2009). It is known that Htt phosphorylation is an important protective mechanism in striatal neurons (Humbert et al., 2002). Phosphorylation of mHtt at serine 421 promotes neuroprotection in HD, by restoring Htt function and the transport of BDNF. Supporting the view that reduced calcineurin may be neuroprotective in HD, increased Htt phosphorylation can be produced by pharmacological inhibition of calcineurin with the immunosuppressor FK506 (also known as tacrolimus and fujimycine) (Pardo et al., 2006), or by overexpression of the regulators of calcineurin RCAN1-1L (Ermak et al., 2009) leading to neuroprotective effects.

Thus, the reduction of calcineurin expression and function would lead to a diminution of its activity, increasing phosphorylated state of key proteins, especially mHtt at S421, that activate survival pathways. These mechanisms may be regarded as a compensatory phenomenon that could retard the progression of striatal degeneration.

PDE1B and PDE10A (Phosphodiesterase 1B and 10A)

Studies on phosphodiesterase (PDE) in HD models have shown preferential reduction of the isoforms PDE1B and PDE10A in HD models, while expression of other PDEs seems relatively maintained (Hebb et al., 2004). The loss is detected before onset of symptoms in R6/2 and R6/1 models. Because, PDE regulates levels of cAMP, which plays a key role in modulation of gene

expression which is altered in HD, the effects of a treatment with a PDE10 inhibitor has been studied in the R6/2 mouse model of HD. Results showed that chronic pharmacological blockade of PDE10 is neuroprotective and reverses a number of transcriptomic anomalies in HD mice (Giampa et al., 2010). In line with this, the characterization of the effects of a pharmacological inhibition of PDE indirectly suggests that the reduction of PDE activity in HD could lead to multiple effects: it up-regulates cAMP-responsive element –dependent transcription, it down-regulates HDAC4 (histone deacetylase 4) mRNA, and could activate Mitogen- and stress-activated kinase-1 (MSK1). These latter effects should contribute to striatal neurons against mHtt toxicity. Thus, the presence of PDE in striatal cells may be considered protoxic, and its decrease in HD could be seen as a compensatory mechanism to counteract the effect of mHtt. Interestingly, further inhibition of the enzyme may allow the triggering of neuroprotective pathway and as such may constitute an interesting pharmacological therapy.

POTENTIAL NEUROPROTECTIVE STRIATAL GENE PRODUCTS

BCL11 (B-cell leukemia/lymphoma 11B)

B-cell leukemia/lymphoma 11B (Bcl11b) (a.k.a. CTIP2) is a transcription factor that has been described to be a key gene for differentiation of medium sized spiny neurons in the striatum. Since MSN represent ∼95% of the neurons in the striatum, Bcl11b likely possesses a central role that determines the architecture and organization of the striatum, and as such its function is likely crucial in HD (Arlotta et al., 2008). Bcl11b mRNA levels are reduced in the HD striatum. The overexpression of Bcl11b has been found neuroprotective in cell models of HD *in vitro* (Desplats et al., 2008). The direct interaction of Bcl11b with mHtt and its possible sequestration in inclusions may further abolish its capacity to regulate the expression of many striatal genes that are crucial for the survival of MSN. In particular, there exists a functional interaction between Bcl11b and BDNF. Chromatin-immunoprecipitation experiment and sequencing (ChIP-seq) indicated that Bcl11b is a regulator of the BDNF signaling pathway (Tang et al., 2011). Thus, the loss of Bcl11b in the striatum may lead to a striatal-selective cascade of events that could explain the preferential vulnerability of MSNs against mHtt.

FOXP1 (Forkhead box protein P1)

FOXP1 is thought to be an important transcription factor regulating cell-cell interaction signaling. FOXP1 shows highly expression in the striatum (Desplats et al., 2006, 2008). Its expression is regulated by Bcl11b. There exist overlaps between the genes that are regulated by FOXP1 in normal neurons and the genes that are deregulated in HD (Tang et al., 2012). No rescue or knockdown experiments have been performed, but FOXP1 seems to interact with mHtt and to be trapped in mHtt-containing aggregates (Tang et al., 2012). Therefore, its reduced expression likely contributes to the preferential vulnerability of the striatum in HD.

MSK-1 (Mitogen- and stress-activated kinase-1)

In healthy conditions, the mitogen- and stress-activated kinase-1 (MSK-1), a striatum-enriched nuclear protein kinase downstream Extracellular Regulated Kinase (ERK), promotes activation of the transcriptional factor kappa-light-chain-enhancer of activated B cells (NF-kappaB) signaling, inducing c-Fos transcriptional activation important for immune and inflammatory responses (Vermeulen et al., 2003). MSK-1 is downregulated in R6/2 HD model mice and in caudate from HD patients (Roze et al., 2008a). Overexpression of MSK-1 in primary culture of striatal neurons expressing a short fragment of mHtt is neuroprotective, whereas knockdown of MSK-1 is protoxic. Interestingly Roze and collaborators found evidence of ERK, Elk-1, and CREB nuclear activation in the striatum of R6/2 mice. This suggested the existence of a possible self-defense response in striatal neurons. However, this response appeared to be blunted, since neither phosphorylation of histone H3 phosphorylation nor c-Fos activation were detected. Indeed, loss of MSK-1 in the striatum in HD mice impeaches activated ERK to produce its downstream effects on transcription. In the normal brain, MSK-1 phosphorylates histone H3, CREB and up-regulates peroxisome proliferator-activated receptor co-activator-1α (PGC-1α), playing role in bioenergetic stability in MSNs. The MSK-1 downregulation likely produces mitochondrial dysfunction rendering MSNs more susceptible to mHtt. Consistent with this hypothesis, MSK-1 overexpression in striatal neurons using lentiviral vectors was neuroprotective against mHtt in mouse models of HD (Martin et al., 2011). Therefore, because MSK-1 shows enrichment in the striatum, its loss would contribute to render the striatum more fragile in HD.

ADORA2 (Adenosine receptor type 2A)

A2A receptors (A2A-R), coded by the *ADORA2A* gene have a highly enriched expression in the striatum. The expression of A2A receptor is down regulated in the striatum of HD patients (Glass et al., 2000) and in several HD mouse models (R6/2,N171-82Q) (Menalled et al., 2000; Chou et al., 2005) These receptors are located at the terminal of cortico-striatal pathway (presynaptic receptors) and in the D2-MSNs (post-synaptic receptors). The mRNA level of A2A-R in the striatum is higher in the striatum than in the cerebral cortex. These two types (pre- and post-synaptic) seem to differ in their contribution to neurodegenerative process. Evidences in HD area suggest that activation of presynaptic A2A-R is pro-toxic for MSNs by modulation of glutamate release whereas activation of post-synaptic A2A-R are protective (Popoli et al., 2007). Both agonists and antagonists were proposed to treat HD symptoms. Interestingly, the A2A-R agonist, CGS21680, produces an opposite effect in WT and symptomatic R6/2 in slices. Field potentials (FP) were recorded with and without NMDA and CGS21680. The NMDA toxicity is observed by the only partial recovery after the FP stimulation. The addition of CGS21680 increases NMDA-mediated toxicity in WT MSNs whereas it decreases it in symptomatic R6/2 mice (Martire et al., 2007). Thus, it seems that complex regulatory mechanisms, possibly compensatory, involve A2A-R in HD mice.

The chronic effect of the presence of A2A-R, especially expressed at high level in MSN is not totally understood. Genetic deletion of the *ADORA2A* gene precipitates motor symptoms and death in HD mice expressing a short N-terminal fragment

of mHtt (Mievis et al., 2011a). In support of the hypothesis that A2A-R may have an impact on the disease progression, a single genetic polymorphism in the *ADORA2A* gene in HD patient can modify the age of onset (Dhaenens et al., 2009). Thus, the loss of A2A-R may be detrimental. These receptors are likely neuroprotective. However, it must be underscored that the exact contribution of presynaptic receptors of the cortico-striatal pathway vs. the post-synaptic receptors expressed by MSN in these experiments remains to be fully elucidated.

CNR1 (Cannabinoid type 1 receptor)

The profound and early loss of striatal type 1 cannabinoid receptors (CB1-R) in the striatum and projection area (substantia nigra reticulate and globus pallidus externus) in HD has been demonstrated by autoradiography studies on post-mortem brain samples from patients at early stages as for the A2A-R (Glass et al., 2000). Loss of CB1 binding sites have been confirmed *in vivo* by PET studies in HD patients (Van Laere et al., 2010). Elegant studies demonstrated that genetic deletion/knockout of CB1 receptors exacerbates the motor phenotype in HD mice (Blazquez et al., 2011; Mievis et al., 2011b). The loss of CB1-R might be due to direct transcriptional deregulation produced by mHtt (via mHtt-induced deregulation of REST) (Blazquez et al., 2011) but also may result from more complex mechanisms. Indeed, exposure of immortalized striatal cells with endogenous cannabinoids produced an increase in CB1-R expression (Laprairie et al., 2013). Treatment of HD striatal cells (Q111/Q111) with cannabinoid markedly increases CB1-R expression. Available results from *in vitro* experiments indicate that the loss of CB1-R in HD would lead to reduced levels of BDNF, which in turn should render striatal cells more vulnerable to mHtt toxicity, possibly through decreases in PGC-1α levels (Laprairie et al., 2013). However, a convincing work recently performed in R6/2 HD mice showed that only the presynaptic CB1-R at the cortico-striatal terminals actually underlie the neuroprotective effects of the CB1-R agonists *in vivo* (Chiarlone et al., 2014). Thus, CB1-R can be considered as neuroprotective. However, the impact of the reduced striatal expression of CB1-R in HD is uncertain.

SCN4B (Sodium channel beta 4b subunit)

SCN4b mRNA expression is down regulated in HD models and HD patients (Oyama et al., 2006; Kuhn et al., 2007; Brochier et al., 2008). Its reduced expression is more severe than that of other sodium channel subunits (Oyama et al., 2006). The function of this sodium channel subunit is unknown. The good correlation between loss of its expression and progression of the disease in R6/2 mice suggested a potential role in striatal vulnerability. In line with this, SCN4b levels seem to be more reduced in regions of the central nervous system that are the most affected by mHtt expression. Interestingly, overexpression of SCN4b in neurons in primary culture produces trophic effects characterized by increased dendritic genesis (Oyama et al., 2006). Thus, SCN4b may be a "neuroprotective" striatal marker whose reduced expression in HD may contribute to the preferential degeneration of the striatal in HD. However, its putative neuroprotective effect needs to be directly assessed against mHtt toxicity.

STEP61(PTPN5 gene, striatal-enriched protein tyrosine phosphatase 61)

Reduced expression of STEP61 mRNA has been found in HD transgenic models and HD brain (Desplats et al., 2006). In different mouse models (YAC1128, TET-HDH94, R6/1, and KI111) the protein is reduced and its level of phosphorylation is increased, which should further contribute to a reduction of its phosphatase activity (Saavedra et al., 2011; Gladding et al., 2014). Convincing results indicate that the loss of STEP61 is globally detrimental to MSN, although it may also partially represent a compensatory mechanism trying to block excitotoxicity in striatal cells. In R6/1 mice, whereas STEP protein levels are reduced in young (excitotoxicity sensitive) mice, its levels of phosphorylation is much increased, leading to its further inactivation (Saavedra et al., 2011). In line with this, intrastriatal injection of a permeable and active form of SETP61 (TAT-STEP), could increase the excitotoxic lesions produced by the NMDA receptor agonist quinolinate. In addition, an increased cleavage of STEP61 has been observed, resulting from increased calpain activation due to entry of Ca^{2+} through NMDA receptors. An accumulation of the breakdown product STEP33 (inactive and unable to dephosphorylate MAPK/p38) is associated with elevated p38 phosphorylation (Saavedra et al., 2011), which his detrimental for cell survival. STEP dephosphorylates ERK, reducing its activation and pro-survival signals. There is an increased activation of pro-survival MAPK/ERK1/2 signaling in older mice resistant to excitotoxicity. In young YAC128CAG HD mice that are sensitive to excitotoxicity, STEP61 levels have also been found reduced, as STEP33 (Gladding et al., 2014). At later stage, when YAC128 mice become resistant to excitotoxicity, the loss of STEP61 may be associated with the induction of ERK1 (blocking excitotoxicity) while maintaining activation of MAPK/p38 that favors cell death pathways. These very interesting studies clearly show the existence of complex "striatum-specific" compensatory mechanisms in HD mice, and their evolution over time, possibly to block sequentially mHtt toxicity.

Thus, the role of STEP61 in striatal vulnerability is ambivalent. Its loss in HD may reduce excitotoxicity, consistent with a neuroprotective compensatory mechanism. In this case STEP61 could be considered as a protoxic actor in MSN. However, its loss also contributes to activate MAPK/p38 pathway. In this latter case, STEP61 may be seen as a neuroprotective agent for MSNs.

ELK-1 (ETS-like gene 1)

In basal condition, Elk-1 is ubiquitously expressed in the brain, but in HD mice models R6/1 and R6/2, and in immortalized HD mouse (Q111/Q111) cells, Elk-1 has a higher protein expression level and phosphorylation, and is found in the nucleus of the MSNs of 30 weeks old R6/1 mice and 12 weeks old R6/2 mice. Elk-1 does not co-localize with mHtt, which suggests a higher transcriptional activity compared to WT mice (Roze et al., 2008a; Anglada-Huguet et al., 2012). The authors suggested that the change in Elk-1 expression may be a compensatory mechanism to protect MSN in response to mHtt-induced stress.

Elk-1 is a member of a subfamily of proteins called ternary complex factors (TCF). Elk-1 is a transcriptional activator, as it interacts with serum response factor to bind jointly to

serum response elements in the promoters of several immediate-early genes (IEGs), such as c-fos and egr-1. In the CNS, Elk-1 is activated by ERKs in response to neurotrophins and neurotransmitters.

Anglada-Huguet et al. have shown that down-regulation of Elk-1 by siRNAs produces caspase 3 cleavage and cell death in immortalized HD mouse (Q111/Q111) cells, but not in wild-type cells (Anglada-Huguet et al., 2012). Thus, the induction of Elk-1 expression in HD may be considered to be a neuroprotective compensatory mechanism. However, transcriptional activity at the *c-fos* promoter was impaired in the striatum of R6/2 transgenic mice, despite activation/phosphorylation of Elk-1 (Roze et al., 2008a). As mentioned above, the reduction of MSK1 in R6/2 mice may partially impair the impact of Elk-1 activation. Elk-1 can be considered as an "inducible" striatal marker in HD, likely producing a neuroprotective self-defense mechanism. Further studies are awaited to better understand how the increase in Elk-1 plays a role in striatal degeneration at late stage in animal models of HD.

NEUTRAL STRIATAL MARKERS

Capucin (a.k.a. Tmem90a)

Capucin, a gene of unknown function is preferentially expressed in the striatum (de Chaldee et al., 2003). Notably, lower capucin mRNA levels have been detected in the R6/1 transgenic mouse model of HD (Desplats et al., 2006), R6/2 and in primary cultures of rat striatal neurons expressing a mutant fragment of human Htt than in the corresponding controls (de Chaldee et al., 2006). However, *in vivo* experiments showed that capucin overexpression is not able to counterbalance mHtt-induced toxicity in the striatum in a lentiviral mouse model of HD (Galvan et al., 2012b). Mice that were knockout for capucin gene had similar susceptibility to mHtt-induced toxicity as wild type age-matched littermates. Size and number of ubiquitin-containing inclusion produced by overexpression of mHtt is these mice were similar to those detected in wild type mice (Galvan et al., 2012b). Capucin downregulation in HD mouse models could be a direct consequence of the transcriptional dysfunction occurring in HD without major consequence on MSN survival. Thus, capucin may be considered as a "neutral" striatal gene.

Hippocalcin

Hippocalcin, a neuronal calcium sensor protein, is also known as p23k. Although the physiological role of hippocalcin is not completely understood, it is implicated in the regulation of neuronal viability and plasticity. Evidences showed that hippocalcin is important for the homeostasis of intracellular calcium levels (Amici et al., 2009). Hippocalcin can protect hippocampal neurons against excitotoxicity induced damage by enhancing Ca^{2+} extrusion and maintaining ideal intracellular Ca^{2+} levels (Masuo et al., 2007).

The decreased expression of hippocalcin in different mouse models of HD suggested a role of this protein in striatal vulnerability. Rudinskiy and collaborators studied this hypothesis in primary culture of striatal neurons (Rudinskiy et al., 2009). Hippocalcin was overexpressed using lentiviral vectors in neurons that expressed mHtt (N-terminal fragments with 82 glutamine repeat). Analysis of different outcomes related to degeneration indicated that hippocalcin was not neuroprotective. In addition, overexpression of hippocalcin did not protect neurons subjected to mitochondrial dysfunction caused by 3-nitropropionic acid or glutamate-induced excitotoxicity, two conditions inducing increase in cytoplasmic Ca^{2+} concentrations (Rudinskiy et al., 2009). Thus, hippocalcin may have deregulated expression, in absence of major consequences in neuronal survival. In this case, as capucin, hippocalcin may be seen as "neutral" striatal marker. However, it cannot be excluded that hippocalcin could have an effect in different HD models, including animal models that express full length mHtt.

OTHER POSSIBLE PATHWAYS TO BE INVESTIGATED

Nowadays, the number of studies trying to decipher the functions of this small number of striatal genes is limited. However, these pioneering studies which tried to understand their roles with regard to mHtt toxicity provided key results indicating that possibly, they are regulators of cell survival, upstream master gene/protein networks of neuronal survival (**Figure 1**). In particular, deregulation of membrane receptors (D1-R, D2-R, CB1-R, A2A-R, SCN4B) involved in neurotransmission in HD could directly modulate cell survival processes through different routes (e.g., MAP Kinase pathway, regulation of PGC1-α etc.). How these different receptors act to positively or negatively regulate striatal cell survival remains to be uncovered. It is likely that, for the GPCR, their effects are related to the activation of heterotrimeric G proteins leading to increased or decreased cAMP levels but could also be mediated through other

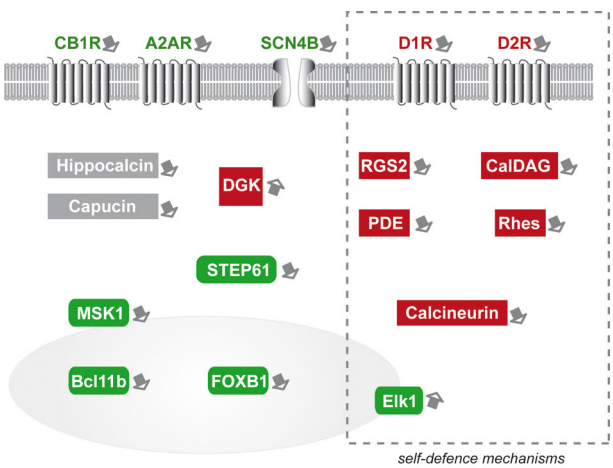

FIGURE 1 | Schematic representation of the striatal markers that have been experimentally studied as potential modifiers of mutant huntingtin toxicity in HD. Green boxes symbolize markers that are "neuroprotective." Red boxes symbolize markers that are "protoxic." Expression changes in markers included in the dotted-line rectangle may represent, at least in part, self-defense-mechanisms. Markers in gray boxes would have altered expression without major consequences on mHtt. Note that striatal gene modifiers have broad biological functions and cellular localization, including neurotransmitters binding, intracellular signaling (kinases and phosphatases), and transcription activators. The nucleus is symbolized by the gray colored round form. MSK1 and Elk1 can be found in the cytoplasm and upon activation translocate in the nucleus.

pathways such as the endocytosis/β-arrestin-mediated pathway and/or interaction of heterotrimeric subunits with transmembrane ion channels (Ritter and Hall, 2009). Increased cAMP levels may be considered neuroprotective while reduction of cAMP should be "protoxic." It is likely that mechanisms converging on cAMP level regulation are important for opposing mHtt toxicity. Indeed PDE which reduce cAMP levels is considered to increase striatal cell vulnerability to mHtt (see below). However, it is probable that the effects of striatal membrane receptors on mHtt toxicity cannot be only explained according to their inherent capability to change cAMP levels. For example, D2-R and D1-R are thought to be coupled to different α subunits (αi/o and αs/olf, leading, when stimulated separately, to a reduction and decrease in cAMP levels respectively) (Beaulieu and Gainetdinov, 2011), but both receptors seem to increase mHtt toxicity. Downstream cAMP changes (and possible through independent mechanisms) the protoxic effects of D2-R may involve inhibition of the pro-survival kinase Akt (Marion et al., 2014) while D1-R effects may involve CDK5 (Paoletti et al., 2008). In line with these complex mechanisms, CB1-R which decrease cAMP levels when stimulated alone, are rather neuroprotective against mHtt through a mechanism that remains to be elucidated. One possibility is that co-activation of D2-R and CB1-R which increase cAMP so that the loss of CB1-R in HD may result in reduced cAMP levels and a protoxic effect which would depend on the presence of D2-R (Glass and Felder, 1997). There also exist a number of very complex cross talks between membrane receptors signaling in striatal neurons that could participate to more complex/integrated biological effects when their stimulation occurs simultaneously. In particular, receptors can heteromerize, which changes their intracellular signaling impact. For example, D1-R/D2-R heteromers act preferentially through Gαq changing signaling as compared to each receptos separately. Another interesting example is related to A2A-R/D2-R heteromers. The activation of A2A-R in these heteromers reduced the binding of dopamine on the D2-R (Ferre et al., 2008). Reciprocally, stimulation of D2-R represses the activation of adenylyl cyclase by A2-R. Other pathways may also be involved. For example, the activation of β-arrestin signaling by A2A-R/D2-R heteromers is stronger and more transient as compared to D2-R alone (Borroto-Escuela et al., 2011). In summary, the mechanisms through which those different membrane receptors act all together on mHtt toxicity (as causal factors or as key actors of compensatory/self-defense mechanisms) are largely unknown but likely involve extremely complex/integrated signaling.

Similarly cytoplasmic signaling proteins (PDE, MSK1, STEP61, DGK) can also act upstream or downstream master regulators of cell survival (CREB, MAPK/Erk1). Other striatal markers seem to be involved in molecular steps between membrane receptor signaling and downstream cytoplasmic effectors. This is the case for RGS2 and CalDAG-GEF1. Other striatal markers may not act directly on signaling processes regulating transcription or survival. Indeed, it is likely that some markers, such as Rhes, may involve key cellular "housekeeping" mechanisms such as SUMOylation of proteins and autophagy. Finally, Bcl11b and FOXP1 are good examples of striatal marker that can be directly implicated in the regulation of transcription, and the inherent state of differentiation of MSN.

The study of the role of striatal markers in striatal vulnerability in HD suggests that these gene products, likely associated with highly specific neurobiological functions (and as such they are markers of highly differentiated non-dividing cells), may be, on the one hand, the most vulnerable targets of mHtt-induced transcription deregulations and, on the other hand, key "switches" of striatal adaptive changes, that may be considered as self-defense mechanisms.

How these different striatal markers functionally interact each other remains to be precisely assessed. It is quite obvious that currently the puzzle is not complete and that many more actors are involved in the vulnerability of the striatum. Indeed, beyond the few striatal markers that have been reviewed above, many others may also act as modifiers of mHtt. When considering striatal markers with relatively stringent criteria (see paragraph above), it clearly appears that only a small proportion of striatal markers has been experimentally studied. It is beyond the scope of this review to provide extensive speculations on every striatal marker that have never been studied in the context of HD research. However, it is worth mentioning that many of them, which abnormal expression in the striatum of HD patients have been observed long ago, have never been studied for their capacity to modify mHtt toxicity. For example, neurotensin, whose expression is high in the striatum as compared to other brain regions, has been found abnormally increased in the HD striatum (Nemeroff et al., 1983). Many newly identified striatal markers have been found deregulated in HD mouse models (Brochier et al., 2008; Mazarei et al., 2010). For example, the upregulation of IDO-1 (indoleamine 2,3-dioxygenase) in YAC128 HD mice may be seen as a risk factor for striatal cells, since deletion of IDO-1 protects the striatum against excitotoxicity (Mazarei et al., 2013b). Since kynurenine pathway likely plays a role in HD pathogenesis (Thevandavakkam et al., 2010), it is possible that IDO-1 is a modifier of mHtt toxicity (Mazarei et al., 2013a). This remains to be further assessed.

CONCLUSION

It is very difficult to know whether a change in expression of a given striatal marker in HD represents a compensatory mechanism, and/or a phenomenon that will contribute to striatal degeneration. This question needs to be experimentally addressed. However, all the gene products that have not yet been explored represent a pool of potential candidate modifiers of mHtt, relevant to striatal vulnerability. Our group and others are currently testing the effects of many newly identified striatal markers of unknown biological functions. Preliminary observations indicate that a majority of them are neuroprotective or protoxic modifiers of mHtt in cell and mouse models. As such, they could represent innovative therapeutic targets. Promoting the activity of the neuroprotective markers or blocking the activity of the protoxic gene products could help to slow the progression of symptoms and degeneration in HD. In addition, since a majority of these striatal markers have ill-defined neurobiological functions, research focused on these striatal gene products could be a unique opportunity to better define the molecular and functional complexity of

the striatum, a brain region which is central stage in a broad spectrum of motor and cognitive functions and is likely implicated in different neurological and psychiatric illnesses.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00295/abstract

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De-regulation of gene expression and alternative splicing affects distinct cellular pathways in the aging hippocampus

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Aging is accompanied by gradually increasing impairment of cognitive abilities and constitutes the main risk factor of neurodegenerative conditions like Alzheimer's disease (AD). The underlying mechanisms are however not well understood. Here we analyze the hippocampal transcriptome of young adult mice and two groups of mice at advanced age using RNA sequencing. This approach enabled us to test differential expression of coding and non-coding transcripts, as well as differential splicing and RNA editing. We report a specific age-associated gene expression signature that is associated with major genetic risk factors for late-onset AD (LOAD). This signature is dominated by neuroinflammatory processes, specifically activation of the complement system at the level of increased gene expression, while de-regulation of neuronal plasticity appears to be mediated by compromised RNA splicing.

Keywords: inflammaging, RNA-editing, innate immune system, RNA-seq, neuroinflammation, synaptic plasticity, learning and memory, gene-environment interaction

INTRODUCTION

Aging is associated with a number of changes that affect cellular homeostasis and impact on the organism's overall health. Aging also leads to a decline of cognitive function including memory formation across species. As such, age-associated memory impairment is observed in invertebrates such as flies as well as in rodents and humans (Horiuchi and Saitoe, 2005; Bishop et al., 2010; Verdaguer et al., 2012). While in humans age is the most significant risk factor for neurodegenerative diseases such as Alzheimer's disease (AD), it is important to note that the degree of cognitive decline varies significantly on an individual level. Thus, some individuals undergo so-called "healthy/successful aging" that is characterized by relatively intact cognitive function, while others develop severe memory impairments and in the most extreme case dementia (Koivisto et al., 1995; Montesanto et al., 2012). In humans it is believed that the manifestation of healthy cognitive aging vs. dementia depends on the variable combinations of genetic pre-disposition and environmental factors an individual experiences throughout lifetime (Fischer, 2014). In order to decipher the molecular signature of age-associated memory impairment it is therefore most suitable to rely on mouse studies in which the genetic background and the environmental factors can be tightly controlled. The mean life span of different mouse strains housed in a laboratory ranges from about 24–30 months (Jucker and Ingram, 1997; Peleg et al., 2010). Previous studies have demonstrated that the onset of age-associated memory impairment in mice can already be observed at 16–18 months of age and is prominent at 24 months of age, while assessment of cognitive function becomes more difficult at more advanced ages due to impaired motor function (Berchtold et al., 2008; Peleg et al., 2010). It has been speculated that age-associated memory decline is correlated to a gene expression signature that dictates cellular plasticity. As such, a number of studies reported altered gene expression in the aging brain using targeted approaches such as qPCR or microarray (Finch and Morgan, 1990; Pletcher et al., 2002; Blalock et al., 2003, 2010; Lu et al., 2004; Verbitsky et al., 2004; Xu et al., 2007; Zahn et al., 2007; Loerch et al., 2008; Pawlowski et al., 2009; Bishop et al., 2010). Unlike these approaches, RNA sequencing is not biased by probe design and in addition to the identification of differential gene expression readily allows the analysis of alternative splicing and RNA editing, two processes intimately linked to cognitive function. RNA sequencing is widely used in other fields and is now also more commonly applied to study brain tissue (Dillman et al., 2013; Mazin et al., 2013; Wood et al., 2013; Stilling et al., 2014). However, the aging hippocampus, a key region for memory formation in rodents and humans that is affected early in age-associated memory decline and AD, has not been studied using RNA sequencing. To this end, we used Illumina next-generation sequencing to compare the hippocampal transcriptomes of 3, 24, and 29-month-old C57BL/6J mice (*3M*, *24M*, and *29M*, respectively). We find that the aging hippocampus is characterized by

a strong neuroinflammatory gene-expression signature that is dominated by differential gene expression but not by differential splicing or RNA-editing. A key component of the neuroinflammatory response was activation of the complement system that has repeatedly been genetically linked to AD (Bertram et al., 2007; Lambert et al., 2009; Brouwers et al., 2012). Taking into account that neuroinflammation is a key mechanism in neurodegenerative diseases, our data supports the view that AD may represent accelerated brain aging due to an unfavorable genetic pre-disposition and exposure to environmental risk factors. On the other hand, we find that compromised synaptic function is linked predominantly to alternative splicing suggesting that, on the level of the transcriptome, age-associated neuroinflammation and decreased synaptic plasticity are mediated by distinct cellular processes.

MATERIALS AND METHODS

ANIMALS

Specific pathogen free (SPF) C57Bl6/J wild type mice were obtained from Janvier SAS. Mice were kept in groups $\leq$5 animals in individually ventilated cages (32 × 16 × 14 cm, Techniplast) on a 12 h light/dark cycle with food and water *ad libitum*. To obtain mice between 28 and 30 months of age, 24 month-old mice were ordered from Janvier SAS and kept in our holding rooms for 4–6 months. All procedures were performed by experienced experimenters and according to protocols approved by the Lower Saxony State Office for Consumer Protection and Food Safety.

NOVEL OBJECT RECOGNITION

Behavioral testing was performed as described previously (Kerimoglu et al., 2013). Animals were habituated individually to a uniform-gray plastic arena (90 × 90 cm, walls 20 cm high) for 5 min on two subsequent days. Animals were then further habituated to two equal objects placed in opposing corners of the arena for 5 min on the next two days. On day 5 objects were exchanged by two new but equal objects (A + A) and animals were allowed to explore the objects for 5 min. Then, mice were sent back to their home cages for 5 min (for short-term memory assessment) and reintroduced to the arena after one object was exchanged (objects A + B). After 24 h, object B was exchanged for object C for long-term memory assessment. Duration of object contacts was measured. Mice that only showed summed contact time of <1 s were excluded from the analysis of this test. Object preference was defined as (novel object)/sum(both objects).

RNA EXTRACTION AND SEQUENCING

Total RNA was extracted using TRI Reagent (Sigma-Aldrich) as previously described (Peleg et al., 2010). In brief, flash-frozen tissue was homogenized on ice with several pestle strokes in 0.5 ml of TRI Reagent (Sigma-Aldrich) After addition of another 0.5 ml of TRI Reagent and 5 min incubation at room temperature (RT) the dissociated homogenate was mixed with 300 µl of CHCl3 and incubated for 15 min (RT) followed by centrifugation at 12,000× g (4°C). The upper aqueous phase was transferred to a new tube, mixed with 500 µl isopropanol and incubated at -20°C for at least 1 h for precipitation. RNA was precipitated by centrifugation at 12,000 g for 30 min (4°C). The pellet was washed twice with 1 ml of 75% ethanol (centrifugation after washing steps: 12,000× g, 5 min, 4°C). The washed pellet was dissolved in 30 µl of RNase-free water. Following DNase1 (life Technologies) treatment to remove residual contaminating genomic DNA for 20 min at 37°C, RNA was purified using phenol-chloroform extraction. Library preparation and cluster generation for mRNA sequencing [single-end libraries for 3M ($n = 5$) vs. 24M ($n = 6$) comparison; paired-end libraries for 3M vs. 29M comparison ($n = 3$)] was performed according to Illumina standard protocols using the TruSeq RNA Sample Prep Kit v2 and the TruSeq Paired-End Cluster Generation Kit v3-cBot-HS (for paired-end mRNA-seq) with subsequent use of the corresponding TruSeq Cluster Generation Kit v3-cBot-HS (for single-end mRNA-seq). Libraries were quality controlled and quantified using a Nanodrop 2000 (Thermo Scientific), an Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit (life Technologies). For the sequencing run, TrueSeq SBS kits were used according to Illumina manuals. Read lengths were 1 × 50 bp for single-end and 2 × 100 bp for paired-end sequencing.

BIOINFORMATIC ANALYSIS PIPELINE

Differential gene expression

Differential gene expression analysis of RNA sequencing data was performed as described previously (Stilling et al., 2014). In brief, library preparation [single-end libraries for 3-month (3M) ($n = 5$) vs. 24-month (24M) ($n = 6$) comparison; paired-end libraries for 3M vs. 29-month (29M) comparison ($n = 3$)] and cluster generation for mRNA sequencing were performed as required by Illumina protocols (TruSeq, Illumina). Downstream analysis steps after read retrieval included quality control (FastQC, www.bioinformatics.babraham.ac.uk/projects/fastqc/) and mapping to reference genome (STAR aligner v2.3.0, Dobin et al., 2013). For calling of differentially expressed genes (DEG), mapped reads were counted with HTSeq v0.5.4p2 (http://www-huber.embl.de/users/anders/HTSeq) (non-default parameters: -m intersection non-empty) and count tables were analyzed independently for both aging groups vs. the 3M young control group using the DESeq2 v1.2.5 R-package (Anders and Huber, 2010). Genes with a $\log_2$(fold-change) ≥ 0.5 and adjusted p-value ≤ 0.05 were considered differentially regulated. All expression data are made publicly available in a GEO SuperSeries (GSE61918). Compressed fastq-files and primary analysis can be found under GEO accession GSE61915, microarray raw data is available under GEO accession GSE61647.

Functional annotation

Functional annotation and category and pathway analysis was carried out using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, v6.7) (Huang et al., 2009a,b). Non-cutoff-based gene set enrichment analysis (GSEA) was performed by using the Broad Institute GSEA application (Mootha et al., 2003; Subramanian et al., 2005). Input lists for GSEA were ranked based on $\log_2$-fold-change.

Transcription factor binding sites

Promoter analysis to search for overrepresented transcription factor binding sites (TFBS) was done using the Pscan web interface

(Zambelli et al., 2009) (http://159.149.160.51/pscan/) scanning the promoter region from −250bp to +50bp from TSS comparing to JASPAR descriptors. All Gene ID conversion was done using BioMart database queries (www.ensembl.org/biomart/).

Differential exon usage

For detection of differential exon usage the DEXSeq R-package was used (Anders et al., 2012) with default parameters. FDR-corrected *p*-value significance level was set to 0.01 for the 3M vs. 24M comparison and to 0.1 for the 3M vs. 29M comparison to adjust for differences in library-type and sample size.

RNA-editing

Known RNA-editing positions in the mouse reference genome (mm10/GRCm38 coordinates) were retrieved from previously described editing sites in the mouse genome from two online databases and two recent publications and compiled to a non-redundant list of 17831 positions (Table S6). This list was used as input for the REDItools algorithm REDItoolKnown.py (Picardi and Pesole, 2013). Further input arguments were a list of splice sites (taken from UCSC table browser), the current genome sequence (GRCm38p2) in fasta format and the most recent GTF file (taken from Ensembl, version GRCm38.e75). Non-default parameters were –C 1000, –c 0, –q 10, –m 10, –v 1, –n 0.001, –t 4. For statistical comparison between groups independent, two-sided Student's *t*-tests were used for each position.

MICROARRAY

RNA quality control, cDNA synthesis, mono-color Cy3-labeling and hybridization to whole mouse genome microarray chips were carried out according to Standard Operating Procedures of the Transcriptome Core facility at University of Göttingen. Total RNA was labeled with Cy3 according to Agilent's Low RNA Input Fluorescent Linear Amplification Kit and later hybridized to Agilent Whole Mouse Genome 4 × 44K G4122F microarrays according to the manufacturer's protocol. Quantity and Cy3-dye incorporation rates of the generated target material were assessed using a NanoDrop ND-1000. Washes were performed according to the Agilent Technologies SSPE protocol (v2.1)—wash solution 3 was replaced by acetonitrile. After that, scanning was performed using an Agilent G2505B scanner. Data analysis was performed as described previously (Peleg et al., 2010; Agis-Balboa et al., 2011; Kerimoglu et al., 2013). In summary, data was analyzed using Agilent Feature Extraction software, version 9.5.3.1 and the Limma (Smyth et al., 2005) package for R/Bioconductor (Gentleman et al., 2004). In order to assure that the intensities had similar distributions across arrays, VSN normalization (Huber et al., 2002) was applied to the intensity values as a method for between-array normalization. To estimate the average group values for each gene and assess differential gene expression, a simple linear model was fit to the data, and group-value averages and standard deviations for each gene were obtained. To find genes with significant expression changes between groups, empirical Bayes statistics were applied to the data by moderating the standard errors of the estimated values (Smyth, 2004). *P*-values were inferred from the moderated t-statistic and corrected for multiple testing using the FDR method (Benjamini and Hochberg, 1995). Afterwards, the final output was filtered for probes showing a change in normalized intensity that was greater than 1.414 – fold [$\log_2$(fold change) $\geq$ 0.5] with an adjusted *p*-value of FDR(p)<0.1.

QUANTITATIVE REAL-TIME PCR (qRT-PCR)

Quantitative real-time PCR (qPCR) was performed as described before (Peleg et al., 2010). In summary, 1 μg of total RNA was used for cDNA synthesis and cDNA was diluted 1:10. Probe-based qRT-PCR (UPL, Roche) was carried out on a LightCycler 480 II (Roche) and analyzed using suppliers software. Primers used for *C4b* amplification: Fwd(5′-TCTCACAAACCCCTCGACAT-3′), Rev(5′- AGCATCCTGGAACACCTGAA-3′), UPL-Probe #10.

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Fluorescent staining of target proteins was performed as previously described (Peleg et al., 2010). In brief, mice were transcardially perfused with 4% PFA, brains isolated and post-fixed for another 16 h in 4% PFA. Free-floating cryosections (30 μm) were incubated with 5% goat serum for blocking and followed by incubation with target-specific primary antibodies (anti-NeuN [A60, MAB377, Merck Millipore, 1:1000, anti-GFAP [G5601, Promega, 1:1000], anti-IBA1 [019-19741, WAKO, 1:1000]). Corresponding secondary antibodies were from life Technologies (anti-mouse Alexa-488 labeled, A11029; anti-rabbit Alexa-633 labeled, A21071). Images were taken on a Leica SP2 confocal microscope. Stereological analysis of the number of cells was performed on 4 serial 40 μm free-floating coronal sections per animal which were analyzed by confocal microscopy to count cells expressing the indicated marker. Cell number was assessed as areal density across the CA1 region. The data was normalized to the 3 month groups.

RESULTS

DIFFERENTIAL GENE EXPRESSION ANALYSIS IN THE AGING HIPPOCAMPUS

Age-associated memory impairment is the result of variable combinations of genetic pre-disposition and environmental factors, which eventually causes detrimental changes in cellular homeostasis. We therefore reasoned that a comprehensive picture of age-related changes in transcription would be most informative about the aging processes occurring in the brain. The hippocampal formation is essential for memory function in rodents and humans and has been linked to cognitive age-associated memory impairment (Fanselow, 2010). Thus, we performed deep sequencing of polyA-enriched RNA extracted from the mouse hippocampus in three different age groups (3-month-old mice, 24-month-old-mice, and 29-month-old-mice). Since C57BL/6J mice in the laboratory have a maximum life span of just above 30 months, 24-month-old mice represent a model of advanced aging, while 29-month-old represent a time point at the end of life-span. We first confirmed that the selected groups of mice indeed show age-associated memory decline (**Figure 1**). Due to severely impaired locomotor activity in 29-month-old mice we had to exclude these mice from any behavioral testing. A commonly employed test for hippocampus-dependent memory

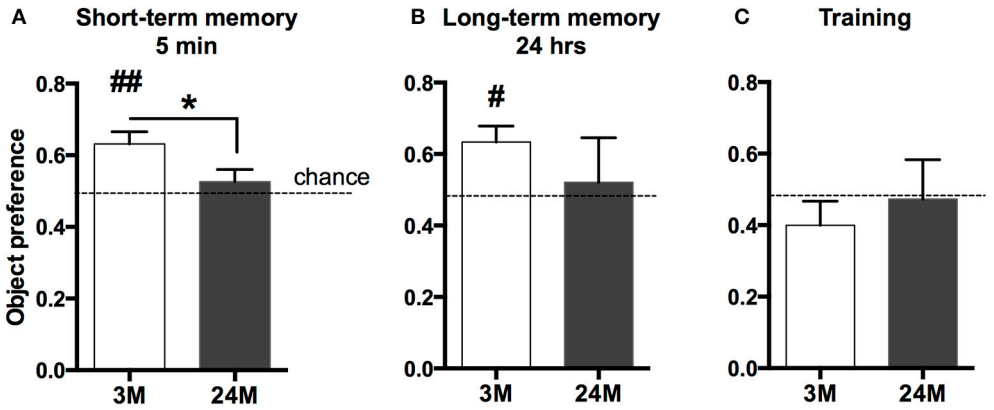

FIGURE 1 | Impaired novel object recognition memory in 24-month-old mice. (A) 3-month-old mice (3M) and 24-month-old mice (24M) were tested in the novel object recognition paradigm. To assay short-term memory, mice were first exposed to two similar objects (A+A) and then re-exposed to the testing area containing one novel object 5 min later (A+B). Preference for the novel object was significantly greater in 3-month-old mice when compared to the 24-month group, which performed at chance level ($^{*}p < 0.05$ between groups, two-sided *t*-test; $^{\#\#}p < 0.01$ vs. chance level, one-sample *t*-test; $n = 11$[3M]/9[24M]). **(B)** To test long-term memory mice were exposed to the arena 24 h later, now containing another novel object (A + C). While object preference in 3M was significantly higher than chance level ($^{\#}p < 0.05$, one-sample *t*-test, $n = 10$), 24M animals ($n = 4$) performed at chance level, indicating impaired long-term object recognition memory in aged mice. Error bars indicate s.e.m. **(C)** During the training session, none of the groups showed preference for any of the two equal objects (A+A).

function in mice is the Morris water maze test. Pilot experiments however showed that in our hands even 24-month-old mice have difficulties to cope with the 2-week-lasting daily training procedure, in which the animals have to swim in a pool filled with opaque water and need to find and climb on a hidden platform. Thus, we decided to subject mice to the novel-object-recognition paradigm that does not depend on advanced motor skills and allows the measurement of short and long-term memory in a non-stressful experimental setting. Moreover, while object recognition learning recruits various brain structures, it also depends on an intact hippocampus (Broadbent et al., 2010; Antunes and Biala, 2012). As expected, we observed that both short (**Figure 1A**) and long-term object-recognition memory (**Figure 1B**) was impaired in 24-month-old mice, when compared to 3-month-old mice. This was not due to altered explorative behavior during the training, since both groups of mice explored the objects presented during the training session to a similar degree (**Figure 1C**).

In the next step, we isolated total RNA from the hippocampi of 3-, 24-, and 29-month-old mice and subjected it to RNA sequencing. In line with previous studies, we observed that aging was not associated with massive changes in cell number (Long et al., 1999) (**Figure S1**). We first compared differential gene expression across the different age groups using the 3-month group as reference. 477 genes were differentially expressed (313 up-regulated, 164 down-regulated) in 24-month-old mice and 323 genes (275 up-regulated, 48 down-regulated) in 29-month-old mice (**Figure 2A**, Table S1) when compared to the 3-month group. In all comparisons, we observed a general trend toward higher numbers of up-regulated genes (∼70%) compared to down-regulated genes (**Figure 2B**). When we compared these lists among each other, we found a significant amount of overlap between genes up-regulated at 24 and 29 months of age (122 genes, **Figure 2C**, Table S1). The overlap between the genes down-regulated in 24- and 29-month-old mice was less pronounced (17 genes, **Figure 2C**, Table S1). When we analyzed the 122 genes commonly up-regulated in 24- and 29-month-old mice for functional pathways we observed inflammatory signaling pathways, namely the "Systemic lupus erythematosus" and the "complement and coagulation pathway" to be highly enriched (**Figure 2D**, Table S2). It has to be noted that several genes of the complement system are linked to systemic lupus erythematosus, which explains the enrichment of this pathway (Table S2). This data suggests that activation of the complement system is one of the key features of the aging hippocampus. Similar results were obtained when we separately analyzed all genes up-regulated in 3 vs. 24-month-old mice (**Figure 2E**, left panel). When we analyzed all up-regulated genes in 3 vs. 29-month-old mice, we found significant enrichment of additional immune-related pathways (**Figure 2E**, right panel, blue bars), suggestive of an even more pronounced immune activation with increasing age. Another group of up-regulated genes was associated with cell adhesion since "cell adhesion molecules" was also identified as a significantly enriched pathway in 24- and 29-month-old mice (**Figure 2E**, Table S2).

These findings, based on analysis of lists of significantly up-regulated genes using the DAVID platform, were further confirmed by GSEA, an unbiased approach to analyze enrichment of functional groups in a given gene list without the need for thresholding or introduction of *p*-value cutoffs. Similar to the previous analysis, we found the enriched immune system pathways, including the top-enriched "complement and coagulation pathway" in both aging groups (**Figure 2F**), as well as several additional pathways associated with immune-system function (**Figure S2**). Interestingly, several of these additional categories were associated with pathological infection by bacteria, viruses and other pathogenic organisms, suggesting that at least part of the neuroinflammatory response may

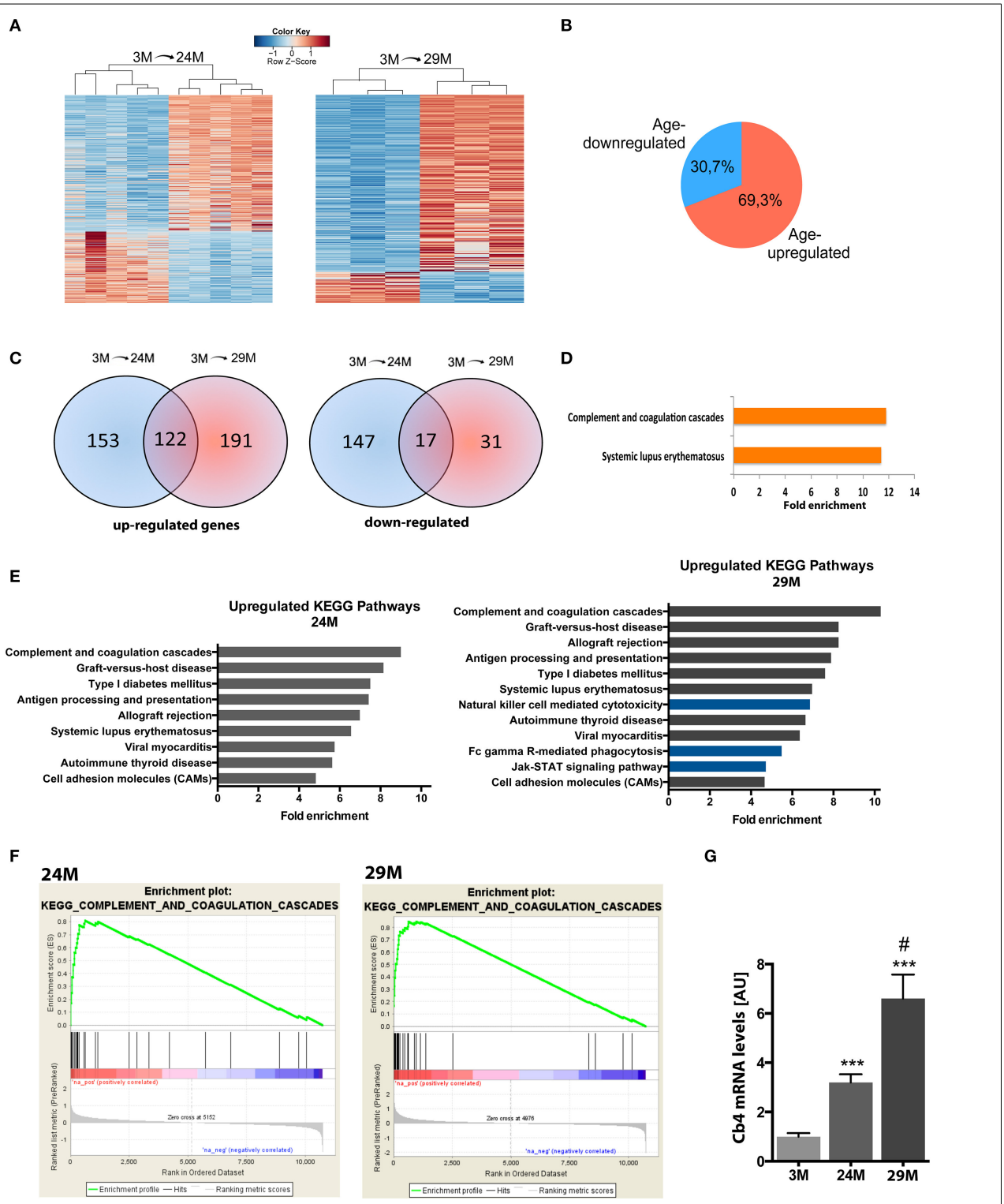

FIGURE 2 | Aging is associated with changes in gene expression and upregulation of immune system functions in the hippocampus. **(A)** Heatmaps of differentially expressed genes in 3-month (3M) and 24-month-old mice (24M, left panel) and 3 and 29-month (29M) old mice (right panel). High expression is marked by the red color spectrum, low expression by blue colors. **(B)** Pie chart showing the percentage of genes up-and down-regulated in 3 vs. 24/29 month-old mice. **(C)** Venn diagrams comparing significantly regulated genes found in the different experiments (3M vs. 24M and 3M vs. 29M). **(D)** Genes up-regulated at 24 and 29 month of age were subjected to functional enrichment analysis for overrepresented biological processes and KEGG pathways. **(E)** Fold enrichment of overrepresented KEGG pathways in up-regulated genes when individually comparing the 3M vs. 24M and 3M vs. 29M groups. **(F)** Using non-cutoff gene set enrichment analysis (GSEA), the top up-regulated KEGG pathway was the complement and coagulation cascade. Shown are enrichment plots along expressed genes, *(Continued)*

FIGURE 2 | Continued
ranked by fold-change [left/red: positive log_2_fold-change (upregulation), right/blue: negative log_2-fold-change (downregulation); a gene contributing to the enrichment score of the selected pathway along the ranked list is marked by a vertical black line and increases the cumulative enrichment score (green line)]. **(G)** qPCR analysis of the *C4b* gene (***$p < 0.001$ vs. 3M group, two-sided *t*-tests; $^{\#}p < 0.05$ vs. 24M group, two-sided *t*-test; $n =$ 5[3M]/4[24M]/8[29M]). Error bars indicate s.e.m.

be attributed to the invasion of parasitic microbes across the blood brain barrier. Furthermore, GSEA found a similar set of additional pathways in the 29-month-old mice compared to the 24-month group as detected by DAVID platform analysis. Thus, GSEA confirmed functional enrichment of immune system-related pathways, which are gradually up-regulated with advancing age.

To further verify these findings we decided to reproduce the results in an additional cohort of 3 and 29-month-old mice using a microarray approach thereby also controlling for any potential experimental bias that might be introduced by RNA sequencing. Albeit DEG differed to some extend, the affected pathways were almost identical and the complement system was the most affected pathway among the up-regulated genes (Table S2).

Within this pathway the *C4b* gene encoding the complement factor 4 (C4) constantly turned up among the most significantly up-regulated genes in all our analyses. Thus, to validate the finding made by genome-wide techniques we sought to directly compare expression levels of this gene among all three aging groups by qRT-PCR. We detected a 3.2-fold increase of *C4b* mRNA levels in the 24-month (24M) group and a further increase in 29-month (29M) group (6.6-fold) when compared to the 3-month (3M) group (**Figure 2G**).

We also determined functional enrichment among down-regulated genes. Interestingly, the down-regulated genes in the 24M group were enriched for genes of the "neuroactive ligand-receptor interaction" and "regulation of transcription" pathways, while no pathways could be detected in the 29M group, even if we combined the data obtained by RNA sequencing and microarray (Table S2). When we combined the genes down-regulated in 24- and 29-month-old mice we observed the "neuroactive ligand-receptor interaction" and the "calcium signaling" pathways to be significantly affected (Table S2).

In addition to protein-coding genes, we could also detect differential regulation of several non-coding RNAs (ncRNAs), including the pseudogene *Pisd-ps1* and the long intergenic non-coding RNA (lincRNA) *Neat1* as well as *Malat1*, also known as *Neat2* (Table S1). While the role of long non-coding RNAs is only beginning to emerge, *Neat1* and *Malat1* are known to accumulate in the nucleus, where they form the RNA backbone of so-called paraspeckles, subnuclear ribonucleoprotein bodies involved in transcriptional regulation, e.g., by nuclear retention of RNAs (Bond and Fox, 2009). Interestingly, we could also detect increased transcription from the *C4a* locus in the 29M group. *C4a* is closely related to the nearby *C4b* but does not encode a protein, according to the ENSEMBL database. Though their functional significance is subject to ongoing research, these results indicate that also regulatory ncRNAs are implicated in hippocampal aging.

Taken together, analysis of differential gene expression revealed that hippocampal aging is markedly characterized by up-regulation of a neuroinflammatory program. Especially activation of the complement system was highly correlated with increasing age.

AGE-ASSOCIATED CHANGES IN GENE EXPRESSION ARE ORCHESTRATED BY A SPECIFIC SET OF TRANSCRIPTIONS FACTORS

To further elucidate potential upstream mechanisms of the identified transcriptional program, we searched the promoters of regulated genes for common TFBS. We found a large number of potential TFBS significantly enriched at promoters of genes that were up-regulated with aging (Table S3). Of these, the TOP20 most strongly enriched TFBS were largely similar in the 24M and 29M group (**Figures 3A,B**). We could identify a number of common transcription factor families that together made up the bulk of the significantly enriched TFBS [*Signal transducer and activator of transcription* (STATs), *Interferon regulatory factor* (IRF), *Spleen focus forming virus proviral integration oncogene* (SPI), Activator protein 1, (AP1, composed of Fos, Jun and ATF family members), *Nuclear factor kappa-light-chain-enhancer of activated B cells* (NF-κB), the GATA and the *E26 transformation-specific* (ETS) family of transcription factors (Table S3)], which are strongly associated with their roles in immune-related signaling (Peng, 2008). Of note, this set of TFs explained 44% of all up-regulated genes in 24- and 53% in 29-month-old mice. In fact, up to 87% of all up-regulated genes could be assigned to the action of enriched TFs (**Figure 3C**, Table S3).

In agreement with functional enrichment of DEG, we observed a higher diversity of enriched TFBS in the 29M group (**Figure 3B**, Table S3). While TFBS of the AP1 family were more abundant in the 24M group, TFBS of the NF-κB and GATA families were more abundant in the 29-month group. Notably, also the Z-scores and significance levels for the STAT and IRF families were considerably higher in the 29M group (**Figures 3A,B** Table S3), which was in line with up-regulation of the *Stat1* gene and several *Irf* genes in the 29M group (Table S1). Together, these results suggest increasing usage of STAT- and IRF-related cellular signaling pathways in the hippocampus with increasing age.

Among down-regulated genes, significant enrichment of TFBS was less pronounced and more heterogeneous. However, binding sites for the E2F family of transcription factors as well as for the specificity protein (SP) and EGR families were identified as common between the two aging groups and enrichment of EGR TFBS among down-regulated genes correlated with down-regulation of *Egr* genes in the 24-month group (Tables S1, S3).

WIDESPREAD AND SPECIFIC ALTERNATIVE EXON USAGE CHANGES IN THE AGING HIPPOCAMPUS

Alternative to the altered expression of genes, co-transcriptional intron excision from the nascent pre-mRNA, known as splicing, is the main mechanism for generation of transcript isoforms and differential exon use in information-dense and complex genomes,

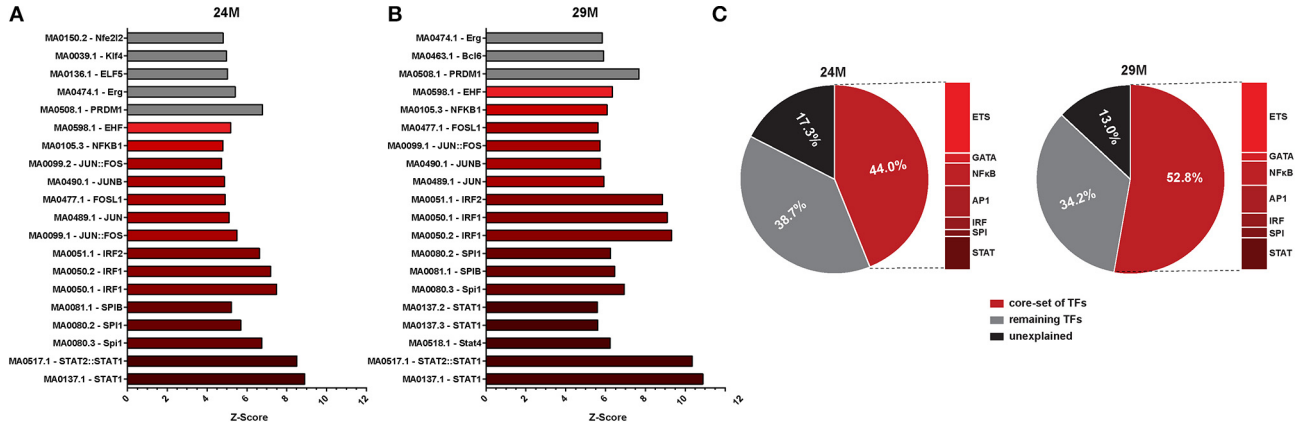

FIGURE 3 | Promoter analysis of aging-regulated genes for overrepresented transcription factor binding sites (TFBS). (A) Z-score (enrichments score) for different position weight matrices of known transcription factors in the set of gene up-regulated in 3 vs. 24-month-old mice. Higher Z-score means higher enrichment. Shown are only the top 20 most significantly enriched matrices. **(B)** The same analysis as described in **(A)** was performed for up-regulated genes in 3 vs. 29-month-old mice. **(C)** Pie charts showing the percentage of differentially expressed genes that can be explained by the selected core set of transcription factors.

thereby adding another important level of gene expression control.

We therefore analyzed our RNA sequencing data with respect to differential exon usage. We found 436 annotated genes with significant changes in exon usage in the 24M group and 80 genes in the 29M group (**Figure 4A**; Table S4). Thus, changes in RNA splicing were, at least in the 24M group, quantitatively comparable to the changes observed in gene expression (**Figure 4A**). However, there was little to no overlap between genes affected by altered splicing and genes that were differentially expressed (**Figure 4B**). In fact, less than 1% of the DEG were also differently spliced in the 24M group, while zero overlap was found in the 29M group. This data indicates that differential gene expression and alternative splicing may affect different signaling pathways. To test this hypothesis, we analyzed the differentially spliced genes for enrichment of functional pathways. Our analysis revealed that there was a significant overrepresentation of genes associated with neuronal function including synaptogenesis, regulation of synaptic transmission, axonogenesis, neuron projection morphogenesis, postsynaptic density and long-term potentiation (**Figure 4C**, Table S5). Of note, none of these pathways was enriched in gene-set of DEG (**Figure 4C**). Vice versa, pathways linked to inflammatory response–which dominated the list of DEG–could not be identified within the group of alternatively spliced genes (**Figure 4C**). These data suggest that inflammatory responses in the aging hippocampus are driven by changes in differential gene expression whereas de-regulation of synaptic plasticity in mainly attributed to differential splicing. One of the genes that was differentially spliced in both 24- and 29-month-old mice when compared to their young counterparts was the *Spectrin* β, *non-erythrocytic 1* gene (*Sptbn1*), that showed a specific upregulation in usage of exon 10 with age (**Figure 4D**). *Sptbn1* is best known for its role in cytoskeleton regulation during neurite outgrowth (Lee et al., 2012), which is in agreement with our previous functional enrichment analysis. The inclusion of exon 10 suggests a shift towards higher expression of *Sptbn1* isoform 2, resulting in a protein that has shorter and distinct N- and C- termini compared to isoform 1 and lacks the pleckstrin homology (PH) domain that is critical for tethering F-actin filaments to the plasma membrane (**Figure S3**).

In addition to these widespread changes, we found differential exon usage in the *amyloid beta (A4) precursor protein* (*App*) and the *beta-site APP cleaving enzyme 1* (*Bace1*) genes (Table S4), both of which are strongly implicated in the etiology of AD. This suggests that also differential-splicing events that occur with aging may be implicated with altered Aβ production in AD.

DIFFERENCES IN RNA-EDITING

A second mechanism to generate alternative transcript isoforms is RNA-editing. Though more subtly changing the coded information as compared to alternative splicing, it also occurs co-transcriptionally and may lead to altered regulation of the transcript or a change in amino acid sequence of the encoded protein. Notably, the mammalian, and especially the human, brain has been identified as the principal site for RNA-editing. Since RNA sequencing is not only able to quantify transcript abundance but also allows visualization of the exact sequence of these transcripts, it is possible to identify changes in transcript sequence from the genomic reference at known positions. We compiled a list of previously described editing sites in the mouse genome from two online databases and two recent publications to yield a non-redundant list of 17831 positions (Table S6).

At 682 of the 17831 known positions, we found RNA-editing in the 3M group (Table S7). Of these 682 positions, we found 14 editing sites in the 24M group and 41 editing sites in 29M that showed significant change in editing frequency, corresponding to 12 and 35 genes, respectively (**Figure 5A**, Table S8). Interestingly, when we compared genes that undergo altered RNA editing in the aging hippocampus to the list of differentially expressed and spliced genes, there was almost no overlap (**Figure 5B**) suggesting that gene expression, splicing and RNA editing control

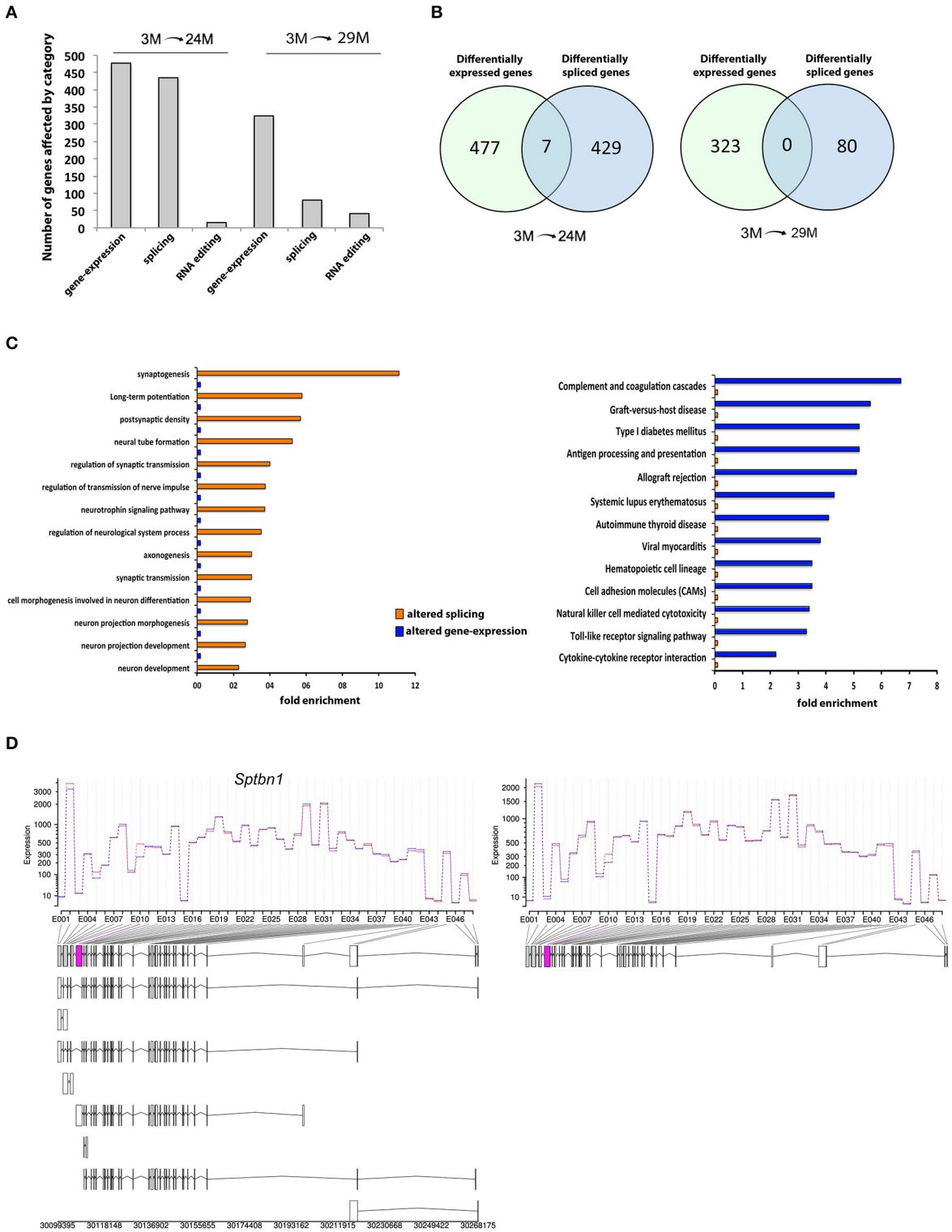

FIGURE 4 | Differential exon usage and alternative splicing changes. **(A)** Number of hippocampal genes affected by differential expression, splicing or RNA-editing during aging. **(B)** Venn-diagrams showing the overlap of genes affected by expression and/or splicing between age groups. Note that there is little to no overlap. **(C)** Cellular pathways affected in 3- vs. 24/29-month-old mice by splicing or expression levels. The data on differential expression is based on all up- or down-regulated genes. **(D)** One of the overlapping genes was *Spectrin* β, *non-erythrocytic 1 (Sptbn1)*, showing higher expression of exon 10 (pink box) in aged mice, suggesting higher abundance of the transcript isoform 2 (see **Figure S2** for details on isoform and domain structure).

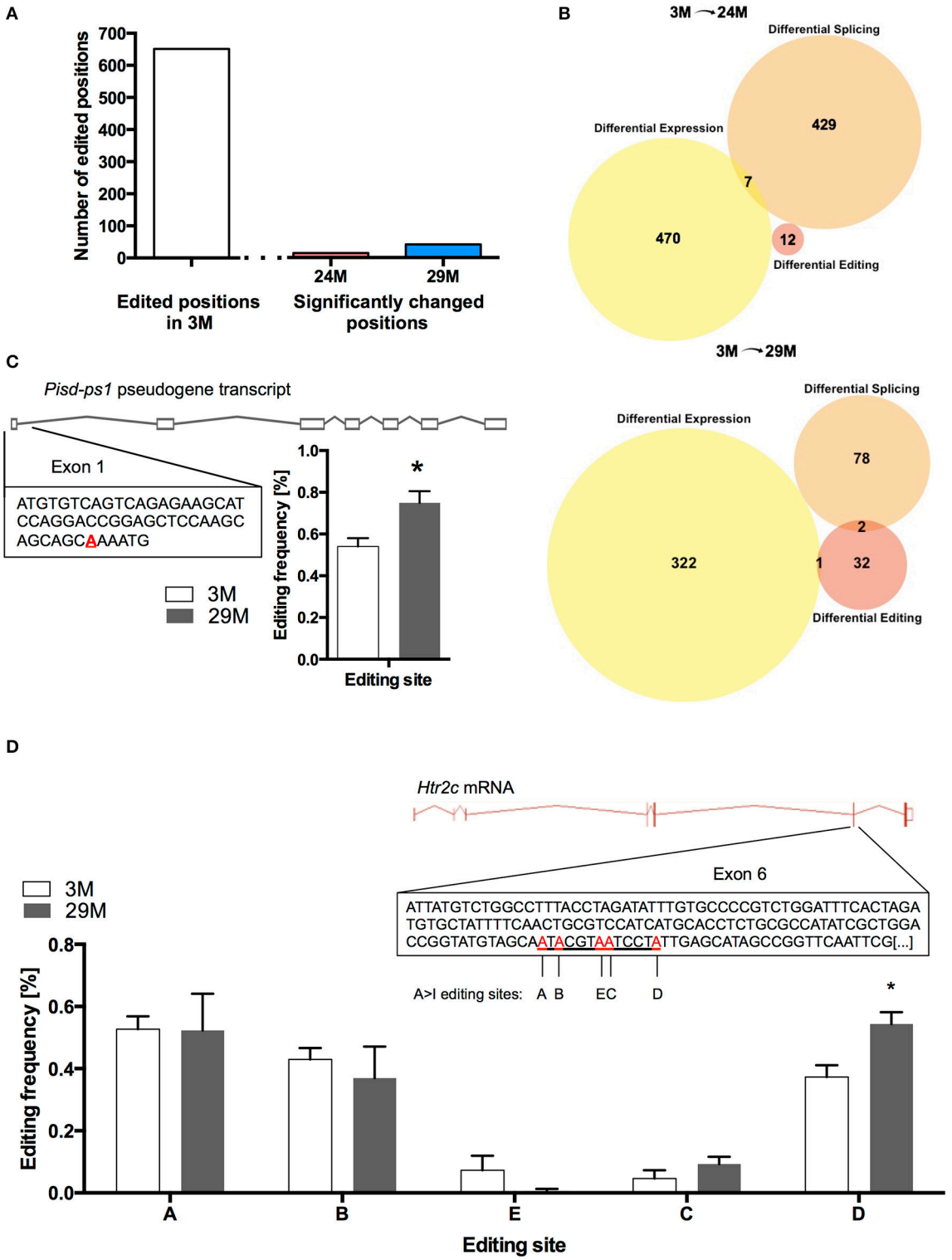

FIGURE 5 | RNA editing in the aging hippocampus. (A) Total number of RNA-editing events detectable in the hippocampus of 3-month-old mice and number of changes occurring during aging. **(B)** Venn diagram showing the overlap of differentially expressed genes, genes affected by splicing and RNA-editing in 24-month-old mice (upper panel) and 29-month-old mice (lower panel). **(C)** Editing of the pseudogene-transcribed ncRNA *Pisd-ps1* within exon 1 was found to be significantly increased in the 29M group **(D)** Within the well-described editing cassette of the *Htr2c* mRNA, editing was significantly upregulated at position D (*$p < 0.05$, $n = 3$ per group). Error bars indicate s.e.m.

distinct cellular pathways in the aging hippocampus. Only one of the genes that was characterized by altered RNA editing in 29-month-old mice was also up-regulated at the gene expression level (**Figure 5C**). This gene (*Pisd-ps1)* codes for a non-coding RNA expressed from a pseudogene with unknown function.

We also found increased editing of the well-established RNA-editing target *serotonin receptor 2c* (*Htr2c*) (**Figure 5D**). It has been shown that increased editing of the *Htr2c* mRNA leads to a decrease in the receptor's signaling fidelity, which is in line with declining function of the serotonergic system with aging (Fidalgo et al., 2013). Moreover, altered RNA-editing of the Htr2c gene was observed in a mouse model for impaired memory function (Stilling et al., 2014).

Another gene that showed higher editing frequency with aging was *Slc7a2*, also known as *Cat2* or *Ctn*. Interestingly, it has been described in the literature that the *Ctn* isoform of this gene undergoes nuclear retention and becomes part of paraspeckles (Bond and Fox, 2009), supporting the previous observation that aging is accompanied by increased nuclear paraspeckle formation.

In summary, our results suggest that hippocampal aging correlates to different degrees with altered gene expression, RNA splicing and RNA editing. Of note all of these processes appear to specifically account for age-associated changes in distinct cellular pathways. Namely, the induction of inflammatory processes is linked to altered gene expression while decline of synaptic plasticity seems to be mainly due to differential splicing.

DISCUSSION

In the present study we performed RNA sequencing in the hippocampus of three different aging groups to determine differential gene expression, exon usage and RNA-editing. Though brain aging has previously been described to go along with transcriptional changes, a detailed, homogenous picture of the transcriptome of the aging mouse hippocampus, especially toward the end of an individual's life span using RNA sequencing, has not been drawn yet.

Several studies have used microarray approaches to investigate age-associated transcriptional profiles of different brain regions in different species and came to heterogeneous results (Finch and Morgan, 1990; Pletcher et al., 2002; Blalock et al., 2003, 2010; Lu et al., 2004; Verbitsky et al., 2004; Xu et al., 2007; Zahn et al., 2007; Loerch et al., 2008; Pawlowski et al., 2009; Bishop et al., 2010), which may in part reflect differences in the technology available and also limited information on the mouse genome and transcriptome at the time the corresponding studies were conducted.

When we analyzed differential gene expression among the different age groups, the most striking effect was the up-regulation of genes linked to inflammatory processes with the complement system being the top ranking pathway identified by gene-set enrichment, as well as pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Increased neuroinflammation is likely the result of multiple up-stream factors such as impaired blood-brain barrier function (Lynch and Johnson, 2012; Erickson and Banks, 2013) leading to increased invasion of peripheral bacteria and viruses as well as cells of the peripheral immune system, which will cause a defense response against these invaders (Blau et al., 2012; Marques et al., 2013). Moreover, increased clearance of damaged or misfolded proteins and other cellular debris becomes necessary with increasing age and these aggregates will activate the immune system (predominantly microglia) (Fonseca et al., 2004; Czirr and Wyss-Coray, 2012).

Of note, up-regulation of neuroinflammatory genes with aging clearly delivers an explanation why neurodegenerative diseases such as AD become increasingly abundant with advanced age. Based on our findings, we derive the hypothesis that disease-related mutations become only detrimental when the respective genes become expressed or activated. As such, aging is the strongest risk factor for AD (Lopez, 2011). The reason why late-onset AD (LOAD) is associated with increasing age is not yet fully understood. However, accumulating evidence and a plethora of genome-wide association studies (GWAS) link LOAD to a fast growing number of genetic risk factors and it seems feasible that these genetic risk factors result in LOAD due to changes at the transcriptome and proteome level. Indeed, in our hippocampal aging study in mice, we found up-regulation of several homologs, interaction partners or other closely related genes to almost all genes that appear to be the top 10 single genetic risk factors for LOAD as designated by the AlzGene database (Bertram et al., 2007). Of particular interest in light of our data is the gene coding for complement receptor 1 (CR1), since this receptor binds processed C4 and C3 complement proteins including the products of the *C4b* gene, which we found to be most prominently up-regulated in the aging hippocampus. Single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) in the human CR1 gene were found to be highly associated with late onset AD in a number of studies and across ethnic groups (Bertram et al., 2007; Lambert et al., 2009; Biffi et al., 2010, 2012; Carrasquillo et al., 2010; Chibnik et al., 2011; Brouwers et al., 2012; Crehan et al., 2012; Hazrati et al., 2012; Keenan et al., 2012; Ma et al., 2014). This makes it very tempting to draw the conclusion that increased expression of C3 and C4 with aging will result in aberrant regulation of the complement cascade, which in turn lead to neurodegeneration in carriers of the "wrong" CR1 allele. Aberrant regulation could lead to neurodegeneration in two different, non-mutual exclusive ways. On the one hand, differential CR1 regulatory activity could lead to less effective Aβ aggregate clearance (Fonseca et al., 2004). On the other hand, de-regulated activation of the complement system could result in collateral damage by overactive inflammation (reviewed in Czirr and Wyss-Coray, 2012). Along this line, CR1 inhibition was shown to prevent microglia activation (Crehan et al., 2013). Of note, mice do not express CR1 but mice that lack the murine ortholog *Cr1-related protein Y* (*Crry*) show reduced inflammatory responses and attenuated increases in AD-related disease progress biomarkers (Killick et al., 2013), further suggesting that activation of the complement system is a key feature of the aging hippocampus that also plays a role in AD pathogenesis.

The exact mechanism of altered CR1 regulatory function in this complex network is still to be elucidated but a beneficial effect of complement inhibition in general has been demonstrated (Rancan et al., 2003; Fonseca et al., 2004; Leinhase et al.,

2006; Kulkarni et al., 2008, 2011; Pillay et al., 2008), yet this data remains controversial (Wyss-Coray et al., 2002; Loeffler, 2004; Maier et al., 2008). Nevertheless, our data support the view that targeting the complement system and other inflammatory pathways poses an intriguing possibility for the treatment of aging-associated diseases and cognitive decline, which has recently been demonstrated for inhibition of NF-κB signaling in a mouse model of AD (Liu et al., 2014). Likewise, physical activity in rodents and humans has been shown to improve cognitive abilities during aging (van Praag et al., 1999; Praag et al., 2005; Erickson et al., 2011). Remarkably, a recent study could show in aged mice that voluntary wheel running leads to a reduction in C4b expression in the hippocampus (Kohman et al., 2011), demonstrating a clear correlation between C4 levels and cognitive abilities, which is further supported by the finding that C4 inhibition by external application of a vaccinia virus complement control protein has beneficial effects on memory performance in mouse models of AD (Kulkarni et al., 2008, 2011).

While it is not entirely clear if the age-associated induction of immune genes is cause or consequence of altered glia cell function that arise on the background of altered activity and/or increased cell number (Lolova, 1991; Amenta et al., 1998; Long et al., 1999; Blalock et al., 2003; Takahashi et al., 2006; Hayakawa et al., 2007) astrocytes and especially microglia are clearly good candidates for a potent source of immune system molecules (Hosokawa et al., 2003). We did not observe significant changes in the number of neurons, astrocytes or microglia in our study. In line with this, the corresponding marker genes for neurons and microglia were not altered in our gene-expression analysis. The astrocyte marker gene gfap was however increased during aging, indicating that the inflammatory response in our study in mainly linked to a change in the active state of glia cells. However, also neurons are known to secrete complement proteins (Shen et al., 1997; Terai et al., 1997; Hosokawa et al., 2003) and future studies should therefore also focus on untangling which cell types are the main contributors to the observed neuroinflammatory program. In any case *C4b* is an interesting gene, since its expression strongly correlated with age and it was in fact one of the most significant changes that have been reported by others and within this study. It was already found to be up-regulated as one of the very few DEGs in the comparison of hippocampal expression in 3-month-old mice with 16-month-old mice (3-fold up- regulation) (Peleg et al., 2010). Interestingly, out of the 12 up-regulated genes found in this independent study, 6 genes were also found up-regulated in the present study. Along with *C4b* and *C3*—another complement component frequently found up-regulated—these were 1700112E06Rik, BC061194, Cox8b and Sult1c2. Hence, these 6 genes obviously belong to a set of genes that are highly associated with murine hippocampal aging. In addition, other studies have observed complement-gene up-regulation in studies of aging in the hippocampus as well as in the prefrontal cortex (Verbitsky et al., 2004; Reichwald et al., 2009; Bordner et al., 2011; Stephan et al., 2013).

To our knowledge, the present study is the first to also specifically report the role of long-non-coding RNAs in the aging hippocampus. We found several long-non-coding RNAs to be differentially expressed. Little is known so far on the role of these RNAs, but the up-regulation of *Neat1* and the associated ncRNA *Malat1* was interesting since *Malat1* is highly abundant in neurons and has been associated with the regulation of gene expression and splicing, but was also found to affect the expression of genes linked to synaptic function (Bernard et al., 2010). Of note, a previous study reported that *Malat1* is increased in the hippocampus of alcoholics (Kryger et al., 2012), a condition clearly linked to cognitive impairment. Another interesting finding was the up-regulation of *Pisd-ps1*. Though the *Pisd-ps1* ncRNA has no annotated function so far, it has been described to be up-regulated with aging (Bordner et al., 2011; Sousa-Victor et al., 2014). *Pisd-ps1* also harbors a known RNA-editing site and was one of the few transcripts that show a higher editing frequency in 29-month-old mice. Thus, this ncRNA might be implicated in brain aging and warrants further research on its regulatory functions.

To obtain more insight on the mechanisms that drive age-associated changes in hippocampal gene expression, we analyzed the promoters of the differentially regulated transcripts. We found high enrichment of TFBS involved in pro-inflammatory signaling cascades. This trend was even more pronounced in 29-month-old mice, which supports our interpretation of increasing neuroinflammation with advancing age as part of the aging program. One of the key factors contributing to the neuroinflammatory gene expression response in the aging hippocampus was STAT1. Interestingly, mice that lack STAT1 show enhanced memory performance and are resistant to memory impairment induced by Aß peptides injected into the hippocampus (Hsu et al., 2014). Thus, activation of STAT1 might be a key step in age-associated memory impairment.

RNA sequencing also allows the analysis of differential splicing events and previous data suggest that differential exon usage undergoes substantial changes during brain development (Tollervey et al., 2011; Mazin et al., 2013). Differential splicing has also been observed in postmortem brain tissue from patients that suffered from sporadic AD (Twine et al., 2011; Mills et al., 2013) and in mouse models for amyloid deposition (Kim et al., 2012). This data is in line with increased protein levels of spliceosome components in AD patients (Bai et al., 2013). However, RNA sequencing has not been used to analyze changes in the aging hippocampus as described in this study. The first interesting observation is that massive changes in RNA splicing occur when we compared the hippocampus of 3-month-old mice to the 24- and 29-month groups. Another highly interesting observation was that there was little to no overlap among the genes differentially expressed and the genes that were differentially spliced during aging. The most striking finding is, however, that genes affected by age-associated changes in splicing were associated with neuronal and synaptic function, including neurite outgrowth and LTP. This is in line with the well-described decline in synaptic plasticity with aging (Blau et al., 2012). A number of interesting candidate genes were identified and await further analysis. For example, we identified *Spectrin β, non-erythrocytic 1* (*Sptbn1*) to be differentially spliced during aging. *Sptbn1* is involved in tethering the actin cytoskeleton to the plasma membrane, which is necessary for neurite outgrowth and possibly spine morphology and motility (Lee et al., 2012). Interestingly, introduction of the

observed exon change in this gene may indicate a shift toward an isoform that cannot perform this function anymore, since it lacks the essential membrane-tethering PH protein domain. Together, these results confirm findings in the literature and make differential exon usage analysis a plausible tool for the study of age-related changes in the transcriptome. The fact that the age-associated inflammatory response is driven by changes in gene expression that could be linked to a number of key transcription factors while genes linked to synaptic plasticity are mainly affected by alternative splicing also suggests that functionally distinct gene expression programs in the adult brain are regulated by specific mechanisms.

In conclusion, our data deciphers at an unprecedented depth the hippocampal transcriptional program linked to aging. While changes in the absolute levels of RNA transcripts are linked to an inflammatory response and especially activation of the complement system, changes in alterative splicing affect genes linked to synaptic plasticity. At the same time RNA editing does not appear to play a major, genome-wide role in the aging hippocampus. This data provides important novel information, and multiple starting points for further analysis of potential therapeutic interventions and strongly suggests a causal relationship between aging-dependent changes in gene expression and the late onset of neurodegenerative diseases, most prominently AD.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00373/abstract

Figure S1 | Immunohistochemical analysis of hippocampal cell number. **(A)** Representative images showing immunostaining for the Neuronal maker protein Neuronal N (NeuN), the Microglia marker Ionized calcium binding adaptor molecule 1 (IBA1) and the astrocyte marker *Glial fibrillary acidic protein (*GFAP) in 3 and 29-month old mice. **(B)** Quantification of A. CA1; hippocampal subregion CA1, cc; corpus calosum. Scale bar: 100 μm.

Figure S2 | Functionally enriched pathway in 3 vs. 24 and 29 month old mice identified on the basis of gene array data. Left panel: Enriched pathways identified on the basis of differentially expressed genes comparing hippocampal RNA from 3- vs. 24-month-old mice. **Right panel**: Enriched pathways identified on the basis of differentially expressed genes comparing hippocampal RNA from 3 vs. 29-month-old mice.

Figure S3 | Genetic architecture and domain structure of Sptbn1. The figure was downloaded from the ENSEMBLE database (version e76). It shows the genomic locus of Sptbn1 and its annotated transcribed isoforms including exon structure and encoded protein domains. Isoform 2 is marked in yellow. This was the isoform that is likely underlying the switch in exon usage detected in RNA-seq data in 24M and 29M groups.

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Transcription factors Foxa1 and Foxa2 are required for adult dopamine neurons maintenance

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The proteins Foxa1 and Foxa2 belong to the forkhead family of transcription factors and are involved in the development of several tissues, including liver, pancreas, lung, prostate, and the neural system. Both Foxa1 and Foxa2 are also crucial for the specification and differentiation of dopamine (DA) neurons during embryonic development, while about 30% of mice with an embryonic deletion of a single allele of the *Foxa2* gene exhibit an age-related asymmetric loss of DA neurons and develop locomotor symptoms resembling Parkinson's disease (PD). Notably, both Foxa1 and Foxa2 factors continue to be expressed in the adult dopamine system. To directly assess their functions selectively in adult DA neurons, we induced genetic deletions of Foxa1/2 transcription factors in mice using a tamoxifen inducible tissue-specific CreERT2 recombinase expressed under control of the dopamine transporter (DAT) promoter (DATCreERT2). The conditional DA neurons-specific ablation of both genes, but not of *Foxa2* alone, in early adulthood, caused a decline of striatal dopamine and its metabolites, along with locomotor deficits. At early pre-symptomatic stages, we observed a decline in aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1) protein expression in DA neurons. Further analyses revealed a decline of aromatic amino acid decarboxylase (AADC) and a complete loss of DAT expression in these neurons. These molecular changes ultimately led to a reduction of DA neuron numbers in the substantia nigra pars compacta (SNpc) of aged *cFoxa1/2*$^{-/-}$ mice, resembling the progressive course of PD in humans. Altogether, in this study, we address the molecular, cellular, and functional role of both Foxa1 and Foxa2 factors in the maintenance of the adult dopamine system which may help to find better approaches for PD treatment.

Keywords: Foxa1, Foxa2, dopamine, dopaminergic neurons, transgenic mice, neurodegeneration, substantia nigra, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is one of the most prevalent age-related movement disorders occurring in about 1% of the population above the age of 60 (Abou-Sleiman et al., 2006; Ferri et al., 2007). PD affects multiple neuronal systems (Braak et al., 2003), however, the major motor symptoms are caused by the degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) (Moore et al., 2005). Current treatment strategies are providing only symptomatic relief to the patients and are not able to prevent the progression of neurodegeneration. Therefore, the identification and characterization of the mechanisms involved in the maintenance of adult DA neurons is critically important for the advance of novel therapies for PD (Meissner et al., 2011).

The development of midbrain DA neurons is a highly orchestrated process involving coordinated action of multiple signaling molecules and transcription factors, such as Shh, Wnt, Otx2, Pitx3, Nurr1 (Nr4a2), Foxa1, and Foxa2 (Perlmann and Wallen-Mackenzie, 2004; Ferri et al., 2007; Smidt and Burbach, 2007; Omodei et al., 2008; Jacobs et al., 2009; Joksimovic et al., 2009; Mesman et al., 2014). Several of these factors continue to be expressed in post-mitotic and also in adult DA neurons contributing to the functional maintenance of this neuronal population. For example, Otx2 expressed in the ventral tegmental area (VTA) DA neurons controls the identity of this neuronal subtype and confers its resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Di Salvio et al., 2010; Di Giovannantonio et al., 2013). A selective ablation of orphan nuclear receptor Nurr1 in adult DA neurons using a tamoxifen (TAM)-inducible Cre/LoxP recombination system leads to the fiber pathology of this neuronal population and loss of

Abbreviations: AADC, aromatic amino acid decarboxylase; Aldh1a1, aldehyde dehydrogenase family 1, subfamily A1; ChIP, chromatin immunoprecipitation; DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPAL, 3,4-dihydroxyphenylacetaldehyde; En1, engrailed 1; HPLC-ED, high-performance liquid chromatography with electrochemical detection; HVA, homovanillic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; post-TAM, after tamoxifen treatment; rpm, rounds per minute; s.e.m., standard error of means; SNpc, substantia nigra pars compacta; TAM, tamoxifen; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

striatal dopamine, recapitulating early stages of PD development (Kadkhodaei et al., 2009). It has recently been shown that Nurr1 regulates the expression of nuclear-encoded mitochondrial genes and is involved in sustaining high respiratory function in adult DA neurons (Kadkhodaei et al., 2013). Transcription factors Foxa1 and Foxa2 play crucial roles not only during the early development and specification (Ferri et al., 2007; Kittappa et al., 2007; Moore et al., 2005), but also in the process of maturation of DA neurons, as has been demonstrated by deleting these factors in post-mitotic DA neurons during late embryonic development (Stott et al., 2013). Interestingly, an asymmetrical degeneration of SNpc DA neurons has been observed in about 30% of aged mice bearing a global heterozygous deletion of *Foxa2* gene allele (Kittappa et al., 2007).

Proteins belonging to Foxa family (Foxa1, Foxa2, and Foxa3) share very high sequence homology within the DNA binding domain, whereas outside of this region they are less similar, and Foxa3 being shorter and more divergent from Foxa1/2 (Lai et al., 1991; Friedman and Kaestner, 2006; Kaestner, 2010). The loss-of-function studies demonstrate that Foxa1 and Foxa2 have partially overlapping functions during embryonic development of DA neurons; both Foxa1 and Foxa2 factors are required for the expression of Lmx1a, Lmx1b (Lin et al., 2009), Nurr1 and engrailed 1 (En1) (Ferri et al., 2007) in immature DA neurons and for the expression of AADC and TH in early post-mitotic DA neurons (Ferri et al., 2007; Stott et al., 2013). Consequently, a combined deletion of Foxa1 and Foxa2 in embryonic DA neurons results in reduced binding of Nurr1 to *Th* and *Aadc* gene promoters leading to a significant loss of TH and AADC expression in the SNpc of embryos and adult mice (Stott et al., 2013).

The expression of both Foxa1 and Foxa2 continues into adulthood (Kittappa et al., 2007; Stott et al., 2013), suggesting that, in addition to their essential role in the development, specification and maturation, both proteins are also involved in the physiological functions of adult DA neurons.

The deregulation of Foxa1/2 may also contribute to demise of DA neurons during PD progression in humans. Indeed, by searching the online databases, such as the National Center for Adult Stem Cell Research Parkinson's review database (Sutherland et al., 2009) and ParkDB (Taccioli et al., 2011) that contain manually curated, re-analyzed and annotated microarray datasets from PD patients and PD models, we found several datasets showing the down-regulation of Foxa1 and Foxa2 expression in the SNpc of PD patients (Hauser et al., 2005; Zhang et al., 2005; Moran et al., 2006; Lesnick et al., 2007).

However, no previous studies have directly addressed the role of Foxa1/2 factors in adult DA neurons. Here we used a tissue-specific TAM-inducible Cre recombination to ablate both the *Foxa1* and *Foxa2* genes selectively in adult DA neurons. This deletion resulted in DA neurons losing their dopaminergic phenotype, which was reflected by the decline in expression of Aldh1a1, AADC, DAT and TH, as well as reduced striatal dopamine leading to the development of locomotor abnormalities, and, ultimately, loss of the neurons in aged *cFoxa1/2*$^{-/-}$ double knockout mice.

MATERIALS AND METHODS

ANIMAL EXPERIMENTS

Mice were maintained in the C57Bl/6N genetic background on a 12 h light-dark cycle with free access to water and food. The *Foxa2*$^{fl/fl}$*DATCreERT2*, *Foxa1*$^{fl/wt}$*Foxa2*$^{fl/fl}$*DATCreERT2*, *Foxa1*$^{fl/fl}$*Foxa2*$^{fl/wt}$*DATCreERT2,* and *Foxa1*$^{fl/fl}$*Foxa2*$^{fl/fl}$*DATCreERT2* mouse lines (referred hereafter as *cFoxa2*$^{-/-}$, *cFoxa1*$^{+/-}$*/2*$^{-/-}$, *cFoxa1*$^{-/-}$*/2*$^{+/-}$, and *cFoxa1/2*$^{-/-}$, respectively) were generated by mating *Foxa1*$^{fl/fl}$ (Gao et al., 2008) and *Foxa2*$^{fl/fl}$ mice (Sund et al., 2000) with *DATCreERT2* (Engblom et al., 2008) mice. Inducible Cre recombinase was activated in 8–10 week-old mice by intraperitonial injections of 1 mg tamoxifen (TAM, Sigma-Aldrich) diluted in sunflower oil twice daily for five consecutive days (Domanskyi et al., 2011; Rieker et al., 2011; Vinnikov et al., 2014). Littermates harboring only floxed alleles were used as controls. All experimental procedures were performed with the approval by the institutional Committee on Ethics of Animal Experimentation and carried out in accordance with the local and European legislation on the protection of animals used for scientific purposes.

HISTOLOGICAL ANALYSES

Mice at the indicated time points after TAM injections (post-TAM) were perfused with 4% paraformaldehyde (PFA); the brains were dissected and fixed overnight in 4% PFA and processed for either paraffin or vibratome sections. Upon dissection, no differences in morphology, weight, or size of the brains were observed in *cFoxa1/2* animals compared to control littermates. Immunohistochemical and immunofluorescent stainings were performed as previously described (Domanskyi et al., 2011; Rieker et al., 2011) using the following antibodies: anti-tyrosine hydroxylase (TH) (1:1000, Millipore #AB1542), anti-aromatic amino acid decarboxylase (AADC) (1:1000, Millipore #AB1569), anti-DAT (1:500, Millipore #MAB369), anti-aldehyde dehydrogenase 1 family, member A1 (Aldh1a1) (1:100, Abcam #ab52492). Fluorescent signals in the brain samples were visualized directly with confocal system TSC SP5 (Leica) or LSM780 (Zeiss).

Quantification of the Aldh1a1- and TH-positive cells on immunostained brain sections was performed either by blinded experimenters or by using MCID Image Analysis software (InterFocus Imaging) as previously described (Isermann et al., 2007; Domanskyi et al., 2011). The SNpc and the VTA were identified according to the anatomical landmarks (Zaborszky and Vadasz, 2001) and the neurons were counted for each mouse from at least five sections covering the ventral midbrain (Domanskyi et al., 2011). For immunofluorescently stained samples, number of TH-positive neurons in the SNpc and VTA in single confocal plane images was determined by blinded investigators, followed by a quantification of the percentage of Aldh1a1-positive neurons within the TH-positive population on the same sections. Quantification of immunohistochemically stained TH-positive neurons in aged *cFoxa1/2*$^{-/-}$ mice was performed in the same way, except that the region of interest was limited by the SNpc and that counting was performed automatically by MCID Image Analysis software.

QUANTITATIVE RT-PCR

Total RNA isolated from ventral midbrain samples served as a template for DNA synthesis using Super-Script III first-strand synthesis kit (Invitrogen). For genomic DNA contamination control, samples with no added reverse transcriptase enzyme were included. Quantitative PCR was performed with a CFX96 Real-Time System (Bio-Rad) using TagMan Gene Expression Assays (Life Technologies) according to the manufacturer's instructions. The mRNA levels of *Hprt1* were measured to control for the equal amount of input cDNA. The following probes were used for detection of *En1*, *Foxa1*, *Foxa2*, *Hprt1*, *Lmx1b*, *Nr4a2*, *Pitx3*, *Th*, and *Ucp2*: Mm00438709_m1, Mm00484713_m1, Mm00839704_mH, Mm01545399_m1, Mm00440209_m1, Mm00443056_m1, Mm01194166_g1, Mm00447557_m1, and Mm00495907_g1, respectively.

BEHAVIORAL ASSAYS

The accelerating and constant speed rotarod assays were performed as previously described (Domanskyi et al., 2011; Rieker et al., 2011). Briefly, for the constant speed rotarod assay mice were initially trained to attain stable baseline levels of performance staying on the rod rotating at 15 rpm for 60 s. After that, the mice received several trials at 25 and 35 rpm rotation speed with 60 s maximum trial length and 5 min intervals between individual trials. Two maximal values per speed per day were used to calculate the average which was used for subsequent statistical analyses. This setting successfully corrects for effects unrelated to motor/balance performance such as re-learning, fatigue, tendencies to learned helplessness or over-performance/hyperactivity.

For the open field test, mice were placed individually into the open arena and monitored for 5 min by a video camera. The resulting data were analyzed using the image processing systems EthoVision 3.0 (Noldus Information Technology) (Chourbaji et al., 2008) and Any-maze 4.82 (Stoelting Co.). For each sample, the systems recorded position, object area and the status of defined events.

MEASUREMENTS OF STRIATAL DOPAMINE AND ITS METABOLITES

After decapitation, the striata were rapidly dissected on ice, weighed, and frozen on dry ice. Measurements of striatal dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were performed by reverse-phase HPLC with electrochemical detection method (HPLC-ED) as previously described (Otto and Unsicker, 1990; Enkel et al., 2014).

STATISTICAL ANALYSES

Statistical significance was calculated by Student's two-tailed unpaired *t*-test or Two-Way ANOVA followed by Bonferroni *post-hoc* test using GraphPad Prism software (GraphPad Scientific, USA). p values less than 0.05 were considered significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) with respect to control groups. Data in text and figures are represented as means ± s.e.m.

RESULTS

Foxa2 DELETION IN ADULT DOPAMINE NEURONS DOES NOT LEAD TO NEURODEGENERATION

To directly investigate the role of Foxa2 in maintenance of adult DA neurons, we crossed *Foxa2*$^{fl/fl}$ mice (Sund et al., 2000) with the *DATCreERT2* line. These transgenic mice provide a tight spatial and temporal control of recombination upon treatment with estrogen receptor antagonist TAM (Engblom et al., 2008). In *cFoxa2*$^{-/-}$ mice harboring deletions of both *Foxa2* alleles in adult DA neurons, no sign of neurodegeneration phenotype was observed at any time point tested (**Figures 1A–C**). Both striatal dopamine content (**Figure 1A**) and motor functions, measured in the accelerated rotarod assay (**Figure 1B**) or in the more sensitive (Monville et al., 2006; Brooks and Dunnett, 2009) constant speed rotarod assay (**Figure 1C**), were at normal levels in *cFoxa2*$^{-/-}$ mice up to 58 weeks after recombination onset by TAM treatment (post-TAM) (**Figures 1A,C**).

The previous works delineating the role of Foxa factors in the liver, pancreas and developing DA neurons (Ferri et al., 2007; Kaestner, 2010; Stott et al., 2013) have detected striking similarities in binding motifs, regulated genes and global functions of Foxa1 and Foxa2 factors, indicating that Foxa1 could compensate for the function of Foxa2 in adult DA neurons of *cFoxa2*$^{-/-}$ mice. Indeed, despite significant down-regulation of *Foxa2* mRNA levels in the ventral midbrain of *cFoxa2*$^{-/-}$ mice, the levels of *Foxa1* in these animals were up-regulated (**Figure 1D**). These results imply the existence of a functional overlap between Foxa1 and Foxa2 in adult DA neurons.

Foxa1/2 DELETION IN ADULT DOPAMINE NEURONS CAUSES LOCOMOTOR DEFICITS

In order to conditionally ablate both Foxa1 and Foxa2 factors in adult DA neurons, we created *cFoxa1/2*$^{-/-}$ mice by crossing *Foxa1*$^{fl/fl}$ (Gao et al., 2008) and *Foxa2*$^{fl/fl}$ (Sund et al., 2000) with *DATCreERT2* (Engblom et al., 2008) animals. The quantitative PCR (qPCR) analysis showed that both *Foxa1* and *Foxa2* mRNA expression in the ventral midbrain decreased in *cFoxa1/2*$^{-/-}$ mice (**Table 1**), confirming the successful deletion of Foxa1 and Foxa2 in these animals. Starting from the 18th week post-TAM, *cFoxa1/2*$^{-/-}$ mice developed locomotor impairments, as determined by the constant speed rotarod assay (**Figures 2A,B**). Importantly, when tested at 21 weeks post-TAM, the mice exhibited a decreased activity in the open field test paralleled by an increased circling behavior (**Figures 2C–E**). The asymmetric circling behavior (**Figure 2D**) in *cFoxa1/2*$^{-/-}$ mice may partly be due to an increased difference in dopamine levels in left and right brain hemispheres (Supplementary Figure 1). Moreover, at 24 weeks post-TAM we observed a significant reduction of the striatal content of dopamine and its metabolites, DOPAC and HVA (**Table 2**) in *cFoxa1/2*$^{-/-}$ mice.

LOSS OF Aldh1a1 EXPRESSION IN THE VENTRAL MIDBRAIN PRECEDES THE ONSET OF LOCOMOTOR IMPAIRMENTS IN *cFoxa1/2*$^{-/-}$ MICE

At the same time point, 24 weeks post-TAM, the numbers of TH-positive neurons both in the SNpc and in the VTA of *cFoxa1/2*$^{-/-}$ mice were normal (**Figures 3A,B**). However, already at 11 weeks post-TAM, we observed a dramatic decrease of the numbers of Aldh1a1-positive DA neurons (**Figure 3C**) that was even more evident at 24 weeks and was stronger in the SNpc than in the VTA (**Figures 3D–G**). Aldh1a1 is neuroprotective in DA neurons (Anderson et al., 2011; Wey et al., 2012; Liu et al., 2014) and, therefore, decline in its expression may render adult

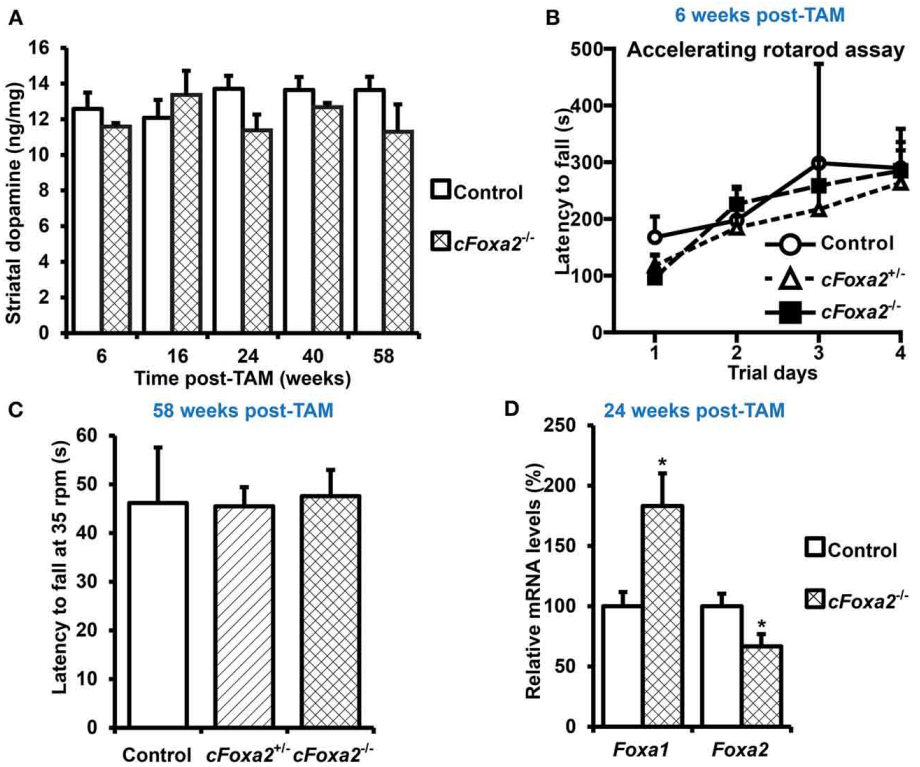

FIGURE 1 | Absence of changes in locomotor activity and striatal dopamine content upon conditional ablation of *Foxa2* gene in adult dopamine neurons. (A) Striatal dopamine content in *cFoxa2*$^{-/-}$ mice at indicated time points after TAM treatment (post-TAM). $n = 4, 3, 3, 7, 14, 10, 6, 6, 5, 3$ for the groups from left to the right on the graph. **(B)** Latency to fall in the accelerating rotarod assay in control, *cFoxa2*$^{+/-}$, and *cFoxa2*$^{-/-}$ mice 6 weeks post-TAM ($n = 4, 3, 3$, respectively). **(C)** Latency to fall in the constant speed (35 rpm) rotarod assay in aged control, *cFoxa2*$^{+/-}$, and *cFoxa2*$^{-/-}$ mice 58 weeks post-TAM ($n = 3, 15, 9$, respectively). **(D)** Reciprocal change of *Foxa1* and *Foxa2* mRNA levels in the ventral midbrain of control ($n = 10$) and *cFoxa2*$^{-/-}$ ($n = 5$) mice 24 weeks post-TAM. $^*p < 0.05$ in comparison to control, as determined by Student's unpaired *t*-test.

Table 1 | Relative levels of *Foxa1* and *Foxa2* mRNA in the ventral midbrain of mice 24 weeks after conditional ablation of these factors in adult dopamine neurons.

Line name	Genotype	*n*	*Foxa1* (%)	*Foxa2* (%)
Control	*Foxa1*$^{fl/fl}$ *Foxa2*$^{fl/fl}$	10	100 ± 11.64	100 ± 10.36
cFoxa2$^{-/-}$	*Foxa2*$^{fl/fl}$ *DATCreERT2*	5	183.24 ± 27.05*	66.56 ± 10.21*
cFoxa1$^{+/-}$*/2*$^{-/-}$	*Foxa1*$^{fl/wt}$ *Foxa2*$^{fl/fl}$ *DATCreERT2*	10	122.32 ± 19.94	61.1 ± 8.24**
cFoxa1/2$^{-/-}$	*Foxa1*$^{fl/fl}$ *Foxa2*$^{fl/fl}$ *DATCreERT2*	5	54.93 ± 13.04*	68.25 ± 7.35*

The data, presented as mean values ± s.e.m., are normalized to the Hprt1 levels and expressed as percentage relative to control; $^*p < 0.05$, $^{**}p < 0.01$ in comparison to control, as determined by Student's unpaired t-test.

DA neurons toward degeneration. Accordingly, even though the numbers of TH-positive neurons at 24 weeks post-TAM *cFoxa1/2*$^{-/-}$ mice did not change, these neurons exhibited a decrease in the ventral midbrain expression of the key proteins in the DA metabolism: TH, AADC, and especially DAT, as detected in immunostaining experiments (**Figures 4A–C**). Furthermore, at 24 weeks post-TAM, when we analyzed the expression of several transcripts important for development and functionality of DA neurons (Smidt and Burbach, 2007), we observed a tendency towards decrease of *En1* and *Th* (Supplementary Figure 2). This data suggests that Foxa1/2 proteins are essential in maintaining the expression of crucial factors in adult DA neurons. Indeed, Foxa2 has previously been shown to regulate En1 expression (Ferri et al., 2007). Moreover, it can directly bind to *Th* gene promoter and cooperate with Nurr1 in regulating the expression of TH and AADC in the ventral midbrain (Lee et al., 2010; Stott et al., 2013). Interestingly, the mRNA level of *Ucp2* encoding a mitochondrial uncoupling protein was up-regulated in *cFoxa1/2*$^{-/-}$ mice (Supplementary Figure 2). Overexpression of Ucp2 has been shown to decrease mitochondrial production of reactive oxygen species (Andrews et al., 2005) and protect DA neurons from MPTP (Conti et al., 2005), and *Ucp2* mRNA

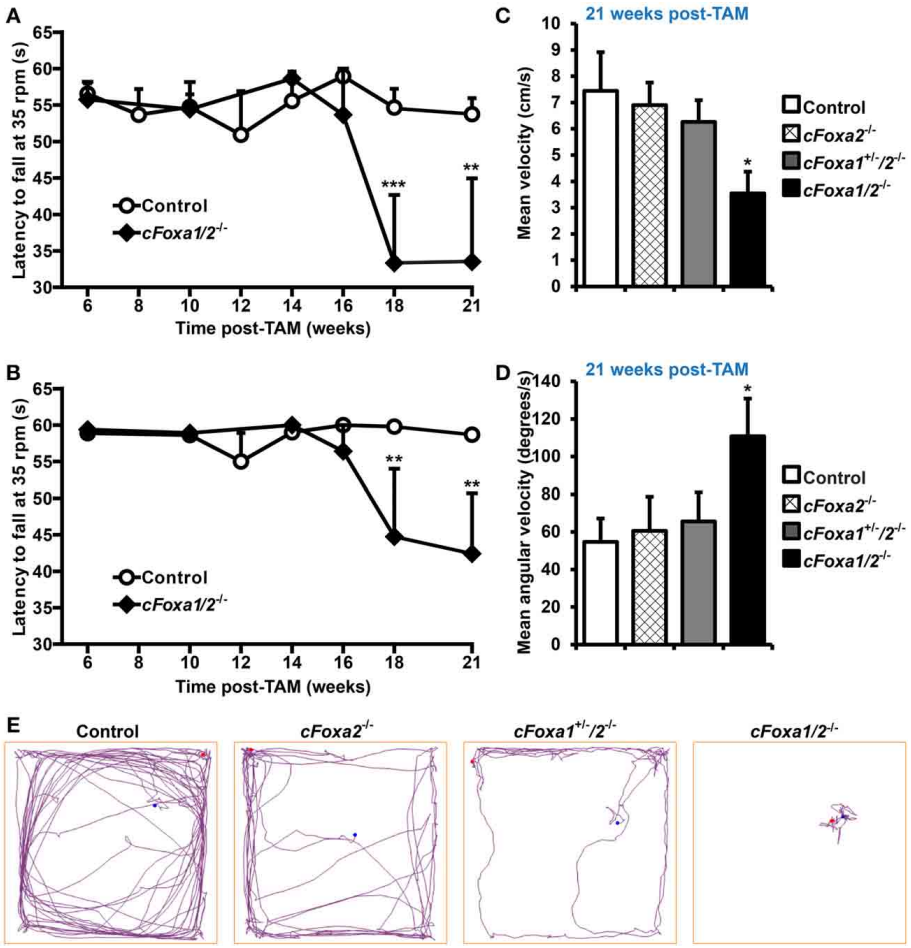

FIGURE 2 | Locomotor impairments in *cFoxa1/2*$^{-/-}$ double mutant mice. (**A,B**) Latency to fall in the constant speed rotarod assay at 35 rpm **(A)** and 25 rpm **(B)** in control and *cFoxa1/2*$^{-/-}$ mice ($n = 13$ and 5, respectively) at indicated time points post-TAM **(C–E)** The quantification of mean velocity **(C)** and mean angular velocity **(D)**, and representative running tracks **(E)** of control, *cFoxa2*$^{-/-}$, *cFoxa1*$^{+/-}$*/2*$^{-/-}$, and *cFoxa1/2*$^{-/-}$ mice ($n = 11$, 6, 10, 5, respectively) in the open field assay performed 21 weeks post-TAM. Length of the open field box side, 60 cm. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison to control, as determined by Student's unpaired *t*-test or Two-Way ANOVA followed by Bonferroni *post-hoc* test. Blue and red dots represent, respectively, the initial and final position of a mouse in the open field assay.

up-regulation may indicate the existence of a compensatory mechanism which might be activated to protect mitochondrial function in *cFoxa1/2*$^{-/-}$ mice.

Ultimately, we observed a down-regulation of striatal dopamine (**Figure 5A**) and the loss of TH-positive neurons in the ventral midbrain of aged *cFoxa1/2*$^{-/-}$ mice 78 weeks post-TAM, visualized by TH and AADC immunostaining and quantification of the TH-positive neurons in the SNpc (**Figures 5B–D**). Thus, in the early adulthood of *cFoxa1/2*$^{-/-}$ mice, when no TH-positive neurons loss has been yet apparent, a decrease in Aldh1a1 (**Figures 3C–G**) and, later, in TH and AADC expression (**Figures 3G, 4A,B**), and a dramatic loss of DAT (**Figure 4C**) predetermined the fatal outcome for the dopamine system in aged *cFoxa1/2*$^{-/-}$ animals (**Figure 5**). Considering that Foxa factors can bind to the 5′-regions of *Aldh1a1*, *Aadc*, and *Th* genes (Lee et al., 2010; Soccio et al., 2011; Stott et al., 2013; Yang et al., 2013), changes in their transcription levels likely represent a molecular mechanism by which Foxa1/2 factors protect adult DA neurons.

The data presented above demonstrate that the deletion of Foxa1/2 ultimately led to a loss of TH-positive neurons in adult mice. To find out whether these results are also clinically relevant, we decided to check if the down-regulation of Foxa1/2 factors and their target genes has been observed in other PD animal models and in PD patients. Online databases of gene expression profiling in clinical PD samples and animal models, namely National Center for Adult Stem Cell Research Parkinson's review database (Sutherland et al., 2009) and ParkDB (Taccioli et al., 2011) provide a comprehensive and constantly updated resource for data mining in a PD-related context. The ParkDB database also allows cross-species comparison of human and mouse expression profiling data. Thus, we searched online both of these databases for the expression changes of *Foxa1*, *Foxa2*, *Aldh1a1*, *Nr4a2*, *Aadc*, *Th*, *En1*, and *Slc6a3*. The levels of these mRNAs were down-regulated

Table 2 | Levels of dopamine and its metabolites in the striata of mice 24 weeks after conditional ablation of Foxa1/2 factors in adult dopamine neurons.

Line name	*n*	Dopamine (ng/mg)	DOPAC (ng/mg)	HVA (ng/mg)
Control	10	13.49 ± 1.0	0.87 ± 0.06	1.24 ± 0.12
cFoxa2$^{-/-}$	6	12.8 ± 0.9	0.86 ± 0.06	1.16 ± 0.09
cFoxa1$^{+/-}$/2$^{-/-}$	10	11.92 ± 0.78	0.67 ± 0.04*	1.02 ± 0.07
cFoxa1/2$^{-/-}$	5	6.27 ± 0.76***	0.38 ± 0.05***	0.67 ± 0.11**

*The data, presented as mean values ± s.e.m., are normalized to the striatal weight; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to control, as determined by Student's unpaired t-test. DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid.*

in several studies comparing expression profiles of the SNpc samples from PD patients and age-matched healthy subjects (Hauser et al., 2005; Zhang et al., 2005; Moran et al., 2006; Lesnick et al., 2007), indicating that not only Foxa1/2 factors, but also several Foxa1/2-regulated genes are affected in PD patients. Interestingly, while the down-regulation of Foxa1/2 has been observed in the SNpc, the levels of these factors did not significantly change in other tissues of PD patients, such as putamen, cerebellum, occipital cortex (Vogt et al., 2006) and whole blood (Scherzer et al., 2007), possibly indicating a specific role of Foxa factors in the SNpc neurons. Notably, the levels of Foxa1/2 also did not change in the SNpc of transgenic mice overexpressing Hsp70 and alpha-synuclein (dataset E-GEOD-4758) (Klucken et al., 2004), suggesting that Foxa1/2 factors might not be contributing to alpha-synuclein-induced pathology.

DISCUSSION

Both Foxa1 and Foxa2 are crucial for the specification and development of DA neurons, as was demonstrated by inactivation of these transcription factors prenatally (Ferri et al., 2007; Kittappa et al., 2007; Stott et al., 2013). Moreover, about 30% of mice heterozygous for Foxa2 develop asymmetric loss of DA neurons in the SNpc late in life (Kittappa et al., 2007). First, we sought to study the role of Foxa2 in adult DA neurons by conditionally inactivating the *Foxa2* gene by a DA neuron-specific inducible CreERT2 recombination (Engblom et al., 2008; Domanskyi et al., 2011; Rieker et al., 2011). However, the ablation of only Foxa2 in the presence of intact Foxa1 alleles led neither to neurodegeneration nor to locomotor impairments in *cFoxa2*$^{-/-}$ mice (**Figures 1A–C**). Moreover, we observed an up-regulation of *Foxa1* mRNA in the ventral midbrain samples from *cFoxa2*$^{-/-}$ mice (**Figure 1D**). This up-regulation may be caused by a yet undiscovered feedback loop mechanism to compensate for the function of Foxa2 in adult DA neurons, explaining the absence of phenotype in *cFoxa2*$^{-/-}$ mice. Indeed, Foxa1 and Foxa2 have been reported to regulate the development of DA neurons in a dose-dependent manner (Ferri et al., 2007). Both Foxa1 and Foxa2 are important "pioneering" factors which open the chromatin for binding of other transcription regulators (Friedman and Kaestner, 2006). Possibly, the Foxa factors have evolved to compensate for the down-regulation of either protein.

In agreement with the redundant functions of Foxa factors, locomotor impairments and movement asymmetry developed only after conditional inactivation of both Foxa1 and Foxa2, but not Foxa2 alone, in adult DA neurons (**Figures 2A–E**). Similar phenotype was observed in our previous studies after conditional ablation of polymerase I transcription initiation factor Ia (TifIa) in adult DA neurons (Domanskyi et al., 2011; Rieker et al., 2011). In *cFoxa1/2*$^{-/-}$ mice, deficits in locomotion became apparent 18 weeks after Foxa1/2 ablation when the double mutant mice exhibited a reduced latency to fall off the rotarod. In the open field test performed 21 weeks post-TAM, *cFoxa1/2*$^{-/-}$ mice showed locally restricted but highly increased circling behavior with almost no forward locomotion. Both behaviors reflect bradykinesia and asymmetric movements, symptoms, the onset of which becomes apparent upon loss of striatal DA content in patients with PD (Taylor et al., 2010). Especially a circling behavior is a classical symptom of unbalanced DA levels in PD, and it is often induced by unilateral lesions (Heuer et al., 2012).

In *cFoxa1/2*$^{-/-}$ mice, there was no apparent loss of TH-positive neurons either in the SNpc or in the VTA (**Figures 3A–B**) up to 24 weeks after Foxa1/2 ablation. However, we detected a significant down-regulation of Aldh1a1 in TH-positive DA neurons of the ventral midbrain of *cFoxa1/2*$^{-/-}$ mice at 11 weeks after recombination (**Figure 3C**), that became even more pronounced at 24 weeks, with TH-positive DA neurons in the SNpc being more affected than those in the VTA (**Figures 3C–G**). Aldh1a1 catalyses the oxidation of 3,4-dihydroxyphenylacetaldehyde (DOPAL) to DOPAC which is then converted to HVA in DA neurons (Marchitti et al., 2007). Thus, down-regulation of Aldh1a1 may also contribute to reduced levels of both these dopamine metabolites that occurred in *cFoxa1/2*$^{-/-}$ mice 24 weeks after recombination (**Table 2**). A protective function of Aldh1a1 in DA neurons has been reported in several studies (Anderson et al., 2011; Wey et al., 2012; Liu et al., 2014); moreover, reduced Aldh1a1 expression and the loss of Aldh1a1-positive DA neurons has been observed in post-mortem brain sections from PD patients (Liu et al., 2014). Interestingly, chromatin immunoprecipitation (ChIP) studies identified two Foxa2 binding sites 5′ to the protein coding sequence of human and mouse *Aldh1a1* gene (Soccio et al., 2011; Yang et al., 2013), suggesting that Foxa factors may directly regulate Aldh1a1 expression. Concomitant with the down-regulation of striatal dopamine, the observed reduction in Aldh1a1 expression was the earliest molecular manifestation of functional disturbances in DA neurons detectable 11 weeks after the conditional ablation of Foxa factors.

Interestingly, even though the number of TH-positive neurons in *cFoxa1/2*$^{-/-}$ mice at 24 weeks after recombination did not change, the TH immunostaining intensity in the ventral midbrain of *cFoxa1/2*$^{-/-}$ mice was lower than that of controls

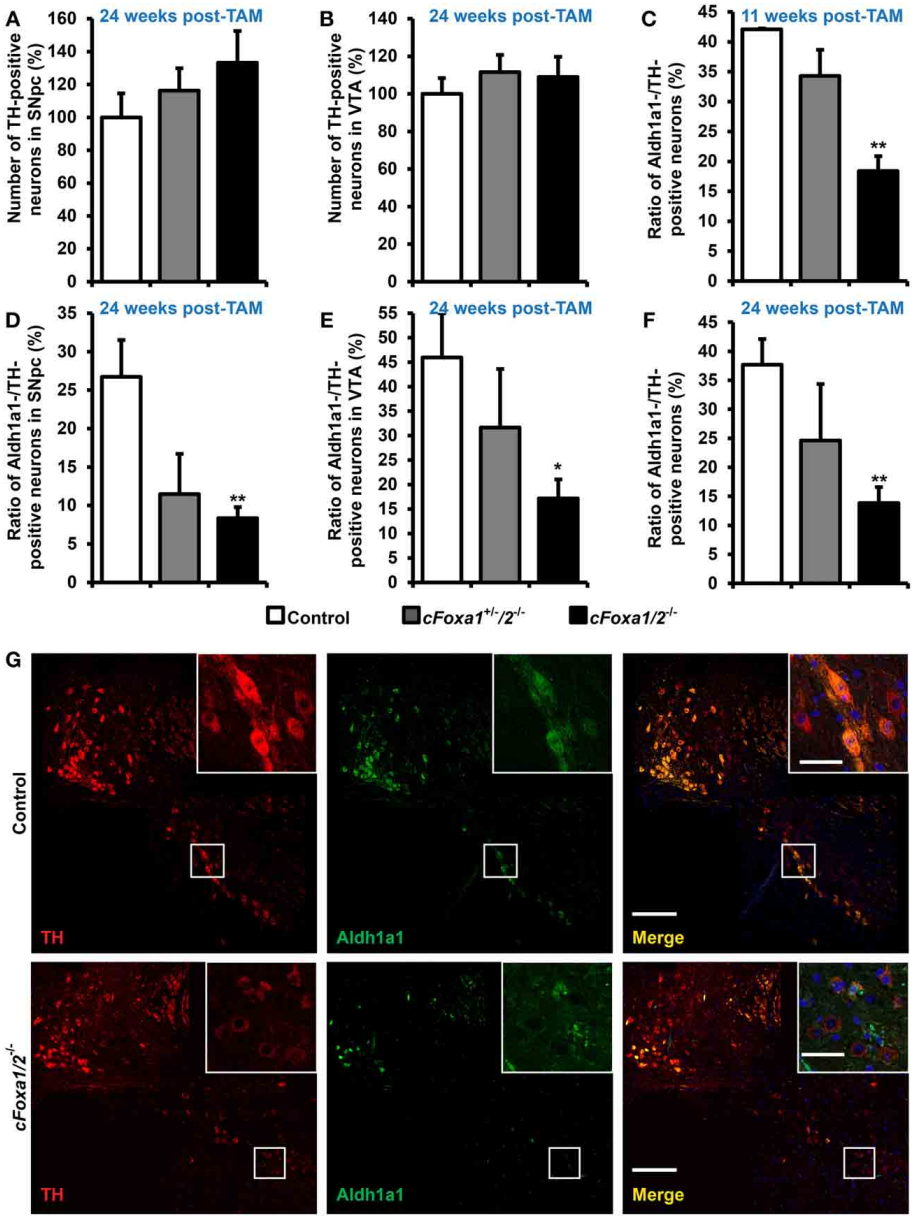

FIGURE 3 | Loss of the Aldh1a1 expression in adult dopamine neurons precedes the onset of locomotor impairments in *cFoxa1/2*$^{-/-}$ mice. (A,B) Quantification of tyrosine hydroxylase (TH)-positive neurons in the SNpc **(A)** or in the VTA **(B)** of control, *cFoxa1*$^{+/-}$*/2*$^{-/-}$, and *cFoxa1/2*$^{-/-}$ mice 24 weeks post-TAM ($n = 4$, 3, and 5, respectively). **(C–F)** Quantification of aldehyde dehydrogenase 1 family, member A1 (Aldh1a1)-positive neurons in the ventral midbrain **(C,F)**, SNpc **(D)** or VTA **(E)** of control, *cFoxa1*$^{+/-}$*/2*$^{-/-}$, and *cFoxa1/2*$^{-/-}$ mice 11 weeks **(C)** and 24 weeks **(D–F)** post-TAM expressed relative to the number of TH-positive neurons ($n = 4$, 3, and 5, respectively). **(G)** Representative microphotographs of TH (red) and Aldh1a1 (green) immunofluorescent staining and co-localization of these proteins and DAPI (blue) in the ventral midbrain sections from control and *cFoxa1/2*$^{-/-}$ mice 24 weeks post-TAM. Scale bar, 200 μm for overviews and 50 μm for insets. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison to control, as determined by Student's unpaired *t*-test.

(**Figures 3G, 4A**) and the *Th* mRNA levels also had a tendency towards a decrease (Supplementary Figure 2). We observed even more pronounced loss of immunostaining intensity for two other markers of DA neurons, AADC and DAT (**Figures 4B,C**), paralleled by altered morphology of these cells (**Figure 3G**), suggesting that the ablation of Foxa1 and Foxa2 caused DA neurons to gradually lose their dopaminergic phenotype. Similar down-regulation of Aldh1a1, TH, DAT, and AADC was also reported in post-mitotic DA neurons after prenatal ablation of Foxa1/2 (Stott et al., 2013) that is in a good agreement with our data. However, in that study, the authors also detected a decrease in TH-positive neurons both pre- and postnatally. On the contrary, in our model, when inactivation of both factors occurred in adult DA neurons, we did not detect any loss of TH-positive cells up to 24 weeks

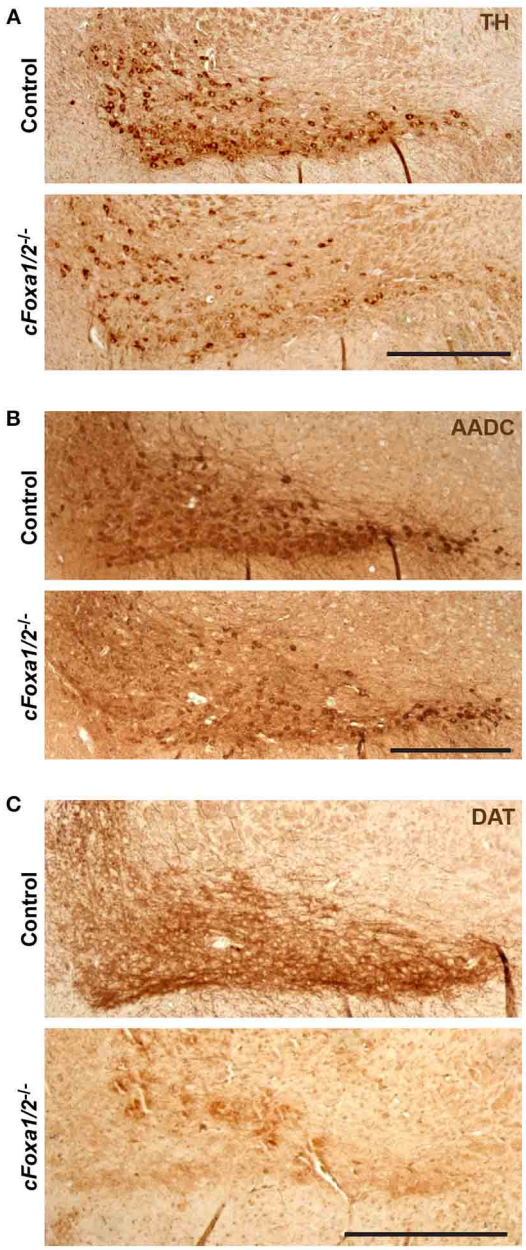

FIGURE 4 | Down-regulation of dopamine neuronal markers in *cFoxa1/2*$^{-/-}$ mice. (A–C) Representative microphotographs of tyrosine hydroxylase (TH) **(A)**, aromatic amino acid decarboxylase (AADC) **(B)** and dopamine transporter (DAT) **(C)** immunostaining in the ventral midbrain of control and *cFoxa1/2*$^{-/-}$ mice 24 weeks post-TAM. Scale bar, 500 μm.

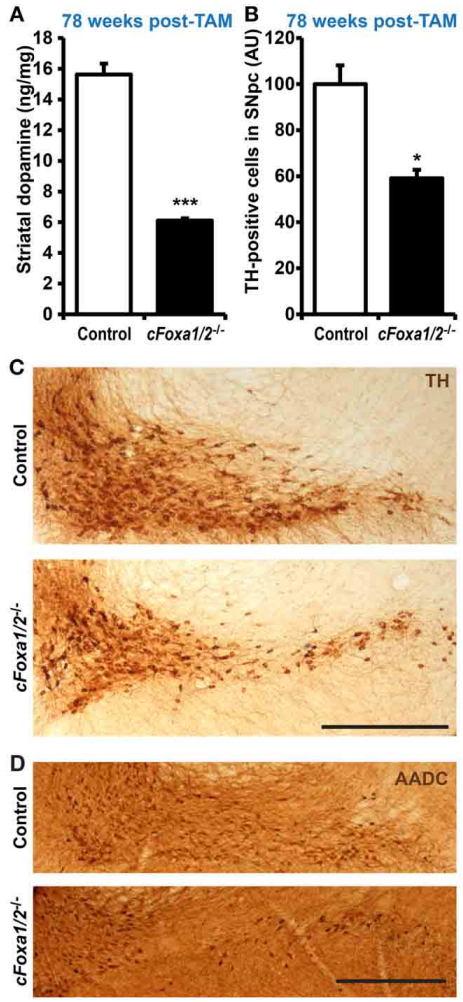

FIGURE 5 | Loss of TH-positive adult dopamine neurons in aged *cFoxa1/2*$^{-/-}$ mice. (A) Striatal dopamine content in control or *cFoxa1/2*$^{-/-}$ mice 78 weeks after TAM treatment ($n = 6$ and 3, respectively). **(B)** Quantification of tyrosine hydroxylase (TH)-positive neurons in the SNpc of control and *cFoxa1/2*$^{-/-}$ mice at the same time point ($n = 3$). **(C,D)** Representative microphotographs of TH **(C)** and aromatic amino acid decarboxylase (AADC) **(D)** immunostaining in the ventral midbrain of control and *cFoxa1/2*$^{-/-}$ mice. Scale bar, 500 μm. $^{*}p < 0.05$, $^{***}p < 0.001$ in comparison to control, as determined by Student's unpaired *t*-test.

post-TAM. These data suggest that Foxa1/2 factors are essential for DA survival during their maturation and specification, while, similarly to PD, additional epigenetic cues (such as environmental factors, mitochondrial stress or aging) are required for the onset of neurodegeneration when Foxa factors are inactivated in mature DA neurons.

Foxa2 can regulate TH expression directly and/or cooperatively with Nurr1 (Lee et al., 2010), and there are three Foxa2 binding sites in the promoter region of the mouse *Aadc* gene identified by ChIP (Soccio et al., 2011; Yang et al., 2013). It has also been shown that Foxa1/2 loss leads to lower occupancy of *Aadc* and *Th* gene promoters by Nurr1 that, without affecting the levels of Nurr1 itself, results in the down-regulation of AADC in post-mitotic DA neurons (Stott et al., 2013). Of note, the loss of Foxa1/2 did not lead to down-regulation of the levels of either Nurr1 or several other factors important for the functions of DA neurons in the ventral midbrain (Supplementary Figure 2). However, consistent with the role of Foxa proteins as "pioneer" factors that increase chromatin accessibility for other transcriptional regulators (Friedman and Kaestner, 2006), the loss of Foxa1/2 might have affected the ability of other transcription

factors, including Nurr1, to bind their target promoters in adult DA neurons, as it was observed in a study with the prenatal ablation of Foxa1/2 factors (Stott et al., 2013).

By mining the available gene expression profiling data, we have found that the expression levels of *Foxa1* and *Foxa2*, as well as *Aldh1a1*, *Nr4a2*, *Aadc*, *Th*, *En1*, and *Slc6a3* were also down-regulated in the SNpc, but not in other brain regions or tissue samples from PD patients. However, the profiling data from patients' SNpc samples should be interpreted cautiously, because the apparent down-regulation of these genes might just reflect the loss of DA neurons expressing them. Nevertheless, in the context of our results and previously published data (Kittappa et al., 2007; Stott et al., 2013), the observed down-regulation of Foxa1/2 in post-mortem samples from PD patients suggests that Foxa1/2 factors and their target genes may have a specific role in the SNpc and contribute to neurodegeneration in PD patients.

In summary, we show that, similar to their role in the embryonic development (Ferri et al., 2007; Stott et al., 2013), Foxa1 can compensate for the loss of Foxa2 in adult DA neurons. Thus, a functional redundancy between Foxa1 and Foxa2 proteins, initially observed during embryonic development, is also evident in adult DA neurons. We have further demonstrated that the ablation of Foxa factors in adult DA neurons initially led to the loss of Aldh1a1 expression accompanied by the loss of striatal dopamine and locomotor impairments in the rotarod and open field tests. Foxa factors may regulate *Aldh1a1* directly by binding to the gene's promoter (Soccio et al., 2011; Yang et al., 2013) and/or indirectly by opening chromatin and facilitating the binding of other transcription factors (Friedman and Kaestner, 2006). This data suggests that Foxa1/2 ablation led to the loss of dopaminergic phenotype in SNpc DA neurons that was further confirmed by the observed down-regulation of AADC and DAT expression in the SNpc. Ultimately, we detected a significant loss of TH-positive DA neurons in aged *cFoxa1/2*$^{-/-}$ mice (**Figures 5B,C**), resembling the course of events during the PD pathology in humans.

Altogether, our data establish a protective role of Foxa factors in the maintenance of dopamine neurons *in vivo*. Drugs targeting cytoprotective pathways in DA neurons of human patients with PD are already effectively used or being tested in clinical studies (Allain et al., 2008; Youdim, 2010; Pahwa and Lyons, 2014). The transcription factors from the Foxa family could become additional candidates for such therapeutic strategies.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00275/abstract

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Compensatory motor neuron response to chromatolysis in the murine hSOD1^{G93A} model of amyotrophic lateral sclerosis

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We investigated neuronal self-defense mechanisms in a murine model of amyotrophic lateral sclerosis (ALS), the transgenic hSOD1^{G93A}, during both the asymptomatic and symptomatic stages. This is an experimental model of endoplasmic reticulum (ER) stress with severe chromatolysis. As a compensatory response to translation inhibition, chromatolytic neurons tended to reorganize the protein synthesis machinery at the perinuclear region, preferentially at nuclear infolding domains enriched in nuclear pores. This organization could facilitate nucleo-cytoplasmic traffic of RNAs and proteins at translation sites. By electron microscopy analysis, we observed that the active euchromatin pattern and the reticulated nucleolar configuration of control motor neurons were preserved in ALS chromatolytic neurons. Moreover the 5′-fluorouridine (5′-FU) transcription assay, at the ultrastructural level, revealed high incorporation of the RNA precursor 5′-FU into nascent RNA. Immunogold particles of 5′-FU incorporation were distributed throughout the euchromatin and on the dense fibrillar component of the nucleolus in both control and ALS motor neurons. The high rate of rRNA transcription in ALS motor neurons could maintain ribosome biogenesis under conditions of severe dysfunction of proteostasis. Collectively, the perinuclear reorganization of protein synthesis machinery, the predominant euchromatin architecture, and the active nucleolar transcription could represent compensatory mechanisms in ALS motor neurons in response to the disturbance of ER proteostasis. In this scenario, epigenetic activation of chromatin and nucleolar transcription could have important therapeutic implications for neuroprotection in ALS and other neurodegenerative diseases. Although histone deacetylase inhibitors are currently used as therapeutic agents, we raise the untapped potential of the nucleolar transcription of ribosomal genes as an exciting new target for the therapy of some neurodegenerative diseases.

Keywords: neurodegeneration, amyotrophic lateral sclerosis, chromatolysis, stress granules, perinuclear region, nucleolus, rRNA transcription, endoplasmic reticulum stress

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by degeneration of motor neurons in the anterior spinal horn, brainstem nuclei, and cerebral cortex (Rowland and Shneider, 2001). An important component in ALS cellular pathophysiology is a chronic stress response at the endoplasmic reticulum (ER) leading to severe disruption of proteostasis. An essential function of the rough ER (RER) is the synthesis of secretory, membrane, and lysosomal proteins, as well as their folding and quality control. Different cellular stressors cause protein misfolding at the RER with inhibition of protein translation, a condition called "ER stress" (Hetz, 2012). As a compensatory mechanism against ER stress, neurons may activate the unfolded protein response (UPR) that, depending on the intensity and nature of the stress stimuli, may lead to either the correct protein folding (neuroprotection) or to neurodegeneration (Hetz and Mollereau, 2014). Neuronal manifestations of chronic ER stress are fragmentation and dissolution of the RER cisterns, an alteration referred to as chromatolysis, and accumulation of misfolded and aberrant proteins in cytoplasmic inclusions. These two cellular events commonly occur in motor neurons of both ALS patients and murine experimental models of the disease (Kusaka et al., 1988; Martin, 1999; Oyanagi et al., 2008; Sasaki, 2010).

Chromatolysis is a prominent neuropathological feature induced by axonal injury, ischemia, neurotoxicity, and several neurodegenerative disorders such as ALS, spinal muscular atrophy, and Alzheimer's disease (Barr and Bertram, 1951; Lieberman, 1971; Wakayama, 1992; Sasaki, 2011; Tapia et al., 2012; Palanca et al., 2014a). Chromatolytic dissolution of the RER clearly reflects a dysfunction of protein synthesis that most often precedes apoptosis

(Martin, 1999). However, neuronal recovery can occur following axotomy and treatment with certain neurotoxic agents (Lieberman, 1971; Palanca et al., 2014a) due to successful activation of neuroprotective mechanisms for neuronal survival.

A major component to maintain the structural and functional integrity of the RER is ribosome biogenesis, which is dynamically accommodated to the cellular demands for protein synthesis. The nucleolus is the nuclear factory for rRNA synthesis, maturing rRNA transcripts, and preribosome subunit assembly (Raska et al., 2006; Boisvert et al., 2007; Grummt, 2013). Beyond its role in ribosome biogenesis, the nucleolus constitutes a central hub for sensing and coordinating cellular stress responses (Olson, 2004; Boulon et al., 2010; Kreiner et al., 2013; Parlato and Liss, 2014). Nucleoli are very prominent in mammalian projection neurons to sustain the high rate of ribosome biogenesis required for protein synthesis. Neurons can accommodate changes in the demand for protein synthesis by regulating the number and size of nucleoli, as well as the transcriptional activity of ribosomal genes (Lafarga et al., 1991; Berciano et al., 2007; Jordan et al., 2007; Hetman and Pietrzak, 2012; Palanca et al., 2014b). In fact, nucleolar dysfunction has been involved in the pathophysiology of several neurodegenerative diseases (Hetman et al., 2010; Baltanas et al., 2011a; Hetman and Pietrzak, 2012; Parlato and Kreiner, 2013; Lee et al., 2014).

An important question in motor neuron diseases is how chromatolytic neurons can survive under conditions of severe disturbance of protein synthesis and ER proteostasis, before the final activation of the apoptotic program. In an experimental model of reversible chromatolysis induced by proteasome inhibition in rat sensory ganglion neurons, we have recently demonstrated that the reorganization of the nuclear envelope environment and the hyperactivity of the nucleolus play a key neuroprotective role in maintaining neuronal survival (Palanca et al., 2014b). In the present work we used an animal model of ALS, the transgenic hSOD1^{G93A} mouse (Gurney et al., 1994; Sunico et al., 2011; Mòdol et al., 2014), to investigate the cellular basis of both chromatolysis and compensatory neuroprotective mechanisms in affected spinal motor neurons. We demonstrate that the dysfunction of the RER is associated with the sequestration of RNA and the preinitiation factor of translation eIF3 in stress granules (SGs). Moreover, to mitigate chromatolysis-mediated inhibition of translation, motor neurons reorganize the protein synthesis machinery at the perinuclear region, preserve the transcriptionally active euchromatin domains, and maintain an active nucleolar transcription for ribosome biogenesis.

MATERIALS AND METHODS

ANIMALS

Transgenic mice [B6SJLTg (SOD1-G93A) 1Gur/J] were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the Animal Service of the University of Cantabria. The colony was maintained by mating heterozygous transgenic males with B6SJLF1/J hybrid females. Real time quantitative PCR of DNA obtained from tail tissue was used for genotyping, with specific primers detecting human SOD1 and the housekeeping mouse gene ApoB. Primer sequences were: SOD1: GGG AAG CTG TTG TCC CAA G and CAA GGG GAG GTA AAA GAG AGC; ApoB: TCA CCA GTC ATT TCT GCC TTT G and GGG AAG CTG TTG TCC CAA G. Transgenic mice and control littermates were housed under controlled temperature and humidity, with a 12-h light/dark cycle and free access to water and food. Animal care and handling was in accordance with Spanish legislation (Spanish Royal Decree 53/2013 BOE) and the guidelines of the European Commission for the accommodation and care of laboratory animals (revised in Appendix A of the Council Directive 2010/63/UE). The experimental plan was preliminarily examined and approved by the Ethics Committee of the University of Cantabria. Animal sacrifice was performed under deep pentobarbital anesthesia (50 mg/kg). In order to evaluate both presymptomatic and symptomatic stage, transgenic and control mice were sacrificed at day 65 and 105 of life.

CASE REPORT

A 53 years-old previously healthy man was admitted to our Hospital referring to a progressive history of gait difficulties during last 3 years. The neurological exam showed a generalized pyramidal syndrome. Once all the complementary exams were performed, a suspicious diagnosis of primary lateral sclerosis was done. Nevertheless 1 year after diagnosis, symptoms and signs of lower motor neuron degeneration were evidenced, being finally diagnosed of ALS. The patient continued deteriorating and died 5 years after the clinical onset. As control subject, a 67-year-old man with no evidence of neurological disorder was used. Post-mortem examination of spinal cord tissue samples was conducted after written consent given by a near kin. In both cases, autopsy was performed 8 h after death.

LIGHT MICROSCOPY AND IMMUNOFLUORESCENCE

For light microscopy analysis, control and transgenic hSOD1^{G93A} mice were perfused under deep anesthesia with pentobarbital (50 mg/kg) with 3.7% paraformaldehyde (freshly prepared) in PBS. After fixation, the lumbar segment of the spinal cord was removed and washed in PBS. Tissue fragments from the anterior horn were transferred to a drop of PBS on a siliconized slide and squash preparations of dissociated neurons were performed following the procedure previously reported (Pena et al., 2001). Human tissue samples from the lumbar segment were fixed with 3.7% paraformaldehyde. Anterior horn tissue fragments were dissected out from 300 μm thick vibratome sections and processed for neuronal dissociation as indicated above.

For immunofluorescence, the samples were, then, sequentially treated with 0.5% Triton X-100 in PBS for 45 min, 0.1 M glycine in PBS containing 1% bovine serum albumin (BSA) for 30 min and 0.05% Tween 20 in PBS for 5 min. The samples were incubated for 3 h with the primary antibody, goat anti-eIF3η (Santa Cruz Lab, sc-16377, 1:200, La Jolla, CA, USA), containing 1% BSA at room temperature, washed with 0.05% Tween 20 in PBS, incubated for 45 min in the specific secondary antibody conjugated with FITC (Jackson, USA), washed in PBS, counterstained with propidium iodide, and mounted with the ProLong anti-fading medium (Invitrogen). Confocal images were obtained with a LSM510 (Zeiss, Germany) laser scanning microscope and using a 63 × oil (1.4 NA) objective. In order to avoid overlapping signals, images were obtained by sequential excitation at 488 and 543 nm. Images were processed using Photoshop software.

The morphometric analysis of the nucleolar size was performed in dissociated motor neurons with diameter larger than 20 μm. Neuronal preparations were stained with propidium iodide, the nucleolar diameter in mononucleolated neurons being measured on confocal images using a 63 × oil objective and the LSM510 software for morphometric analysis. At least 100 neurons for each experimental group (control, presymptomatic ALS mice, and symptomatic ALS mice) were analyzed. Data were analyzed using one-way ANOVA followed by Bonferroni tests for comparisons. Statistical significance was set at $p < 0.05$. All the analyses were carried out using GraphPad software for Windows.

ELECTRON MICROSCOPY

For conventional ultrastructural examination of motor neurons, control and transgenic hSOD1^{G93A} mice ($n = 3$ per group) were perfused under deep anesthesia with 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The lumbar segment of the spinal cord was removed. The anterior horn was dissected from 300 μm thick Vibratome sections. Human tissue fragments from the lumbar segment of the anterior horn were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer. Mice and human tissue fragments were rinsed in 0.1 M phosphate buffer, post-fixed in 2% osmium tetroxide, dehydrated in acetone, and embedded in araldite (Durcupan, Fluka, Switzerland). Semithin sections (1 μm thick) stained with toluidine blue were used for light microscopy studies and for the quantitative analysis of the frequency of motor neurons having perinuclear caps of RER. Three animal per animal group (wild type, and asymptomatic and symptomatic ALS mice) and 100 neurons per animal were used. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a JEOL 201 electron microscope.

RUN ON TRANSCRIPTION ASSAY WITH 5′-FLUOROURIDINE

Active transcription sites were labeled by the incorporation of 5′-fluorouridine (5′-FU) into nascent RNA, as previously reported (Casafont et al., 2006). Briefly, under anesthesia both control and transgenic hSOD1^{G93A} mice ($n = 3$ per group) were given an intravenous injection of 5′-FU (Sigma, UK) at doses of 5 μl/g of a stock solution of 0.4 M 5′-FU in 0.9% saline. All animals were sacrificed after 45 min post-injection of the halogenated nucleotide and fixed by perfusion with 3.7% paraformaldehyde in 0.1 M cacodylate buffer for 15 min at room temperature. Small tissue fragments of the anterior horn were washed in 0.1 M cacodylate buffer, dehydrated in increasing concentrations of methanol at -20°C, embedded in Lowicryl K4 M at -20°C and polymerized with ultraviolet irradiation. Ultrathin sections (60 nm thick) were mounted on nickel grids and sequentially incubated with 0.1 M glycine in PBS for 15 min, 5% BSA in PBS for 30 min, and the primary mouse monoclonal anti-BrdU antibody (clone BU-33, Sigma-Aldrich, UK), diluted 1:25 in 50 mM Tris–HCl, pH 7.6, containing 1% BSA and 0.1 M glycine, for 1 h at 37°C. After washing, the sections were incubated with the specific secondary antibodies coupled to 15-nm gold particles (BioCell, UK; diluted 1:50 in PBS containing 1% BSA). Following immunogold labeling, the grids were stained with lead citrate and uranyl acetate and examined with a JEOL 201 electron microscope. As controls, ultrathin sections were treated as described above but without using the primary antibody.

The quantitative analysis of labeling density was performed on electron microscopy images, recorded at a magnification of 25,000×, of nucleus and nucleoli of motor neurons from three wild type and three symptomatic transgenic hSOD1^{G93A} mice. Labeling density values of gold particles lying on euchromatin and nucleoli, expressed as number of particles per 1 μm^2, were determined using the ImageJ software (US National Institutes of Health, Bethesda, MD, USA). Electron micrographs from 15 motor neurons per animal were sampled.

RESULTS

STRESS GRANULES ARE INDUCED IN MOTOR NEURONS OF BOTH AFFECTED TRANSGENIC hSOD1^{G93A} MICE AND THE ALS PATIENT

Spinal motor neurons in wild type (controls) and transgenic hSOD1^{G93A} mice were examined in preparations of dissociated neurons (Pena et al., 2001) and 1 μm semithin sections. The cytochemical staining for nucleic acids with propidium iodide revealed prominent rRNA-rich nucleoli and Nissl bodies in control neurons. At the symptomatic stage (105 days old), a disintegration of Nissl bodies associated with vacuolar degeneration of perikaryal cytoplasm and neuronal processes were cytological hallmarks in hSOD1^{G93A} neurons, although the prominent nucleoli were preserved (**Figures 1A–C**).

Since disruption of the protein synthesis machinery (Nissl bodies) with accumulation of misfolded proteins is a manifestation of the ER stress response in the ALS-linked SOD1 mutants (Kikuchi et al., 2006), we analyzed whether this mutation induced the formation of SGs containing stellated translational initiation complexes (Kedersha et al., 2013). Co-staining for nucleic acids and the immunocytochemical marker of SGs eIF3η, the largest initiation factor of translation (Malys and McCarthy, 2011), revealed a diffuse cytoplasmic eIF3η immunostaining in control neurons and the concentration of this factor in numerous SGs in transgenic hSOD1 neurons. The presence of eIF3η-positive SGs was also confirmed in motor neurons of the ALS patient (**Figures 1M–O**). Sudan black was used to remove endogenous autofluorescence due to lipofuscin in both mouse and human neuronal samples (Liu-Yesucevitz et al., 2010). Interestingly, SGs also sequestrated RNAs not associated with polyribosomes as a result of the translational inhibition. The quantitative analysis of the proportion of motor neurons containing SGs revealed a significant increase from the asymptomatic (65 days old) to symptomatic (105 days old) stages in ALS mice, while they were rarely found in control neurons (**Figure 1P**).

CHROMATOLYTIC DISRUPTION OF THE RER IN hSOD1^{G93A} MOTOR NEURONS

Although previous studies have demonstrated the induction of chromatolysis with fragmentation of the RER in motor neurons of both ALS patients and murine models of ALS (Kusaka et al., 1988; Oyanagi et al., 2008; Sasaki, 2010), we have investigated the cellular basis of RER alterations that leads to translational inhibition in the mutant SOD1 mice. Electron microscopy of motor neurons showed a dense cytoplasm with numerous typical stacks of RER cisterns in control neurons in contrast to the pale cytoplasm

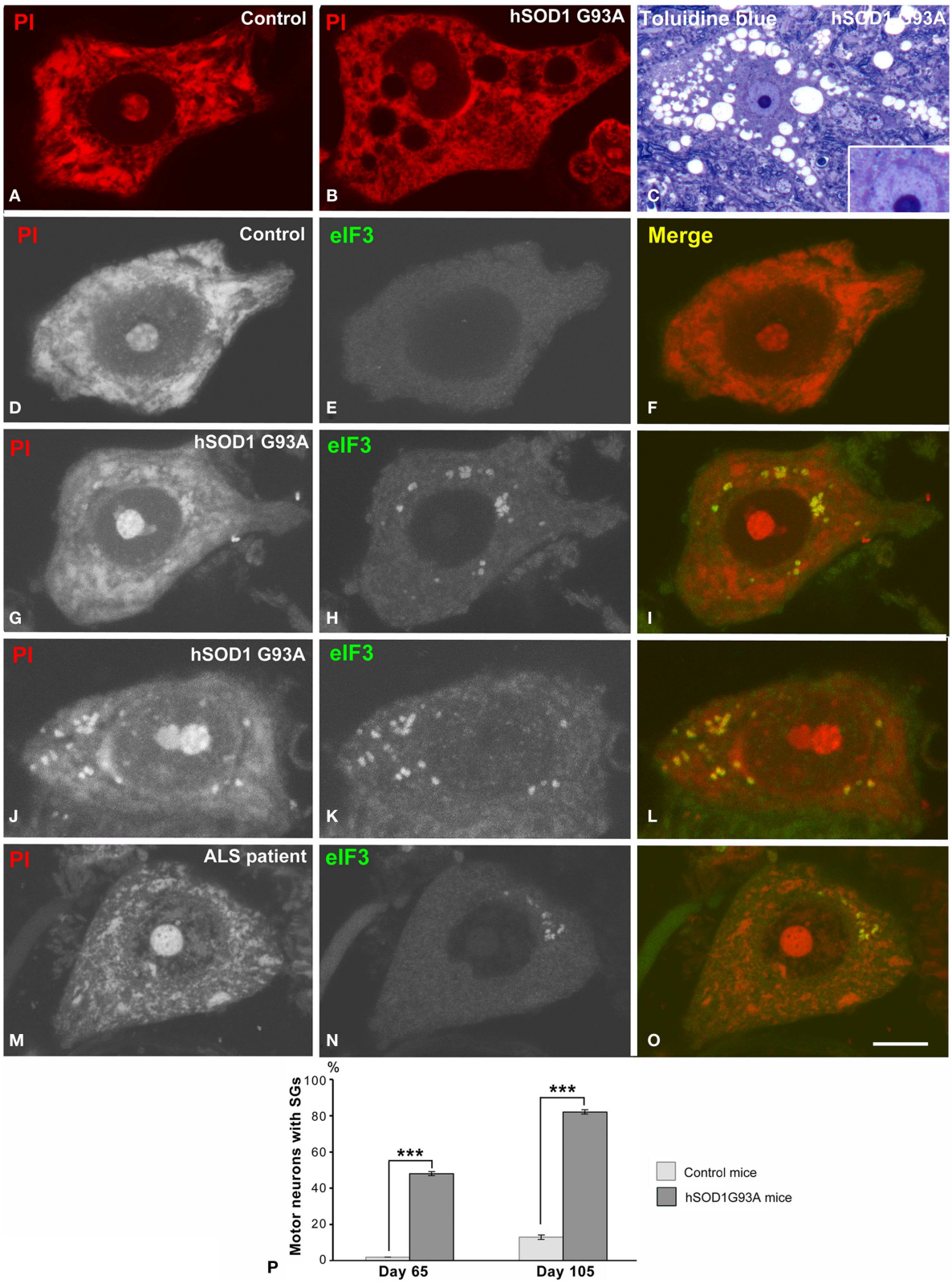

FIGURE 1 | (A,B) Dissociated spinal motor neurons stained with propidium iodide (PI) from control **(A)** and hSOD1^{G93A} mice **(B)**. Note the prominent Nissl bodies and nucleolus in the control neuron **(A)**. The hSOD1^{G93A} motor neuron exhibits dispersion of the Nissl substance, excluding a perinuclear cap strongly stained with PI, and vacuolar cytoplasmic degeneration.

(Continued)

FIGURE 1 | Continued
(C) Toluidine blue staining of an ALS motor neuron illustrating the extensive vacuolar degeneration. Note the prominent nucleolus, the pale euchromatic nucleus, and the basophilic perinuclear cap (inset). **(D–L)** Dissociated motor neurons from control **(D–F)** and hSOD1^{G93A} mice immunolabeled for eIF3 and costained with PI. eIF3 shows a diffuse cytoplasmic staining in the control neuron **(E)**, but it is concentrated in SGs in ALS motor neurons **(H,K)**. Note the colocalization of eIF3 and RNA (stained with PI) in SGs. **(M–O)** Representative example of a motor neuron from the ALS patient illustrating the presence of eIF3-positive granules. Scale bars: **(A,B,D–L)** = 7 μm; **(C)** = 18 μm, inset 10 μm; **(M–O)** = 8.5 μm. **(P)** Quantitative analysis of the percentage of motor neurons from control and hSOD1^{G93A} mice with eIF3-positive SGs. Data are mean ± SE from three independent experiments, $***p < 0.001$.

produced by the paucity of RER elements in chromatolytic transgenic neurons (**Figures 2A,B**). Detailed ultrastructural analysis of RER showed two characteristic alterations. The first consisted of focal dilatations of the RER cisterns with partial detachment of membrane-associated polyribosomes that ultimately leads to fragmentation of the cisterns in numerous small cytoplasmic vesicles ranging in diameter from 200 to 500 nm (**Figures 2C,D**). The RER origin of these vesicles was also supported by the presence of remnants of polyribosomes attached to the cytosolic face of the membrane (**Figure 2D**). The accumulation of RER-derived vesicles is a common finding in chromatolytic areas of transgenic hSOD1^{G93A} neurons. A second manifestation was the formation of whorl and stack arrays of RER containing collapsed cisterns with occlusion of the lumen (**Figures 2E,F**). Moreover, polyribosomes tended to be detached from collapsed cisterns, indicating that they are not engaged in active translations. This ultrastructural organization is distinct from the RER-derived lamellar bodies described in motor neurons of sporadic ALS patients (Sasaki, 2010). Taken together, these RER alterations reflect a severe disruption of the protein synthesis machinery.

COMPENSATORY RESPONSE OF THE PROTEIN SYNTHESIS MACHINERY TO CHROMATOLYSIS

Next, we investigated possible cellular mechanisms involved in neuronal survival under conditions of severe chromatolysis in ALS motor neurons. In particular, we analyzed the reorganization of the RER. Both cytochemical staining with propidium iodide and toluidine blue staining showed the frequent presence of fluorescent or basophilic perinuclear caps enriched in RNA in motor neurons of the hSOD1^{G93A} mice at both presymptomatic and symptomatic stages (**Figures 1B,C**). The ultrastructural counterpart was the local accumulation of RER cisterns and free polyribosomes in close proximity to the nuclear envelope (**Figures 3A,B**). While in the majority of control neurons, the perinuclear cytoplasm commonly displayed scattered polyribosomes, Golgi complexes and mitochondria (**Figure 3C**), two main perinuclear arrangements of the protein synthesis machinery were found in the ALS mouse model. The first consisted of concentric arrays of RER cisterns in close proximity of the nuclear envelope (**Figure 3D**). The second were perinuclear caps of free polyribosome with some isolated cisterns of RER (**Figure 3E**). Perinuclear caps were most commonly observed in motor neurons with severe chromatolysis and abnormal accumulations of neurofilaments, and frequently occurred at a wrinkled nuclear pole in which the infoldings of the nuclear envelope were filled with polyribosomes and RER cisterns (**Figures 3B,E**). Tangential sections of the nuclear envelope at the nuclear infoldings showed high density of nuclear pores and their spatial association with polyribosomes (**Figure 3F**, inset). The quantitative analysis of the proportion of motor neurons having perinuclear caps of RER revealed a significant increase in ALS mice compared to wild type, with a notable higher frequency in symptomatic than in asymptomatic stages of the ALS (**Figure 4E**).

Interestingly, a similar reorganization of the protein synthesis machinery was observed in human motor neurons of the sporadic ALS patient. **Figures 4A,B** illustrate representative examples of a control motor neuron with typical Nissl bodies stained with propidium iodide, and an ALS patient motor neuron with chromatolysis and a strong fluorescent perinuclear cap. Electron microscopy analysis of chromatolytic neurons confirmed the perinuclear accumulations of RER cisterns and polyribosomes, which frequently appeared in nuclear infolding domains (**Figures 4C,D**). The quantitative analysis of the proportion of motor neurons with chromatolysis and/or perinuclear caps of RER showed a significant increase in the ALS patient as compared to the control patient (**Figures 4F,G**). Collectively, these findings suggest that the reorganization of the protein synthesis machinery at nuclear infoldings, which provide an increased nuclear surface studded with numerous nuclear pores, may facilitate nucleo-cytoplasmic traffic in chromatolytic neurons.

NUCLEOLAR CONFIGURATION AND TRANSCRIPTIONAL ACTIVITY IN MOTOR NEURONS OF THE SOD1 MUTANT MICE

Since ribosome biogenesis is an essential step to sustain protein synthesis activity, we analyzed the response of nucleolus to severe chromatolysis in motor neurons of the ALS transgenic SOD1 mice. Light microscopy cytochemical staining with propidium iodide clearly illustrated prominent nucleoli in motor neurons from both control and ALS mice (**Figure 1**). The morphometric determination of the nucleolar diameter demonstrated that the nucleolar size was preserved in motor neurons from ALS mice (**Figure 5I**).

To determine whether the dysfunction of the protein synthesis caused by the severe chromatolysis modified the nucleolar architecture in motor neurons, we performed ultrastructural analysis. Both control and ALS mice nucleoli in motor neurons exhibited a typical reticulated configuration (**Figures 5A–C**) of neurons with high transcriptional activity (Peters et al., 1991; Casafont et al., 2006; Palanca et al., 2014b). This nucleolar organization is characterized by the presence of numerous fibrillar centers surrounded by a shell of dense fibrillar component and variable masses of granular component. The reticulated nucleolar architecture was well preserved even in motor neurons with nuclear eccentricity and severe chromatolysis of symptomatic hSOD1^{G93A} mice (**Figures 5A**). Moreover, nucleolar macrosegregation of dense fibrillar and granular components, an alteration of the neuronal nucleolus associated with the inhibition of the nucleolar transcription (Casafont et al., 2006, 2007; Baltanas et al., 2011a), was not observed.

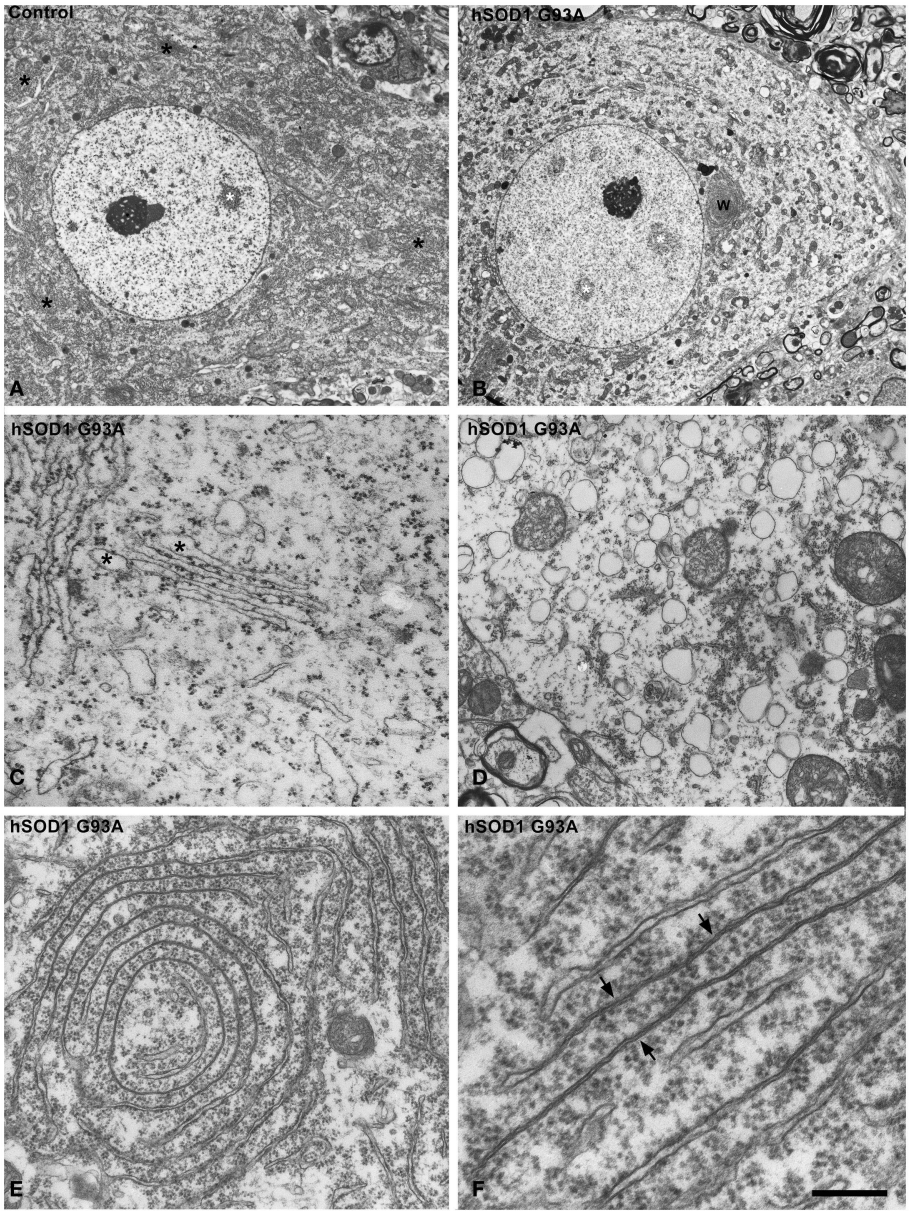

FIGURE 2 | (A,B) Electron micrographs of motor neurons from control **(A)** and hSOD1^{G93A} mice **(B)**. While control neuron exhibits numerous Nissl bodies throughout the cytoplasm (black asterisks), the ALS neuron shows a pale extensive chromatolytic area free of Nissl bodies. Note, however, that both neurons display large euchromatic nuclei with several interchromatin granule clusters (white asterisks) and a prominent nucleolus. W: whorl of RER. **(C,D)** Disruption of the protein synthesis machinery in ALS motor neurons from the hSOD1^{G93A} mice. Note in **(C)**, the partial detachment of polyribosomes from RER cisterns, with numerous polyribosomes scattered throughout the cytosol, and the focal dilations of cisterns (asterisks). **(D)** illustrates a cytoplasmic area with massive accumulation of RER-derived vesicles with remnants of membrane-bound ribosomes. **(E,F)** Whorl **(E)** and parallel **(F)** arrays of RER cisterns in motor neurons from hSOD1^{G93A} mice. Note the presence of long segments with obliteration of the cisternal lumen and partial detachment of membrane-bound polyribosomes. Scale bars: **(A,B)** = 5 μm; **(C,D)** = 1 μm; **(E)** = 0.7 μm; **(F)** = 250 nm.

Next, we investigated whether nucleolar transcription was affected in motor neurons of the hSOD1^{G93A} mice. We performed an *in situ* transcription assay at the ultrastructural level based on the incorporation of the RNA precursor 5′-FU into nascent RNA, following a 45 min pulse of intravenous administration of the halogenated nucleotide. Nucleolar and chromatin sites of 5′-FU incorporation were detected with the monoclonal anti-BrdU antibody and using a secondary antibody conjugated with gold particles. As illustrated in **Figures 5D,E**, a similar pattern of distribution of immunogold particles was observed in reticulated nucleoli of motor neurons from control and symptomatic ALS mice. Thus, immunogold particles preferentially decorated the threads of dense fibrillar component.

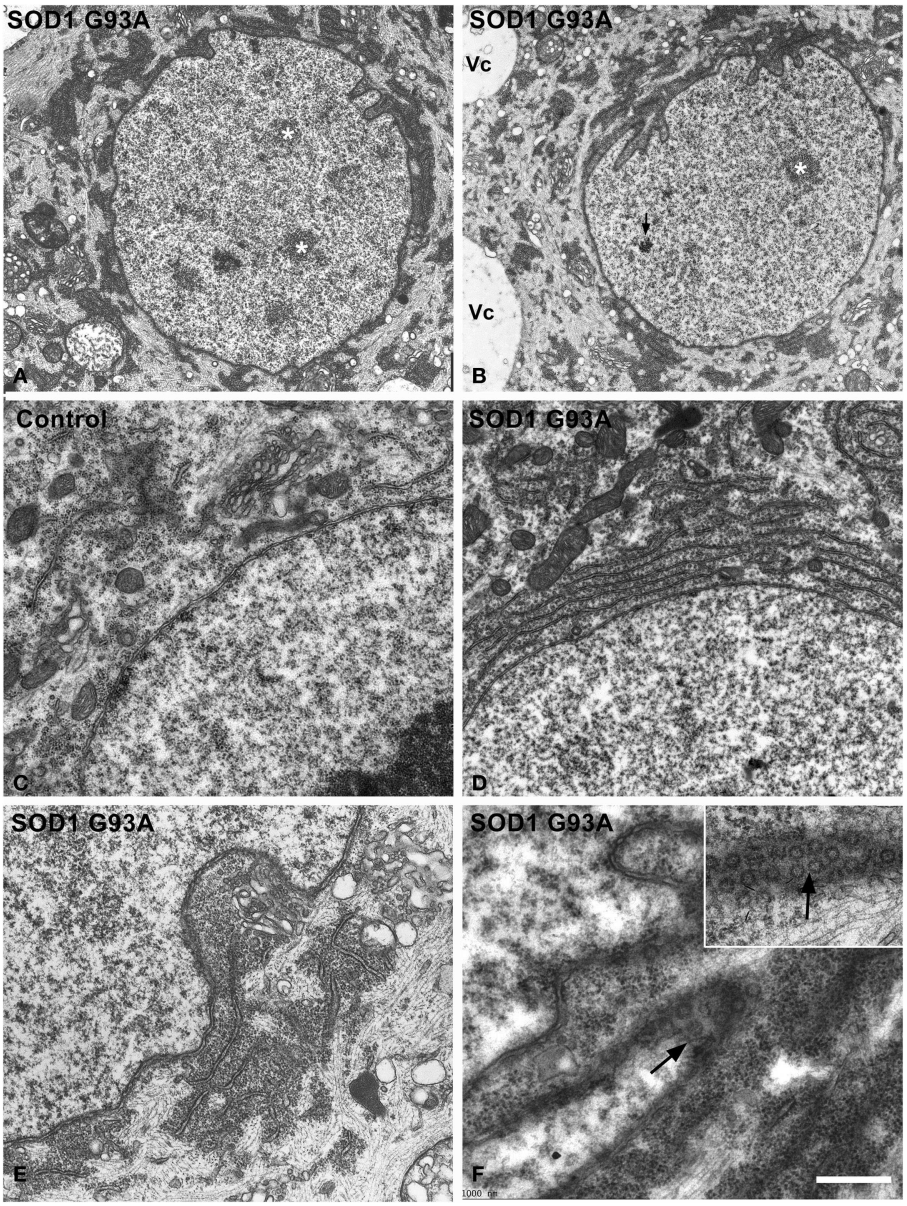

FIGURE 3 | (A–F) Electron micrographs illustrating the organization of the perinuclear region in motor neurons from control **(C)** and hSOD1^{G93A} mice **(A,B,D–F)**. **(A,B)** Low magnification images of chromatolytic neurons showing the euchromatic nuclei with several interchromatin granule clusters (white asterisks) and infoldings of the nuclear envelope. The arrow indicates a Cajal body. Electron-dense cytoplasmic areas, corresponding to local accumulations of free polyribosomes and RER cisterns, appear concentrated at the perinuclear region. Note in **(B)**, a perinuclear cap of polyribosomes at the wrinkled nuclear pole and the presence of large cytoplasmic vacuoles (Vc). **(C–F)** Detail of the perinuclear region in control **(C)** and ALS **(D–F)** motor neurons. The perinuclear cytoplasm exhibits scattered polyribosomes, Golgi cisterns and some mitochondria in the control neuron **(C)**. In contrast, perinuclear accumulations of either concentric arrays of RER cisterns **(D)** or combinations of free polyribosomes and RER cisterns **(E)** are observed in an ALS motor neurons. Note in **(E,F)** that nuclear infoldings contain a great abundance of polyribosomes that fill the depressions of the nuclear envelope. Tangential sections of the nuclear membranes illustrate the great density of nuclear pores at the polyribosome-rich nuclear infoldings [arrows in **(F)** and inset]. Scale bars: **(A,B)** = 2.8 μm; **(C–E)** = 0.9 μm; **(F)** and inset = 600 nm.

Another important point related to global transcriptional activity in motor neurons was the configuration of chromatin. Interestingly, ALS motor neurons with severe chromatolysis and vacuolar degeneration preserved the typical pale euchromatic nucleus of control neurons (**Figure 1**). This chromatin organization was confirmed by electron microscopy analysis in which only the interchromatin granule clusters, nuclear sites of accumulation of splicing factors, and post-transcriptional pre-mRNA processing (Melcák et al., 2000; Spector and Lamond, 2011), stand out on the predominant pattern of euchromatin (**Figures 2A,B**, **3A,B**, and **5A**).

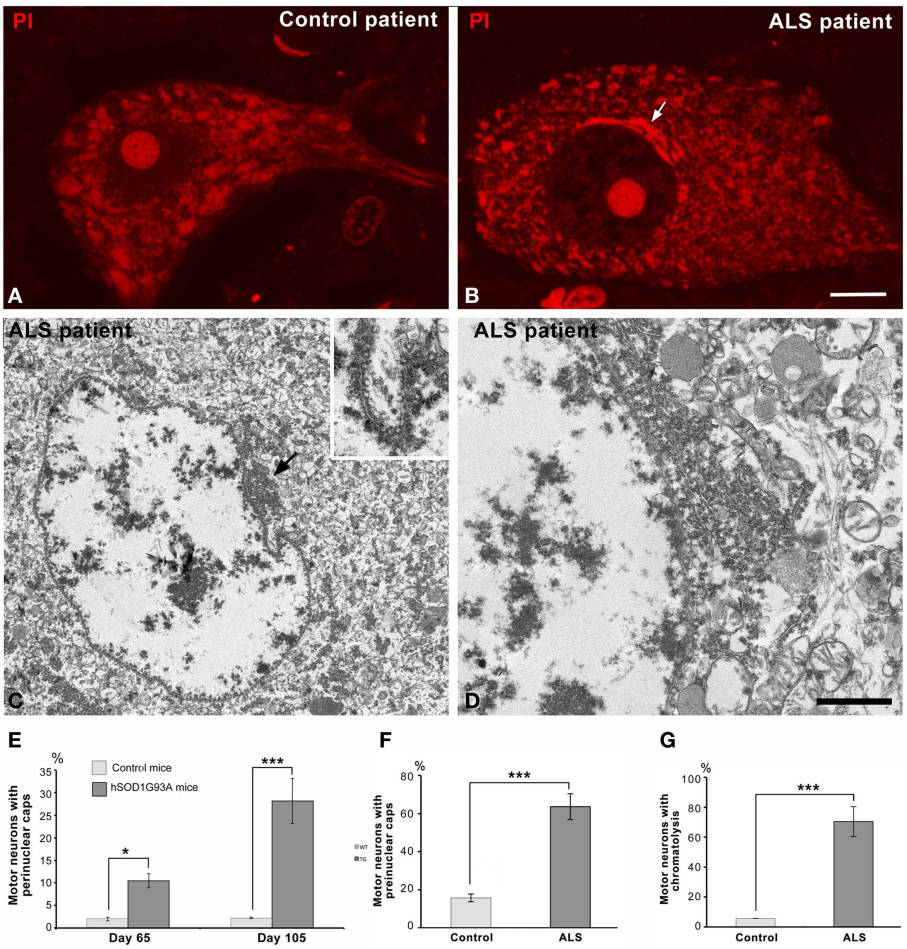

FIGURE 4 | (A,B) Representative examples of human spinal motor neurons from the control **(A)** and the ALS patient **(B)** stained with propidium iodide. Note the typical organization of Nissl bodies in the control neurons and the perinuclear cap (arrow) in the chromatolytic ALS neuron. Both neurons exhibit a prominent nucleolus. Scale bar: **(A,B)**: 8 μm. **(C,D)** Electron micrographs of chromatolytic spinal motor neurons from a patient with sporadic ALS. Nuclei show a predominant euchromatin organization with some small aggregates of heterochromatin at the nuclear interior and associated with the nuclear envelope. Local accumulations of free polyribosomes and RER cisterns appear in the perinuclear cytoplasm, most often at nuclear infolding sites enriched with nuclear pores (arrow). Scale bar: **(C)** = 3 μm; **(D)** = 1 μm; inset: 0.85 μm. **(E)** Proportion of wild type and transgenic ALS motor neurons having perinuclear caps of RER. Data are mean ± SD; $*p < 0.05$, $***p < 0.001$. **(F,G)** Quantitative analysis of the percentage of human control and ALS motor neurons with perinuclear caps **(F)** and chromatolysis **(G)**. At least 100 neurons from the control and the ALS patient were examined.

Furthermore, the 5′-FU transcription assay clearly demonstrated that the extranucleolar transcriptional activity was preserved in ALS motor neurons, as indicated the presence of numerous immunogold particles decorating perichromatin fibrils (Cmarko et al., 1999) throughout euchromatin domains (**Figures 5F,G**). As negative control, we illustrated the conspicuous absence of immunogold particles of 5′-FU incorporation in transcriptionally silent perinucleolar heterochromatin masses (**Figures 5D,E**). The quantitative analysis of the labeling density over the nucleolus and euchromatin, expressed as numbers of gold particles per 1 μm^2, showed no significant differences in both transcription compartments between wild type and ALS motor neurons (**Figure 5J**). As expected, labeling density was higher in the nucleolus than in the euchromatin in both transgenic and wild type animals.

Finally, it is noteworthy that the presence of some immunogold particles decorating the polyribosomes that filled nuclear infoldings, indicating that this perinuclear domain of protein synthesis machinery contains newly synthesized RNAs exported from the nucleus (**Figure 5H**).

DISCUSSION

Neuropathological hallmarks in ALS spinal motor neurons include two main sequential stages: chromatolysis, with its associated vacuolar degeneration of perikaryal cytoplasm and neuronal processes, and apoptosis (Martin, 1999; Oyanagi et al., 2008; Sasaki, 2010). These neuronal alterations appear at the asymptomatic ALS and proceed faster during the symptomatic stage of the disease.

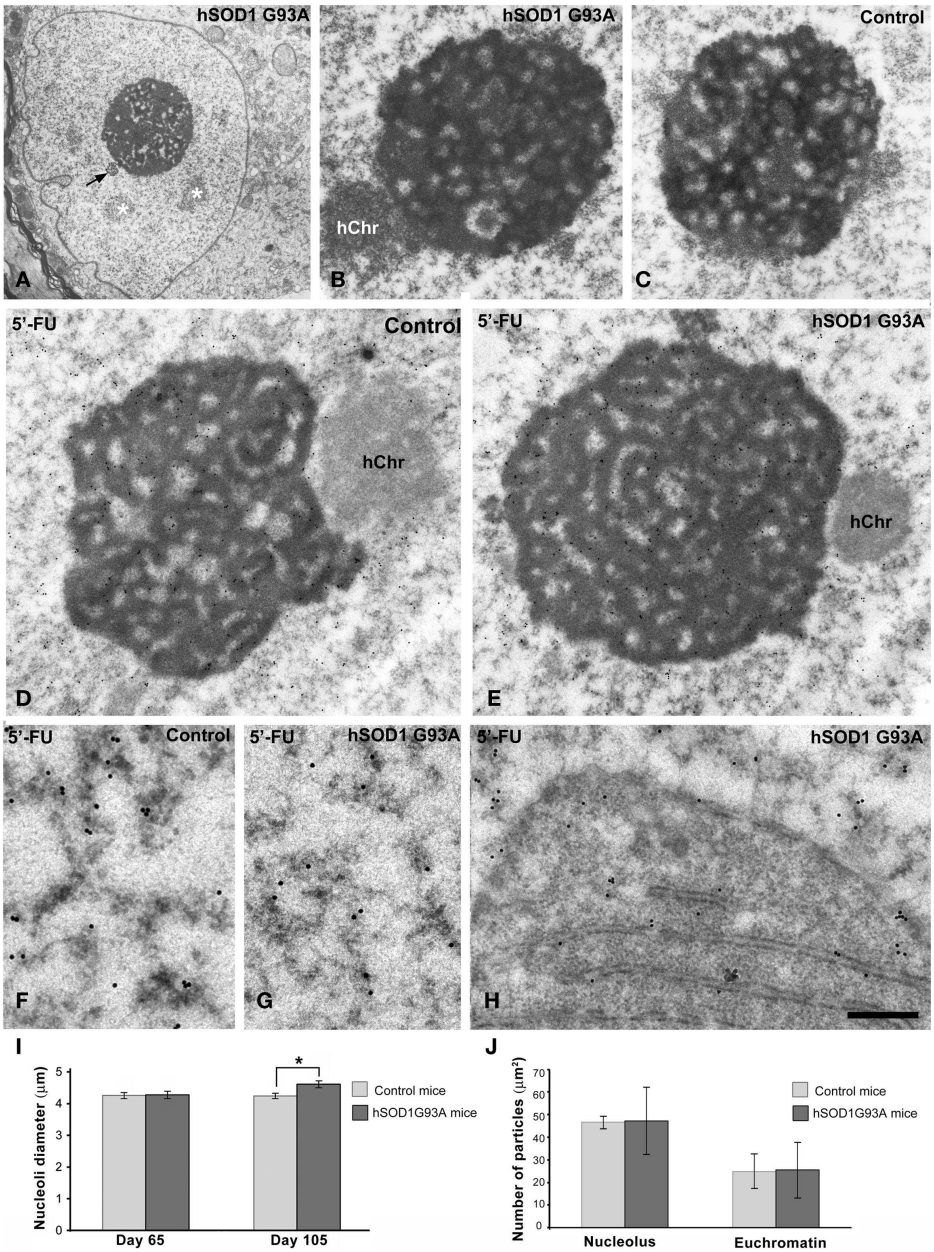

FIGURE 5 | (A–C) Nucleolar organization in motor neurons from control **(C)** and ALS **(A,B)** hSOD1^{G93A} mice. **(A)** ALS chromatolytic neurons with nuclear eccentricity and a prominent nucleolus. Note the presence of interchromatin granule clusters (white asterisks) and a Cajal body (arrow). **(B,C)** In both control and ALS motor neurons, the nucleolus exhibits a typical reticulated configuration with numerous fibrillar centers surrounded by the dense fibrillar component and intercalated masses of the granular component. hChr: nucleolus-associated heterochromatin. **(D,E)** Transcription assay with a 45 min pulse of 5′-FU incorporation into nascent RNA. A similar pattern of 5′-FU incorporation is detected in nucleoli of both control and ALS motor neurons. Immunogold particles of 5′-FU incorporation in nucleolar transcription sites preferentially decorate the dense fibrillar component. Transcriptional activity is also detected throughout the euchromatin, while the transcriptionally silent heterochromatin (hChr) lacks of immunogold particles. **(F,G)** Detail of euchromatin regions from control and ALS motor neurons shows the localization of the extranucleolar transcription sites in perichromatin fibrils, which appear decorated with immunogold particles. (**H**) Some immunogold particles decorate newly synthesized RNA on polyribosomes accumulated at the perinuclear region, within a nuclear invagination, in a motor neuron from the hSOD1^{G93A} mouse. Scale bars: A = 2 μm; B,C = 0.8 μm; D,E = 0.75 μm; F–H = 325 nm. **(I)** Morphometric analysis of the nucleolar diameter in mononucleolated motor neurons from control and hSOD1^{G93A} mice. Data are mean ± SE from three independent experiments; $*p < 0.05$. At least 100 neurons per animal group were sampled. **(J)** Labeling density of gold particles detecting 5′-FU incorporation in euchromatin and nucleolar compartments. Data are mean ± SD (see additional information in Materials and Methods).

Our results in motor neurons of the hSOD1^{G93A} mouse and in the ALS patient indicate that the progression of chromatolysis associates with formation of SGs enriched in eIF3, a signature component of SGs required for their assembly (Ohn et al., 2008). Under physiological conditions translation initiation and translational silencing rates are in equilibrium and most cytoplasmic mRNA is located in polyribosomes (Anderson and Kedersha, 2008). Several studies indicate that ER stress shift this balance resulting in increased rate of translational silencing and sequestration of the excess of mRNAs released from polyribosomes in SGs (Kedersha et al., 2013). Recent biochemical studies have shown activation of ER stress pathways during motor neuron degeneration in the hSOD1^{G93A} mouse model (Kikuchi et al., 2006; Nagata et al., 2007; Saxena et al., 2009; Sasaki, 2010). Thus, the formation of SGs reported here in ALS motor neurons is consistent with an ER stress-induced chromatolytic disassembly of polyribosomes and subsequent recruitment of some released mRNAs into SGs, as being suggested by their cytochemical staining with propidium iodide. Moreover, previous studies in ALS cellular models and brain tissues from ALS patients have reported the recruitment of TDP-43 and FUS to SGs, two RNA-binding proteins involved in ALS pathogenesis (Volkening et al., 2009; Liu-Yesucevitz et al., 2010; Bentmann et al., 2012).

Previous electron microscopy studies of the RER in motor neurons of ALS patients have reported various types of alterations, such as cisternal distension with ribosomal detachment, intracisternal accumulations of amorphous material and, occasionally, formation of lamellar bodies, RER arrays with intercisternal electron-dense material (Oyanagi et al., 2008; Sasaki, 2010). Our ultrastructural analysis in motor neurons of the hSOD1^{G93A} mouse model suggest that, in addition to polyribosome detachment, the massive fragmentation of RER cisterns in numerous small vesicles with remnants of membrane-bound polyribosomes is a major structural component in chromatolytic areas of the cytoplasm. RER-derived small vesicles are clearly distinguishable from larger vacuoles of several micrometers in diameter characteristic of the vacuolar degeneration in ALS motor neurons (Sasaki et al., 2004). Another alteration frequently found in RER arrays is the occlusion of the cisternal lumen accompanied by polyribosome detachment. This structural modification can prevent protein synthesis and peptide translocation and processing in the RER lumen. Taken together, these RER alterations provide new structural basis for the disruption of Nissl bodies and translational arrest in chromatolytic neurons.

However, it is worth noting that in some experimental models of axotomy and neurotoxicity chromatolysis is a reversible dysfunction of protein synthesis machinery, indicating the activation of neuroprotective mechanisms leading to neuronal recovery of function (Kinderman and Jones, 1993; Palanca et al., 2014a). In the current hSOD1^{G93A} mouse model, we show that chromatolysis induces compensatory neuronal mechanisms, which might allow chromatolytic motor neurons tolerate a severe dysfunction of proteostasis until the final activation of apoptosis. Such mechanisms include the perinuclear reorganization of the protein synthesis machinery, the higher-order chromatin organization in predominant active euchromatin, and the nucleolar activity in ribosome biogenesis.

An important observation in motor neurons from both hSOD1^{G93A} mice and the ALS patient is the preferential perinuclear reorganization of protein synthesis machinery, in close proximity to the nuclear envelope. It is well known that the nuclear envelope environment provides a specialized region for a wide range of cellular functions, such as signal transduction from cytoskeleton to nucleus, regulation of nuclear morphology, transcription at the nuclear periphery and nucleo-cytoplasmic traffic (for a review, see Mekhail and Moazed, 2010; Wilczynski, 2014). A similar perinuclear reorganization of RER and free polyribosomes has been reported in recovery neurons from axotomy or proteasome inhibition (for a review, see Lieberman, 1971; Palanca et al., 2014a). This perinuclear organization is particularly prominent at the dendritic nuclear pole of normal Purkinje cells (Palay and Chan-Palay, 1974), where the local accumulation of the protein synthesis machinery seems to facilitate the transfer of new-synthesized proteins and mRNAs to the dendritic tree. In the case of ALS, motor neuron accumulation of RER and free polyribosomes frequently occur at sites of nuclear infoldings, where increased nuclear surface is studded with high density of nuclear pores. This spatial arrangement decreases diffusion distances of RNAs from the nucleus to protein synthesis machinery, likely facilitating perinuclear translation at the proximity of nuclear pores. In fact, we have observed that newly synthesized RNAs are rapidly exported to the protein synthesis machinery that filled nuclear infoldings after a short pulse of 45 min of 5′-FU administration. Moreover, in an animal model of hippocampal neurons stimulation, the induction of nuclear infoldings has also been related to enhancing transcription at the proximity of nuclear pores (Akhtar and Gasser, 2007; Wittmann et al., 2009). In this context, we consider that the perinuclear reorganization of protein synthesis machinery reflects a compensatory reactive response to chromatolysis, in order to preserve the translational activity required for neuronal survival. The molecular mechanisms underlying ER biogenesis at the perinuclear region in response to the disturbance of ER proteostasis are unknown. The ER is a dynamic compartment that can be expanded according to cellular demands of secretory proteins, although the molecular mechanisms that regulate the synthesis of phospholipid and proteins required for its biogenesis are poorly understood (Sriburi et al., 2004). In the context of ER stress in neurodegenerative disorders, the adaptive outcome of the UPR includes the activation of the spliced form of the transcription factor XBP1s (X-box binding protein) that has neuroprotective activity (Casas-Tinto et al., 2011; Hetz and Mollereau, 2014). Interestingly, cellular and biochemical studies have demonstrated that this factor links mammalian UPR to phospholipid biosynthesis and ER biogenesis (Sriburi et al., 2004). Further studies will be needed to determine whether XBP1s is involved in the perinuclear reorganization of ER observed in ALS motor neurons.

Regarding chromatin organization in ALS motor neurons, what is noteworthy is their predominant euchromatin pattern, a chromatin architecture associated with permissive transcriptional activity (for a review, see Wilczynski, 2014). Indeed, the 5′-FU

incorporation assay clearly demonstrated that the transcriptional activity is preserved in the extensive euchromatin domains where immunogold particles decorate perichromatin fibrils, nuclear sites of transcription, and cotranscriptional pre-mRNA processing enriched in nascent pre-mRNA transcripts (Cmarko et al., 1999; Casafont et al., 2006; Spector and Lamond, 2011). Maintenance of euchromatin architecture and transcriptional activity in ALS motor neurons could be essential for the activation of genes involved in the neuronal stress response to the disturbance of ER proteostasis (Steffen et al., 2010; Hetz and Mollereau, 2014) and consequently for neuronal survival. In contrast, in the *pcd* ("Purkinje cell degeneration") mouse model of neurodegeneration, large-scale chromatin condensation is associated with transcriptional silencing, absence of cellular signs of neuroprotection, and rapid induction of apoptosis in Purkinje cells (Baltanas et al., 2011b).

Concerning the nucleolar response to chromatolysis in motor neurons of the hSOD1^{G93A} mice, it is noteworthy that the preservation of the size, reticulated configuration, and high transcriptional activity of the nucleolus under conditions of severe chromatolysis. Similarly, a high rate of rRNA synthesis has been reported in experimental models of neuronal injury with severe chromatolysis, such as axotomy and treatment with proteasome inhibitors, which trigger a neuroprotective compensatory response to promote functional recovery (Kinderman and Jones, 1993; Wells and Vaidya, 1994; Palanca et al., 2014b). Interestingly, motor neurons in the ALS mouse model preserve a reticulated nucleolar configuration with numerous fibrillar centers, a reliable feature of transcriptionally very active neurons (Lafarga et al., 1991; Peters et al., 1991; Pena et al., 2001; Berciano et al., 2007), in addition to a high nucleolar incorporation of 5′-FU into nascent rRNAs. These results support an active participation of nucleolar transcription as compensatory mechanisms to chromatolysis. These findings are also consistent with our recent study (Palanca et al., 2014b) demonstrating high nucleolar incorporation of 5′-FU and upregulation of genes encoding UBF, fibrillarin, and B23, three essential proteins for rRNA synthesis and processing (Boisvert et al., 2007; Hernandez-Verdun et al., 2010; Grummt, 2013), as a neuroprotective compensatory response to proteasome inhibition. In contrast, in Parkinson's disease and other neurodegenerative disorders with severe nucleolar dysfunction, the impairment of rRNA transcription and disruption of nucleolar integrity cause nucleolar stress (Baltanas et al., 2011a; Hetman and Pietrzak, 2012; Parlato and Kreiner, 2013; Parlato and Liss, 2014).

In this scenario, the high rRNA transcription observed in ALS motor neurons of the hSOD1^{G93A} mice could be a compensatory attempt by chromatolytic neurons to enhance ribosome biogenesis required for neuronal survival. Epigenetic activation of chromatin and nucleolar transcription could have important therapeutic implications for neuroprotection in ALS and other neurodegenerative disorders. Although histone deacetylase inhibitors are currently used as therapeutic agents, we raise the untapped potential of the nucleolar transcription of ribosomal genes as an exciting new target for the therapy of some neurodegenerative diseases.

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RNA-mediated pathogenic mechanisms in polyglutamine diseases and amyotrophic lateral sclerosis

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Gene transcription produces a wide variety of ribonucleic acid (RNA) species in eukaryotes. Individual types of RNA, such as messenger, structural and regulatory RNA, are known to play distinct roles in the cell. Recently, researchers have identified a large number of RNA-mediated toxicity pathways that play significant pathogenic roles in numerous human disorders. In this article, we describe various common RNA toxicity pathways, namely epigenetic gene silencing, nucleolar stress, nucleocytoplasmic transport, bi-directional gene transcription, repeat-associated non-ATG translation, RNA foci formation and cellular protein sequestration. We emphasize RNA toxicity mechanisms that involve nucleotide repeat expansion, such as those related to polyglutamine (polyQ) disorders and frontotemporal lobar degeneration-amyotrophic lateral sclerosis.

Keywords: polyglutamine disease, Drosophila models, C9orf72, repeat expansion-associated non-ATG translation, nucleolin

INTRODUCTION

Ribonucleic acids (RNAs) are polymeric macromolecules composed of a wide variety of nucleotide building blocks. Various classes of RNA have been reported to date, of which major examples are messenger RNAs (mRNAs), ribosomal RNAs (rRNAs), transfer RNAs, and non-coding RNAs. Each RNA class is distinct in terms of synthesis, properties and functions. It is widely acknowledged that most RNAs have cellular roles in gene regulation and protein translation. However, a few RNAs are known to have less common functions such as serving as genetic materials for RNA viruses. In cells, RNAs are transcribed by RNA polymerase according to sequences on the DNA template. A subset of RNAs is further modified through various processing steps such as cleavage, base modification and editing. Such modifications are crucial to the generation of mature and fully functional RNA molecules.

In addition to the well-established roles of various classes of RNA molecules in neuronal (Iyengar et al., 2014) and brain (Follert et al., 2014) development, many RNAs are known to be involved in neural pathologies (Cooper et al., 2009). Recent investigations have provided a more thorough understanding of the pathogenic roles of RNAs in neurological disorders. The dysregulation of the cellular processes that govern RNA metabolism is now known to contribute to neuronal dysfunctions and diseases. Such perturbation may be caused by the alteration of RNA transcription, splicing, editing and/or nuclear export due to genetic predisposition or as a consequence of normal aging (Cooper et al., 2009; Da Cruz and Cleveland, 2011; Johnson et al., 2012; Singh, 2012; Belzil et al., 2013b; Nalavade et al., 2013; Caillet-Boudin et al., 2014). Genome microsatellite instability, particularly nucleotide repeat expansion, has been shown to cause a number of human genetic diseases (Mirkin, 2007), many of which are neurological diseases such as amyotrophic lateral sclerosis (ALS), Huntington's Disease (HD) and spinocerebellar ataxias (SCAs; **Figure 1**; Cruts et al., 2013; Nalavade et al., 2013; Tsoi and Chan, 2014). The toxic effect of nucleotide repeat expansion has been demonstrated to be caused by to a gain-of-function mechanism. Mutant RNA molecules that harbor expanded repeat sequences tend to form intracellular RNA foci, which are pathogenic hallmark of many of the diseases listed above (Wojciechowska and Krzyzosiak, 2011). The involvement of many RNA-binding proteins in human diseases is unsurprising, as RNAs often take the form of ribonucleoprotein complexes in cells (Lukong et al., 2008). Protein sequestration and RNA foci formation have been reported to play important roles in the gain-of-function pathogenic mechanisms that produce neuronal RNA toxicity. Mutant RNAs may also interfere with gene transcription via RNA-mediated gene silencing mechanisms (Colak et al., 2014). In addition, mutant RNAs may confer neurotoxicity at the protein level through a repeat-associated non-ATG translation mechanism (Cleary and Ranum, 2014). In this review, recent advances in research on the above mentioned RNA toxicity mechanisms will be discussed.

NUCLEOTIDE REPEAT EXPANSION AS A PATHOGENIC MECHANISM OF NEURODEGENERATION

In 1991, the mutations causing fragile-X syndrome (Kremer et al., 1991; Verkerk et al., 1991) and spinobulbar muscular atrophy/Kennedy's disease (La Spada et al., 1991) were identified. Incidentally, the molecular pathogenic mechanisms of

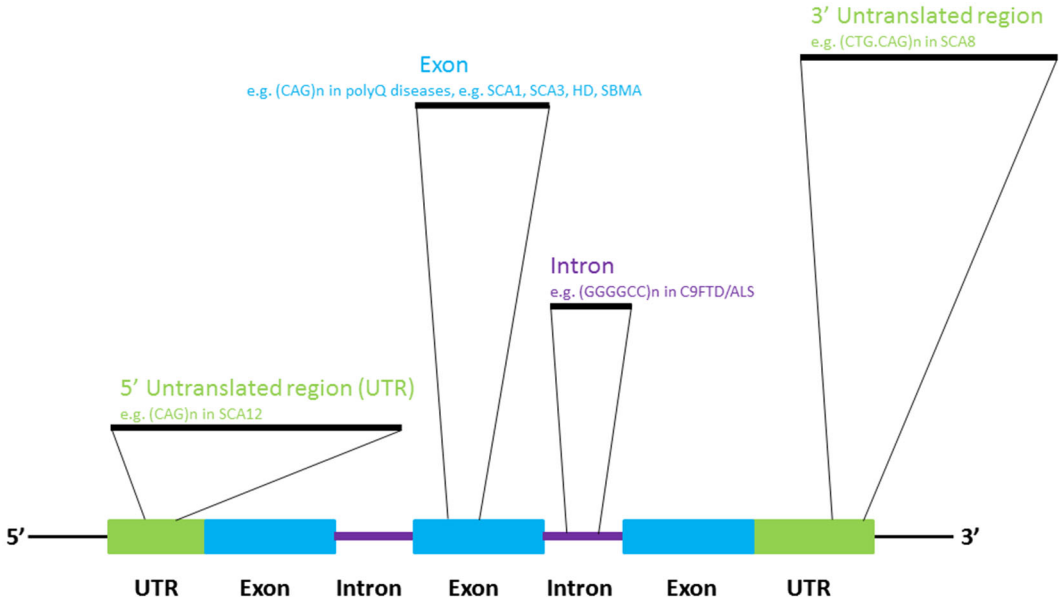

FIGURE 1 | Select diseases that display RNA repeat expansion-mediated toxicities.

both diseases stem from the inter-generational expansion of genomic triplet nucleotide repeat sequences. To date, more than 20 neurological diseases have been reported to be caused by microsatellite sequence expansion mechanism (Polak et al., 2013), such as HD (Macdonald, 1993), several types of spinocerebellar ataxia (Rüb et al., 2013) and frontotemporal lobar degeneration-amyotrophic lateral sclerosis (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Gijselinck et al., 2012; **Figure 1**). We now know that repeat-expansion mutation is closely associated with the unique structural features of repetitive DNA sequences, and perturbs cellular DNA replication, repair and recombination (Mirkin, 2007).

ASSOCIATION BETWEEN PATHOGENIC PATHWAYS AND CAG REPEAT EXPANSION IN THE PROTEIN-CODING REGION

The expansion of CAG trinucleotide repeats in the protein-coding regions of the human genome is the pathogenic mechanism for many neurological diseases (Nalavade et al., 2013). Protein translation leads to the production of proteins that harbor expanded stretches of glutamine amino acid residues. Various mouse models of polyglutamine (polyQ) diseases have been developed (Figiel et al., 2012) to examine the role of expanded polyQ proteins in disease pathogenesis. Since the beginning of the new millennium, *Drosophila* disease models have offered an alternative means of investigating the genetic pathogenic pathways involved in nucleotide-expansion diseases (McLeod et al., 2005), such as polyQ diseases (Chan and Bonini, 2000; Yu and Bonini, 2011). To investigate the pathogenic role of expanded CAG sequences at the RNA level, Li et al. (2008) expressed an artificial *DsRed* reporter that carried an expanded allele of CAG sequence located in the 3′ untranslated region of the transgene in the nervous system, and observed neurodegeneration in the retina and the brain. Intriguingly, toxicity was reduced when the CAG repeat continuity of the mutant allele was intermittently disrupted by a CAA codon. This indicates that the repeat continuity of CAG triplets is essential to RNA toxicity (Li et al., 2008). Expanded CAG RNA toxicity was also observed in *Caenorhabditis elegans* (Wang et al., 2011a) and mouse (Hsu et al., 2011) models.

EXPANDED CAG RNA PATHOGENIC MECHANISMS

ALTERNATIVE SPLICING OF RNAs

Expanded CAG RNAs form foci in cell and animal models, and in patient cells (Li et al., 2008; de Mezer et al., 2011; Hsu et al., 2011; Wang et al., 2011a; Wojciechowska and Krzyzosiak, 2011). Muscleblind-like (MBNL) proteins are a group of RNA-binding proteins that contain four zinc-finger domains, and are involved in the regulation of RNA alternative splicing (Konieczny et al., 2014). Artificial expanded CAG RNA binds to MBNL1 with a high affinity (~11 nM, as determined by filter-binding assay; Yuan et al., 2007). It was reported that MBNL1 protein sequestered to CAG RNA foci formed by *artificial CAG*, *ataxin-3* (*ATXN3*) and *huntingtin* (*htt*) transcripts (de Mezer et al., 2011; Hsu et al., 2011; Mykowska et al., 2011; Wang et al., 2011a). A group of MBNL1-regulated genes examined in cell models demonstrated alternative splicing alterations, such as neuronal cell lines that expressed expanded CAG constructs and SCA3 patient fibroblasts (Mykowska et al., 2011). The overexpression of MBNL1 may partially restore artificial expanded CAG RNA-induced alternative splicing defects in Mykowska et al. (2011). This demonstrates a correlation between MBNL1 recruitment to CAG RNA foci and MBNL1 dysfunction in the RNA toxicity of polyQ diseases (**Figure 2**). However, alternative splicing defects were not observed in an *in vivo DsRed-CAG*$_{270}$ transgenic *Drosophila* model (Li et al., 2008). The overexpression of MBNL ortholog in a *C. elegans* expanded CAG RNA model may partially mitigate the

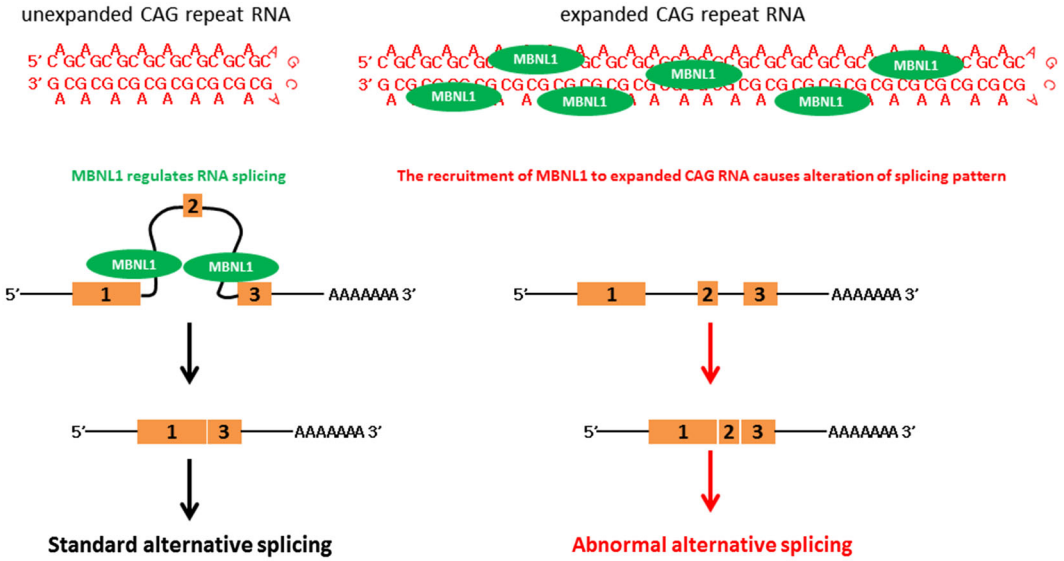

FIGURE 2 | CAG triplet repeat RNA causes aberrant RNA splicing.

expanded artificial CAG RNA phenotype of transgenic worms (Wang et al., 2011a).

TOXICITY OF HAIRPIN AND DOUBLE-STRANDED CAG REPEAT RNAs

In addition to alternative splicing defects, small CAG RNAs are responsible for HD toxicity (Bañez-Coronel et al., 2012; **Figure 3**). Although these CAG RNA repeat sequences do not form foci in cells, they bind MBNL1 with an affinity similar to that of long CAG RNAs (Mykowska et al., 2011). Unexpanded short CAG repeat RNAs (sRNAs), such as $(CAG)_7$ or $(CAG)_{20}$, have been shown to induce alternative splicing defects in mammalian cells (Mykowska et al., 2011). More recently, Bañez-Coronel et al. (2012) detected *htt* small RNAs comprising approximately seven CAG repeats in cell models, mouse HD disease models and patient brain samples. The production of such sRNAs causes caspase activation and cytotoxicity and the number of CAG-repeat sRNAs is dependent on the CAG repeat length (Bañez-Coronel et al., 2012). Dicer is a RNase III family ribonuclease responsible for cleaving double-stranded precursor RNAs to generate sRNAs, and plays a key role in RNA interference (Bernstein et al., 2001). Expanded CAG RNAs form double-stranded RNA hairpins with A-A mismatches (Sobczak et al., 2003; Kiliszek et al., 2010; de Mezer et al., 2011; Busan and Weeks, 2013; Yildirim et al., 2013). Bañez-Coronel et al. (2012) showed that expanded CAG RNAs are good substrates of Dicer. A knockdown of Dicer expression reduces the level of CAG sRNAs in HD and SCA type 1 (Krol et al., 2007; Bañez-Coronel et al., 2012), which further indicates the involvement of RNAi machinery in the production of CAG sRNAs. Cleaved sRNAs participate in posttranscriptional gene silencing. The role of CAG sRNAs in RNA interference relies on their ability to interact with complementary CUG RNA tracts in cellular RNAs. The genes *MEIS2* and *ADORA2* have also been reported to be down-regulated in HD, and their transcripts have been predicted to form RNA duplexes with CAG sRNAs (Hodges et al., 2006). The overexpression of CAG sRNAs in cells reduces endogenous levels of *MEIS2* and *ADORA2* (Bañez-Coronel et al., 2012). This explains the role of CAG sRNAs in silencing cellular gene expression in CAG triplet repeat expansion diseases in general.

In addition to expanded CAG hairpin RNA, the transgenic expression of two complementary expanded CAG and CUG RNA sequences in the *Drosophila* eye causes deleterious external eye morphology (Lawlor et al., 2011; Yu et al., 2011). In contrast with CAG RNAs which adopt a double-stranded RNA hairpin structure with A-A mismatches (Sobczak et al., 2003; Kiliszek et al., 2010; de Mezer et al., 2011; Busan and Weeks, 2013; Yildirim et al., 2013), duplex CAG/CUG RNAs are expected to form perfectly-matched double-stranded CAG-CUG RNAs. In addition, Lawlor et al. showed that neither the expression of CAG nor CUG RNA elicits toxicity in flies (Lawlor et al., 2011), and that the rough eye phenotype induced by CAG-CUG co-expression was dependent on Dicer activity (Lawlor et al., 2011; Yu et al., 2011; **Figure 3**). sRNAs were reported in flies expressing single-stranded CAG hairpin RNAs and CAG-CUG duplex RNAs (Lawlor et al., 2011). However, CAG sRNAs were observed to be more prominent in the CAG-CUG dsRNA-expressing flies. At the molecular level, Lawlor et al. (2011) also detected altered miRNA levels in the CAG-CUG dsRNA-expressing flies. It is worth noting that miRNAs have been implicated in neurodegeneration (Abe and Bonini, 2013).

NUCLEOLAR STRESS AND RNA TOXICITY

The results of a biochemical study conducted by our group indicate that in contrast with unexpanded CAG transcripts (both *ATXN3* and *artificial CAG* RNA), RNAs that carry expanded CAG repeats are enriched in the nuclear fraction (Tsoi et al., 2011). This finding suggests that nucleocytoplasmic transport of the mutant

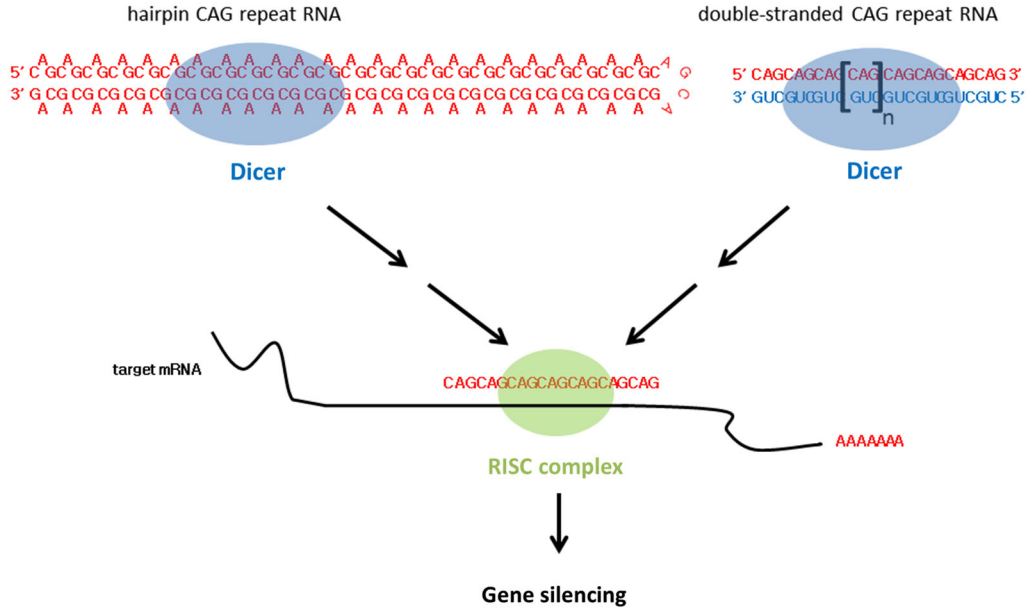

FIGURE 3 | CAG triplet repeat RNA leads to gene silencing.

RNAs is perturbed. A follow-up genetic investigation using *Drosophila* model identified *U2AF50*, *Drosophila* ortholog of U2 small nucleoprotein auxiliary factor 65 (U2AF65; Blanchette et al., 2004), as a genetic modifier of expanded CAG RNA nucleocytoplasmic localization. The export of unexpanded *ATXN3* and pure CAG repeat transcripts was not affected. Knockdown of *U2AF50* expression in flies was found to intensify the neurodegenerative phenotype in flies (Tsoi et al., 2011). Tsoi et al. also identified a ribonucleoprotein complex consisting of mutant *ATXN3* RNA, U2AF65 and nuclear RNA export factor 1 in mammalian cells. This complex is responsible for mediating mutant RNA export. Knockdown of *U2AF65* expression in cells leads to the nuclear retention of mutant *ATXN3* RNA and triggers caspase activation (Tsoi et al., 2011). This finding confirms the involvement of the nucleus in expanded CAG RNA toxicity. Interestingly, the results of a HD mouse model study indicate that the level of developmental expression of U2AF65 decreases with age, and that this decline attenuates the nuclear export of mutant *htt* RNAs (Tsoi et al., 2011). This suggests that the nuclear accumulation of mutant *htt* RNA is a direct consequence of developmental decline in the cellular abundance of U2AF65, which coincides with the progressive degeneration of HD.

Within the nuclear compartment, expanded CAG RNAs, including *ATXN3*, co-localize the nucleolar protein fibrillarin (Tsoi et al., 2012), suggesting that mutant RNAs interfere with nucleolar function (**Figure 4**). The cell nucleolus is the site of production of ribosome subunits, and the dysregulation of ribosome biogenesis has been shown to cause disease (Kressler et al., 2010). RNA polymerase I is responsible for the transcription of rRNA precursors (pre-rRNAs). rRNA is an essential component of the ribosome, a ribonucleoprotein complex responsible for protein translation. The inhibition of pre-rRNA transcription has been shown to cause apoptosis in neurons (Kalita et al., 2008). "Nucleolar stress" denotes the cellular pathway used by the nucleolus to communicate with cytosolic compartments (Boulon et al., 2010), such as the mitochondria (Lindenboim et al., 2011) and thereby initiate apoptosis. It is a universal and efficient mechanism for eliminating cells that are defective in protein synthesis due to ribosome biogenesis failure. Our group recently demonstrated a link between nucleolar stress and RNA toxicity in polyQ diseases, including SCA type 3 and HD (Tsoi et al., 2012; Tsoi and Chan, 2013, 2014). We found that nucleolin (NCL), a nucleolar protein that regulates rRNA transcription (Durut and Sáez-Vásquez, 2014), interacts directly and specifically with expanded CAG RNAs, and that such binding is mediated by the RNA-recognition motifs of NCL (Tsoi et al., 2012). This RNA/protein interaction prevents NCL protein from binding to the *upstream control element* (*UCE*) of the rRNA promoter, which results in *UCE* DNA hypermethylation. This leads to the down-regulation of rRNA transcription. We further showed that p53 protein becomes stabilized in cells and concentrated in the mitochondria. This causes the release of cytochrome c from the mitochondria, caspase activation and apoptosis induction (Tsoi et al., 2012). Although restoring the efficient nuclear export of mutant RNAs would alleviate nucleolar stress-mediated RNA toxicity (Tsoi and Chan, 2014), the exported cytosolic expanded CAG RNAs could cause the synthesis of expanded polyQ disease protein which might in turn trigger protein-mediated toxicity in cells (Williams and Paulson, 2008). To ensure a suitable therapeutic treatment for polyQ diseases, both RNA and protein toxicities must be effectively handled.

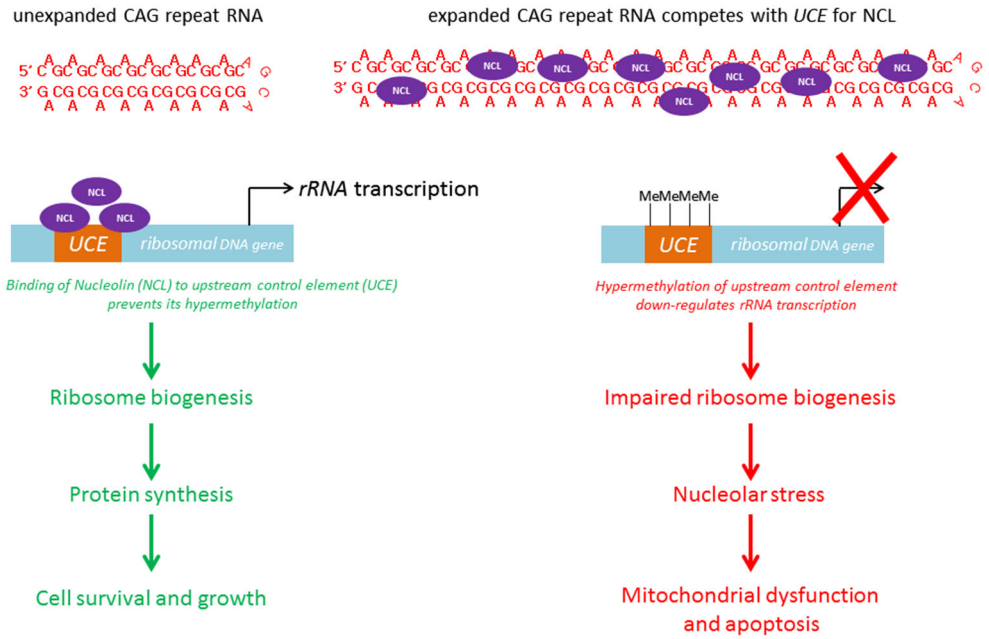

FIGURE 4 | CAG triplet repeat RNA induces nucleolar stress.

BI-DIRECTIONAL TRANSCRIPTION AND REPEAT-ASSOCIATED NON-ATG TRANSLATION OF CAG EXPANSION TRANSCRIPTS

Bi-directional transcription has been reported in the human genome, yielding both sense and anti-sense transcripts of complementary sequences. The anti-sense transcripts are now known to play important regulatory roles in eukaryotic gene expression (Faghihi and Wahlestedt, 2009). Spinocerebellar ataxia type 8 (SCA8) is caused by a CTG.CAG repeat expansion in the 3′ untranslated region of the *ataxin-8* gene (*ATXN8*; **Figure 5**; Koob et al., 1999). On gene transcription, the CAG-containing *ATXN8* and CUG-containing *ATXN8 opposite strand* (*ATXN8OS*) transcripts were detected in SCA8 patients (Moseley et al., 2006), and nuclear CUG *ATXN8OS* foci were found in HEK293 cells (Chen et al., 2009), the SCA8 mouse model and SCA8 patients (Daughters et al., 2009). Further, ribonuclear *ATXN8OS* foci were found to be co-localized with MBNL1 and displayed an alternative splicing pattern in both SCA8 mice and SCA8 patients (Daughters et al., 2009), demonstrating a gain-of-function role for expanded CUG *ATXN8OS RNA* in the pathogenesis of SCA8.

Under normal physiological conditions, protein synthesis is mediated by ATG initiation codon-dependent mRNA translation. Recently, Zu et al. (2011) discovered a repeat expansion-associated non-ATG translation (RAN) mechanism that facilitates protein synthesis in repeat expansion diseases. Interestingly, when an *ATXN8* transgene carrying a mutated ATG initiation codon was expressed in HEK293 cells, the CAG-containing region of the mutant *ATXN8* CAG transcripts was still able to produce protein products carrying a polyQ, a polyalanine or a polyserine domain (Zu et al., 2011). These homopolymeric proteins were translated from all three different reading frames of the ATG-defective *ATXN8* transcripts through the RAN mechanism (**Figure 5**). Long CAG repeat-containing transcripts are prone to frameshift error during protein translation and have been reported to cause the production of polyalanine and polyserine proteins in HD mouse model and HD patients (Davies and Rubinsztein, 2006), and polyalanine proteins in SCA3 patients (Gaspar et al., 2000). Both *in vitro* and *in vivo* analyses showed that these homopolymeric proteins are toxic and thus play a role in pathogenesis (Zu et al., 2011; van Eyk et al., 2012).

NON-TRANSLATING EXPANDED CAG REPEAT SEQUENCES UP-REGULATE GENE TRANSCRIPTION

Spinocerebellar ataxia type 12 is caused by the expansion of CAG triplet sequences in exon 7 of the brain-specific regulatory subunit of the protein phosphatase 2A gene *PPP2R2B* which constitutes part of the non-coding region of the gene (Holmes et al., 1999). The *PPP2R2B* promoter, which carries an expanded CAG allele, demonstrated greater transcriptional activity (Lin et al., 2010), and the transgenic overexpression of the human *PPP2R2B* gene in *Drosophila* caused neurodegeneration (Wang et al., 2011b), indicating that, in addition to glutamine-coding CAG triplet expansion, non-translating CAG repeat sequences can cause toxicity via the up-regulation of gene expression.

DOUBLE-STRANDED RNA-DEPENDENT PROTEIN KINASE BINDS MUTANT *htt* TRANSCRIPTS AND ITS ACTIVITY IS INDUCED IN HD

Protein kinase R (PKR), also known as double-stranded RNA-dependent protein kinase, regulates protein translation by

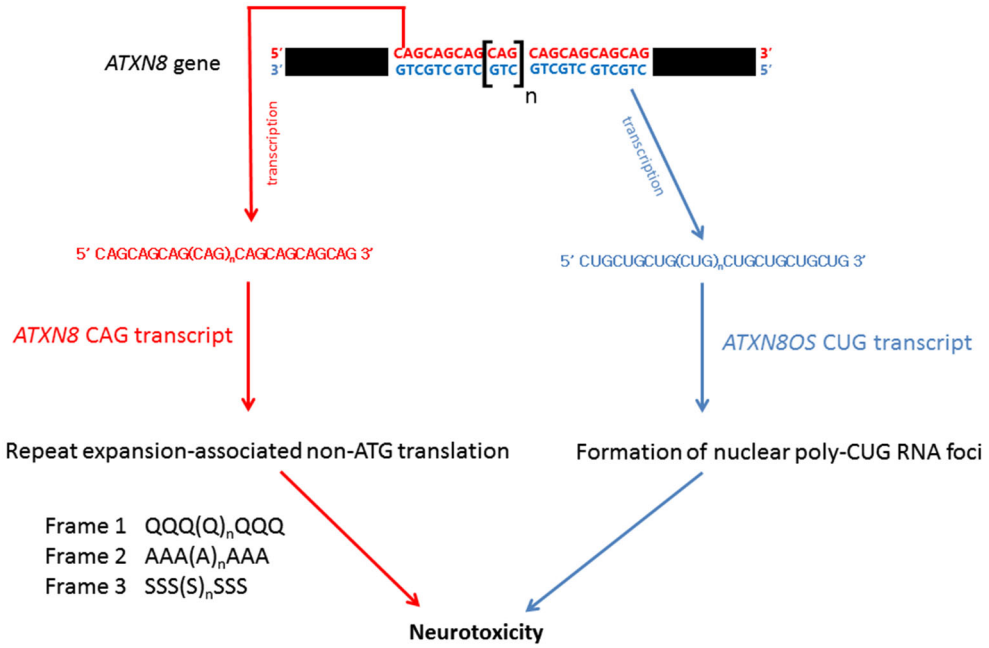

FIGURE 5 | RNA-mediated pathogenic pathways that associate with spinocerebellar ataxia type 8.

mediating the protein phosphorylation of eukaryotic initiation factor 2α (eIF2α; Marchal et al., 2014). Double-stranded RNAs, such as viral RNAs, have been shown to be the main activators of PKR. Expanded CAG transcripts, including *htt* and the *androgen receptor* (for spinobulbar muscular atrophy) RNAs, were reported to form double-stranded hairpin structures (de Mezer et al., 2011; Busan and Weeks, 2013). When *in vitro* transcribed biotinylated mutant *htt* RNA was incubated with human brain extracts, PKR was identified as the *htt* RNA-binding protein (Peel et al., 2001), demonstrating that *htt* transcripts are able to interact with PKR. The autophosphorylation of PKR is an indication of its activation (Marchal et al., 2014), and phospho-PKR immunoreactivity was detected in a HD transgenic mouse model and HD patients (Peel et al., 2001; Bando et al., 2005). As PKR activation causes the phosphorylation of eIF2α and subsequently leads to the induction of apoptosis (Peel, 2004), the binding of mutant *htt* transcripts to PKR may serve as a trigger to initiate neuronal cell death in HD.

OTHER PATHWAYS

The results of recent genome-wide microarray and genetic analyses (Shieh and Bonini, 2011; van Eyk et al., 2011) have shown that in addition to alternative splicing (Mykowska et al., 2011), mRNA down-regulation (Bañez-Coronel et al., 2012), miRNA alteration (Lawlor et al., 2011) and nucleolar stress (Tsoi et al., 2012; Tsoi and Chan, 2013), other gene pathways are responsible for expanded CAG RNA toxicity. However, detailed mechanisms of these pathways are yet to be fully elucidated.

ASSOCIATION BETWEEN PATHOGENIC PATHWAYS AND GGGGCC REPEAT EXPANSION IN NON PROTEIN-CODING REGIONS

The expansion of non-coding GGGGCC repeats in a gene named *Chromosome 9 open reading frame 72 (C9orf72)* has been identified as a cause of ∼40% of hereditary ALS and 25% of familial frontotemporal dementia (FTD) cases. Together, these conditions are generally termed C9FTD/ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Recent findings indicate that gain-of-function toxicity contributes significantly to C9FTD/ALS pathogenesis. Here, we discuss some recent advances in RNA-mediated gain-of-function toxicity in C9FTD/ALS (**Figure 6**).

INFLUENCE OF EPIGENETIC REGULATION AND G-QUADRUPLEX DNA FORMATION ON *C9orf72* GENE EXPRESSION

The *C9orf72* gene product belongs to the guanine nucleotide exchange factor protein family and has been implicated in intracellular membrane trafficking (Zhang et al., 2012; Levine et al., 2013). The down-regulation of *C9orf72* gene expression leads to toxicity due to a loss-of *C9orf72* function in C9FTD/ALS. The GGGGCC-repeat region lies between two alternatively spliced non-coding exons of the *C9orf72* gene (DeJesus-Hernandez et al., 2011; Belzil et al., 2013a). The findings of various independent studies indicate that the expression level of *C9orf72* transcripts is reduced in C9FTD/ALS patients (DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Belzil et al., 2013a; Fratta et al., 2013). Recently, Waite et al. (2014) detected a reduction in C9ORF72 protein levels in C9FTD/ALS patients with the GGGGCC repeat expansion. This finding further supports the hypothesis that C9FTD/ALS entails a loss-of-protein function.

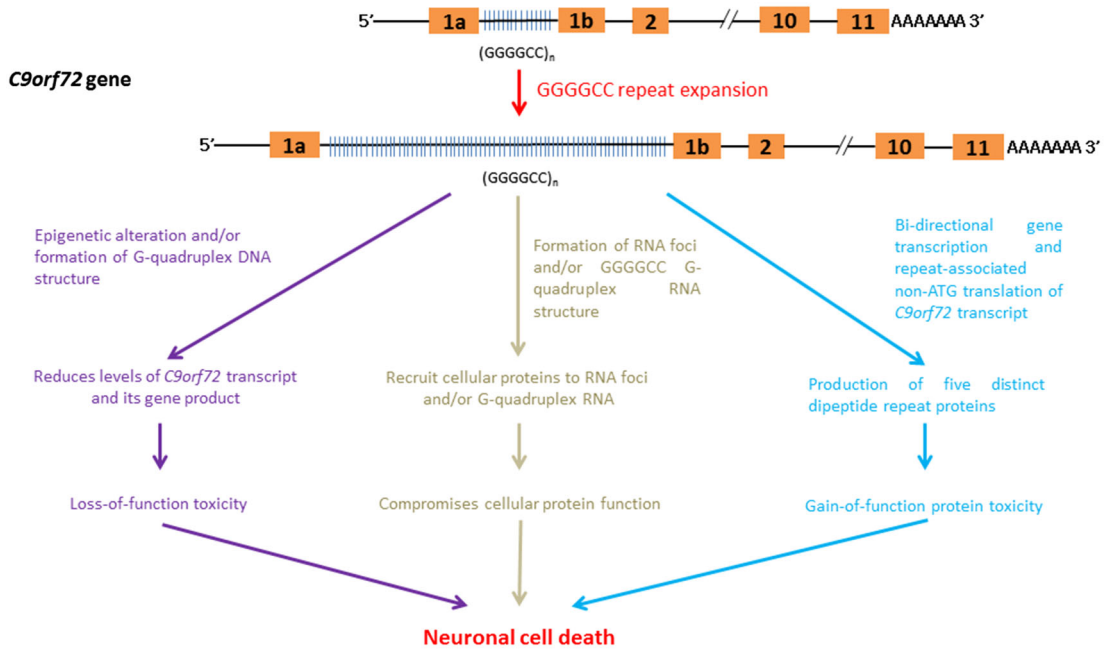

FIGURE 6 | RNA-mediated pathogenic pathways that associate with GGGGCC hexanucleotide repeat expansion in C9FTD/ALS.

Post-translation modifications of the core histone proteins, H3 and H4, are known to modulate gene regulation. Trimethylation of H3 and H4 core histones at particular lysine residues are associated with gene silencing (Barski et al., 2007). Belzil et al. (2013a) showed that the gene expression level of *C9orf72* was reduced in patients who presented with H3K9, H3K27, H3K79 and H4K20 trimethylation. In addition to histone modifications, the intrinsic biophysical properties of *C9orf72* GGGGCC hexanucleotide repeat sequences also help to reduce the gene expression of *C9or72*. The appearance of four consecutive guanine nucleotides in DNA, such as in the case of GGGGCC in *C9orf72*, leads to the formation of a meta-stable DNA secondary structure termed a G-quadruplex (Haeusler et al., 2014). The formation of G-quadruplex DNA has been shown to play regulatory roles in various cellular processes, such as gene transcription control (Lam et al., 2013). Haeusler et al. (2014) recently demonstrated that the formation of GGGGCC DNA G-quadruplex structure halts the transcription of *C9orf72*, which provides an alternative explanation for the down-regulation of *C9orf72* gene expression observed in C9FTD/ALS.

C9orf72 RNA FOCI AND CELLULAR PROTEIN SEQUESTRATION

C9orf72 RNA containing GGGGCC repeats has been reported to form RNA foci (DeJesus-Hernandez et al., 2011; Almeida et al., 2013; Donnelly et al., 2013; Gendron et al., 2013; Lagier-Tourenne et al., 2013; Mizielinska et al., 2013; Sareen et al., 2013; Zu et al., 2013). Mizielinska et al. (2013) also identified an inverse correlation between RNA foci and age-at-onset of C9FTD/ALS. Biochemical and microscopic investigations showed that *C9orf72* RNA is capable of recruiting cellular proteins to RNA foci (reviewed by Vatovec et al., 2014). Among these proteins are RNA-binding proteins such as hnRNPs (Lee et al., 2013; Mori et al., 2013b; Sareen et al., 2013) and RNA export factors (Sareen et al., 2013; Cooper-Knock et al., 2014). The recruitment of these proteins to *C9orf72* RNA foci compromises their cellular functions, leading to C9FTD/ALS pathologies.

RECRUITMENT OF CELLULAR COMPONENTS TO *C9orf72* DNA AND RNA G-QUADRUPLEXES

G-quadruplex structures (Haeusler et al., 2014) have been detected in DNAs and RNAs (Kikin et al., 2008). Recently, GGGGCC repeat sequences have been found to promote the formation of *C9orf72* RNA G-quadruplex (Fratta et al., 2012), whose structure is sequence- and GGGGCC repeat length-dependent (Reddy et al., 2013). Haeusler et al. (2014) identified a series of cellular proteins that interact specifically with GGGGCC RNA G-quadruplexes (Haeusler et al., 2014). The direct interaction of nucleolar protein NCL with GGGGCC RNA G-quadruplexes was confirmed (Durut and Sáez-Vásquez, 2014). More importantly, the subcellular localization of NCL was found to be altered in patient cells, and NCL was shown to become more diffusely localized outside the nucleolar region in cells expressing expanded GGGGCC *C9orf72* RNA (Haeusler et al., 2014). The subcellular mislocalization of NCL also affects pre-rRNA processing in patient cells. This finding is indicative of nucleolar stress activation (Boulon et al., 2010), and provides a direct molecular link between *C9orf72* GGGGCC RNA G-quadruplexes and C9FTD/ALS toxicity. In addition to NCL, GGGGCC G-quadruplex DNA and RNA structures have also recently been reported to possess heme-binding activity (Grigg et al., 2014).

Heme is composed of ferrous iron and protoporphyrin, and serves as a prosthetic group of many cellular proteins, such as redox enzymes. Its role in neurodegeneration is well illustrated in Alzheimer's Disease. Amyloid beta peptide has been found to bind with heme (Atamna and Frey, 2004), leading to heme deficiency (Atamna et al., 2001). It is obvious that the recruitment of cellular components to GGGGCC G-quadruplex structures triggers neurotoxicity in C9FTD/ALS via various pathogenic pathways.

BI-DIRECTIONAL TRANSCRIPTION AND REPEAT-ASSOCIATED NON-ATG TRANSLATION OF *C9orf72*

Although GGGGCC repeat expansion is located in non-coding exon of *C9orf72* (DeJesus-Hernandez et al., 2011; Belzil et al., 2013a), Mori et al. (2013a) showed that GGGGCC repeats are transcribed bi-directionally. More recently, both the sense and anti-sense GGGGCC *C9orf72* transcripts have been reported to generate five different dipeptide repeat (DPR)-containing proteins composed of GA, GP, GR, PR and AP amino acid repeats (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013a,c; Zu et al., 2013) via the RAN mechanism (Zu et al., 2011). Microscopic DPR protein aggregates have been detected in C9FTD/ALS patients (Liu et al., 2014; May et al., 2014; Proudfoot et al., 2014). It has been shown that DPR protein aggregates are heterogeneous in nature, and co-localize with cellular proteins such as the proteasome degradation marker p62 (Mackenzie et al., 2013; Mann et al., 2013; Mori et al., 2013a,c; May et al., 2014), ubiquitin (Zhang et al., 2014) and transport factor Unc119 (May et al., 2014). The co-localization of cellular proteins to DPR aggregates is expected to cause varying degrees of loss-of-function in the cellular proteins, which in turn contributes to C9FTD/ALS pathogenesis.

The neurotoxicity of DPR proteins is determined by their subcellular localization and by the sequestration of cellular proteins to DPR aggregates. Kwon et al. (2014) demonstrated that the nucleolar localization of DPR aggregates impairs pre-rRNA biogenesis and causes cell death. The results from an independent study conducted by Zhang et al. (2014) indicate that cytosolic DPR aggregates impair the ubiquitin-proteasome system (UPS) and induce ER stress. As both the UPS and ER stress pathways are involved in protein homeostasis, these findings highlight the role of protein misfolding in C9FTD/ALS. In addition to cell culture models, *Drosophila* models have been used to demonstrate the neurotoxicity of DPR polypeptides (Mizielinska et al., 2014). In other words, DPR protein toxicity has been confirmed using an *in vivo* animal disease model.

THERAPEUTIC DEVELOPMENT TO COMBAT NUCLEOTIDE REPEAT EXPANSION RNA TOXICITY

Based on our current understanding of nucleotide repeat expansion disease pathogenesis pathways, various therapeutic approaches have been developed. Oligonucleotide-based therapeutics (Fiszer and Krzyzosiak, 2014) such as antisense oligonucleotides (ASOs) have been shown to reduce *C9or72* RNA expression and foci formation (Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Sareen et al., 2013), and thereby to reduce RNA toxicity in C9FTD/ALS. Small molecules capable of targeting toxic RNA-protein interaction can pharmacologically correct splicing defects associated with sequestration of MBNL1 in expanded CAG RNA (Kumar et al., 2012), and hnRNPA1 in expanded GGGGCC *C9orf72* RNA (Zamiri et al., 2014). In addition, another class of small molecules that target GGGGCC RNAs has been reported to be capable of reducing RNA foci formation and RAN-mediated DPR protein production (Su et al., 2014) in C9FTD/ALS. Prior to clinical trials, animal disease models will be used to test these novel compounds and approaches. It is expected that the identification of more nucleotide repeat expansion pathogenic pathways will enable more therapeutic approaches to be developed in the future.

OUTLOOK

Although protein toxicity was considered to play a major role in the pathogenesis of repeat expansion diseases, we now know that mutant transcripts also induce cell dysfunction and death via multiple mechanisms, such as the alteration of gene expression levels and splicing patterns, the generation of small RNAs, the induction of nucleolar stress, the promotion of bi-directional transcription and repeat-associated non-ATG translation of the disease locus, the activation of apoptotic signaling, and the sequestration of cellular components to RNA foci. The above RNA-mediated mechanisms are predicted to operate in conjunction with the protein-mediated pathways to confer overall neurotoxicity. We now know that mutant RNAs confer cytotoxicity by sequestering cellular components to RNA foci (Wojciechowska and Krzyzosiak, 2011). The identification of the individual polyQ protein species, including the monomer, oligomer, protofibril, fibril and inclusion body, has greatly facilitated the study of polyQ protein toxicity (Hands and Wyttenbach, 2010). Technologies and strategies that result in a detailed classification of individual mutant RNA species will allow more in-depth and systematic investigations of the RNA-mediated pathogenesis of neurodegeneration.

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Expression analysis of the long non-coding RNA antisense to Uchl1 (AS Uchl1) during dopaminergic cells' differentiation *in vitro* and in neurochemical models of Parkinson's disease

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Antisense (AS) transcripts are RNA molecules that are transcribed from the opposite strand to sense (S) genes forming S/AS pairs. The most prominent configuration is when a lncRNA is antisense to a protein coding gene. Increasing evidences prove that antisense transcription may control sense gene expression acting at distinct regulatory levels. However, its contribution to brain function and neurodegenerative diseases remains unclear. We have recently identified AS Uchl1 as an antisense to the mouse Ubiquitin carboxy-terminal hydrolase L1 (*Uchl1*) gene (AS Uchl1), the synthenic locus of UCHL1/PARK5. This is mutated in rare cases of early-onset familial Parkinson's Disease (PD) and loss of UCHL1 activity has been reported in many neurodegenerative diseases. Importantly, manipulation of UchL1 expression has been proposed as tool for therapeutic intervention. AS Uchl1 induces UchL1 expression by increasing its translation. It is the representative member of SINEUPs (SINEB2 sequence to UP-regulate translation), a new functional class of natural antisense lncRNAs that activate translation of their sense genes. Here we take advantage of FANTOM5 dataset to identify the transcription start sites associated to S/AS pair at Uchl1 locus. We show that AS Uchl1 expression is under the regulation of Nurr1, a major transcription factor involved in dopaminergic cells' differentiation and maintenance. Furthermore, AS Uch1 RNA levels are strongly down-regulated in neurochemical models of PD *in vitro* and *in vivo*. This work positions AS Uchl1 RNA as a component of Nurr1-dependent gene network and target of cellular stress extending our understanding on the role of antisense transcription in the brain.

Keywords: antisense transcription, long non-coding RNA, Parkinson's disease, Nurr1, dopaminergic cells

Introduction

Large genomic projects such as ENCODE (Derrien et al., 2012) and FANTOM (Forrest et al., 2014) have shown that the majority of the mammalian genome is transcribed, thus generating

a previously underestimated complexity in gene regulatory networks. A vast repertory of different classes of transcripts includes non-coding RNAs and RNAs of Transposable Elements (TEs), such as LINE (long interspersed nuclear element) and SINEs (short interspersed nuclear element) (Katayama et al., 2005; Faulkner et al., 2009; Kapranov et al., 2010; Fort et al., 2014). Long non-coding RNA (lncRNA) genes seem to represent the majority of cellular transcriptional output. The FANTOM project has cataloged more than 30,000 putative lncRNA transcripts by full length cDNA cloning (Katayama et al., 2005) while NONCODEv4 currently contain 46,475 lncRNA genes (Xie et al., 2014; Quek et al., 2015).

Antisense (AS) transcripts are RNA molecules that are transcribed from the opposite strand to sense (S) genes forming S/AS pairs. These are estimated to include the large majority of protein encoding genes and about one third of lncRNAs (Chen et al., 2004; Katayama et al., 2005; Engstrom et al., 2006; Derrien et al., 2012). The most prominent class of S/AS pair is when a protein-encoding gene presents a lncRNA on the opposite strand. In a growing number of cases, AS lncRNAs have been proved to be required for proper regulation of sense genes, carrying genetic information acting at distinct regulatory levels (Yu et al., 2008; Spigoni et al., 2010; Tripathi et al., 2010). Understanding their mode of action may be also relevant for gene expression manipulation *in vivo* since lncRNAs may become in the near future a new class of RNA-based drugs for therapeutic intervention.

The contribution of AS lncRNAs to neurodegenerative diseases is still unclear although some significant examples in Alzheimer's disease (AD) (Faghihi et al., 2008, 2010), spinocerebellar ataxia type 7 (Sopher et al., 2011) and Huntington's disease (Chung et al., 2011) may suggest they play a prominent role in neuronal homeostasis and dysfunction.

Parkinson's disease (PD) is a slowly progressive degenerative disorder of the central nervous system (CNS) that is classically defined in terms of motor symptoms. The neuropathological hallmark in *post-mortem* brains is the selective degeneration of specific subsets of mesencephalic dopaminergic (DA) cells and the formation of cytoplasmic aggregates called Lewy bodies. The current model of toxicity of DA neurons includes mitochondrial dysfunction, oxidative stress and alterations in protein turnover. This stems from the observation on *post-mortem* PD brains as well as from the identification of genes associated to rare forms of early-onset familial PD. Some of these features are recapitulated in a neurochemical model of the disease that takes advantage of the selective accumulation of toxic MPP^+ species in DA neurons.

So far, relevant examples for lncRNAs antisense to genes involved in PD have been restricted to a transcript associated to PINK1/PARK6 locus (Scheele et al., 2007).

Recently, we have identified a lncRNA that is antisense to the mouse Ubiquitin carboxy-terminal hydrolase L1 (*Uchl1*) gene (AS Uchl1), the synthenic locus of UCHL1/PARK5 (Carrieri et al., 2012).

Uchl1 encodes for one of the most abundant proteins in the brain. It acts as deubiquinating enzyme, ubiquitin ligase or monoubiquitin stabilizer, thus regulating ubiquitin turnover (Liu et al., 2002). Dysfunction in UCHL1 has been reported in many neurodegenerative diseases. A missense mutation in UCHL1/PARK5 has been associated to rare cases of early-onset familial PD. Inactivating oxidative modifications of UchL1 protein have been reported in PD *post-mortem* brains where it correlates with the formation of protein aggregates (Choi et al., 2004; Barrachina et al., 2006; Gong et al., 2006). In this context several evidences position UchL1 as a major regulator of α-synuclein degradation and toxicity (Liu et al., 2009). Lack of hydrolase activity has also been found in recessive cases of a childhood-onset progressive neurodegeneration (Bilguvar et al., 2013). An in-frame deletion in the Uchl1 gene, as observed in the *gracile axonal dystrophy* mice, leads to axonal dystrophy and premature death (Saigoh et al., 1999). Reduced UCHL1 protein levels were also found in sporadic AD brains. Recently, UCHL1 overexpression has been shown to accelerate lysosomal degradation of APP, inhibit plaque formation and improve memory deficits in AD transgenic model mice (Gong et al., 2006). These data proves UchL1 activity is required for proper brain function. Furthermore, they suggest that increasing UCHL1 expression *in vivo* may be a safe and effective disease-modifying strategy to treat neurodegenerative diseases. It is therefore important dissecting all the molecular events involved in Uchl1 gene regulation.

AS Uchl1 is a 5′ head to head, 1.2 kb long transcript that initiates within the second intron of Uchl1 and overlaps the first 73 nts of the sense mRNA including the AUG codon. The non-overlapping part of the transcript also contains an embedded repetitive sequence SINEB2 of the B3 subclass in the inverted orientation. AS Uchl1 is expressed in mouse mesencephalic DA neurons, the site of degeneration in PD. In physiological conditions AS Uchl1 RNA is nuclear-enriched. Upon rapamycin, it shuttles from the nucleus to the cytoplasm and specifically targets Uchl1 mRNA to heavy polysomes for translation (Carrieri et al., 2012). AS Uchl1 is the representative member of SINEUPs (SINEB2 sequence to UP-regulate translation), a new functional class of natural antisense lncRNAs that activate translation of their sense genes (Zucchelli et al., submitted).

Cap Analysis of Gene Expression (CAGE) is a technology based on the generation of short sequence tags from the 5′ end of full-length cDNAs followed by high-throughput sequencing. When mapped to a reference genome, CAGE tags survey transcription start site (TSS) activity of specific promoters and measure expression levels on a massive scale (Gustincich et al., 2003; Shiraki et al., 2003; Carninci et al., 2006). The FANTOM5 (Functional Annotation of Mammals 5) project has developed a simplified CAGE protocol adapted to single-molecule HeliScope sequencer (hCAGE) (Kanamori-Katayama et al., 2011) to decrease PCR biases and improve depth of sequencing. hCAGE technology was applied to a wide range of human and mouse tissues providing an unprecedented dataset for promoter usage analysis (Forrest et al., 2014).

Abbreviations: Uchl1, Ubiquitin Carboxy-terminal Hydrolase L1; S, sense; AS, antisense; hCAGE, Heliscope Cap Analysis of Gene Expression; TSS, transcription start site; lncRNA, long non-coding RNA; PD, Parkinson's disease; DA, dopaminergic; TH, tyrosine hydroxylase; Vmat2, vescicular monoamine transporter 2; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine.

Here we take advantage of FANTOM5 dataset to map TSSs and analyze the expression of the S/AS pair at the Uchl1 mouse locus. This led to the identification in the AS Uchl1 promoter region of a binding site for Nurr1, a transcription factor required for DA cells differentiation. Chromatin immuno-precipitation and quantitative RT-PCR proved that AS Uchl1 expression is under the control of Nurr1 activity. Finally, we show that transcription of S/AS Uchl1 RNA is regulated in neurochemical models of PD *in vitro* and *in vivo*.

Materials and Methods

Cell Lines

Murine dopaminergic MN9D cells with doxyciclin- inducible induction of Nurr1 transcription factor (MN9D Nurr1 Tet-ON stable cells, or iMN9D cells) were maintained in culture as previously described (Hermanson et al., 2003). Nurr1 expression was obtained by culture with 2.5 μg/ml doxycycline for 12 h or longer, as required. For *in vitro* neurochemical model of PD, iMN9D cells were treated with 100 μM MPP^+ for 16 h.

ChIP Assay

Chromatin immunoprecipitation (ChIP) was performed with magnetic beads (Dynabeads, Invitrogen) following the protocol as described (Schmidt et al., 2009). For each ChIP, one confluent 100 mm plate of iMN9D cell was treated with doxycicline 2.5 μg/ml. Nurr1 expression upon doxycicline treatment was followed by western blot. 1 μg of ChIP-grade anti-Nurr1 antibody was used (sc-990 X). Rabbit IgG were used as negative control (Cell signaling #2729).

qPCR was performed with primers for DNA binding regions of indicated targets and distal primers were designed for an unrelated region 6000 bps upstream the AS Uchl TSS:

VMAT NBRE F: 5′-ATTGTGCTAACATTTATTCCAGAG-3′
VMAT NBRE R: 5′-AGGGCTTCCTACGTGACC-3′
OCN NBRE F: 5′-CCACAACACGCATCCTTT-3′
OCN NBRE R: GGACTTGTCTGTTCTGCA-3′
AS Uchl1 NBRE F: 5′ CTTCCCATACAGCTTAGTTCC-3′
AS Uchl1 NBRE R 5′-TTGCGTCTCTGCCAGATG-3′
Distal F 5′-TCATCCAGCCACAAGGTCAGAG-3′
Distal R 5′- CCAGCAGGCACACTGTTGAAC-3′

Enrichment of chromatin binding was calculated relative to total input, as described previously (Guccione et al., 2006).

RNA Isolation, Reverse Transcription and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from iMN9D cells or dissected mouse ventral midbrain using TRIZOL reagent (Invitrogen) and following manufacturer's instructions. RNA was treated with DNAse I (Ambion) before use. Single strand cDNA was prepared from 1 μg of purified RNA using the iSCRIPT™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions. qRT-PCR reaction was performed using SYBR-Green PCR Master Mix (Applied Biosystem) and an iCycler IQ Real time PCR System (Bio-Rad). Oligonucleotide sequences of primers used in this study were previously described (Carrieri et al., 2012). qRT-PCR for Nur77 was performed with the following primers:

Nur77-FWD CCTCATCACTGATCGACACG
Nur77-REV CCTCCAACTTGAGGCAAAAG

MPTP Treatment

Mice used in this study were treated according to the NIH guidelines for Care and Use of Laboratory Animals. MPTP use and safety precautions were as described previously (Jackson-Lewis and Przedborski, 2007). All animal experiments were performed in accordance with European guidelines for animal care and following SISSA Ethical Committee permissions. Mice were housed and bred in SISSA non-SPF animal facility, with 12 h dark/light cycles and controlled temperature and humidity. Mice had *ad libitum* access to food and water.

Eight-week-old, male, TH-GFP mice (Sawamoto et al., 2001) were subjected to a sub-acute MPTP regimen (Jackson-Lewis and Przedborski, 2007). Animals received one intra-peritoneal injection of MPTP-HCl (free base suspended in saline; Sigma-Aldrich) at 20 mg kg^{-1} dose every 2 h for a total of four doses over an 8 h period (Tatton and Kish, 1997; Miller et al., 2004; Gibrat et al., 2009). Injection of saline solution was used in control mice. Animals were sacrificed 2 days or 7 days after last injection, as these time-points were previously associated to variations in Uchl1 and Nurr1 expression (Miller et al., 2004; Gibrat et al., 2009). 300 DA neurons were purified by laser capture microdissection as described previously (Biagioli et al., 2009; Carrieri et al., 2012) and used for RNA extraction and qRT-PCR.

Bioinformatic Analysis

Analysis of FANTOM5 collection of mouse libraries was performed using the Zenbu browser genomic tool (Severin et al., 2014) and publicly available FANTOM5 datasets (http://fantom.gsc.riken.jp/5/) (Forrest et al., 2014). A specific script was designed to extract expression values from graphical tables in Zenbu Genome Browser and convert into Excel spreadsheet for further analysis (Paolo Vatta, unpublished). Expression values for S/AS Uchl1 were calculated for a window of about ±800 base pairs around main TSS. Selection of brain-specific libraries was done by manual annotation.

For co-expression analysis, average expression values were calculated for S/AS transcripts in libraries that express both and divergence from average was considered.

For ChIP experiments, identification of NGFI-B binding elements was performed with the Genomatrix program (http://genomatrix.de) and the TRANSFAC database (Knuppel et al., 1994). The mouse *AS Uchl1* genome region from kb −3000 to +1000 was the reference sequence. Trascriptional binding factor motifs were chosen on the basis of core similarity (score 1.0) and matrix similarity (above 0.80).

Results

Evidence of AS Uchl1 Transcription in Mouse FANTOM5 Collection of Cell Lines, Primary Cells and Tissues

We first interrogated FANTOM5 expression data for almost 400 mouse samples, covering cell lines, primary cells and tissues. These dataset are build from hCAGE libraries and

are based on sequencing cDNA copies of the 5′ ends of mRNAs, of which the integrity is inferred by the presence of their cap. These sequences—referred to as *tags*—are sufficiently long to be aligned in most cases at a single location in the genome. The first position of this alignment identifies a base pair where transcription is initiated defining a TSS. The number of times a given tag is represented in a library gives an estimate of the expression level of the corresponding transcript.

Since antisense lncRNAs are typically expressed at much lower level than overlapping protein-coding transcripts (Derrien et al., 2012; Forrest et al., 2014) (Zucchelli et al., FANTOM5 satellite, submitted), we used FANTOM5 mouse datasets in which no expression cutoff was applied to detect values as low as 1 count per library. Tags were positively scored if mapping to a region of about 800 bp around the main TSS of Uchl1 (**Figures 1A**, **3**). Expression was measured as Tag Per Million (TPM).

While sense mRNA is expressed in almost all analyzed samples (90% of libraries), only 40% of them score positive for antisense transcription proving AS Uchl1 expression is relatively restricted (**Figure 1B**). Sense mRNA is present at higher levels as compared to its antisense (**Figure 1C**). Both transcripts are highly expressed in the brain (**Figure 1D**). In particular AS Uchl1 RNA is present in the cortex, striatum and hippocampus (TPM 3.2-2.2) although its highest level is measured in testis (TPM 5.38). Within FANTOM5 dataset, about 50 libraries were prepared from blood-derived primary cells (B cells, T cells, macrophages,

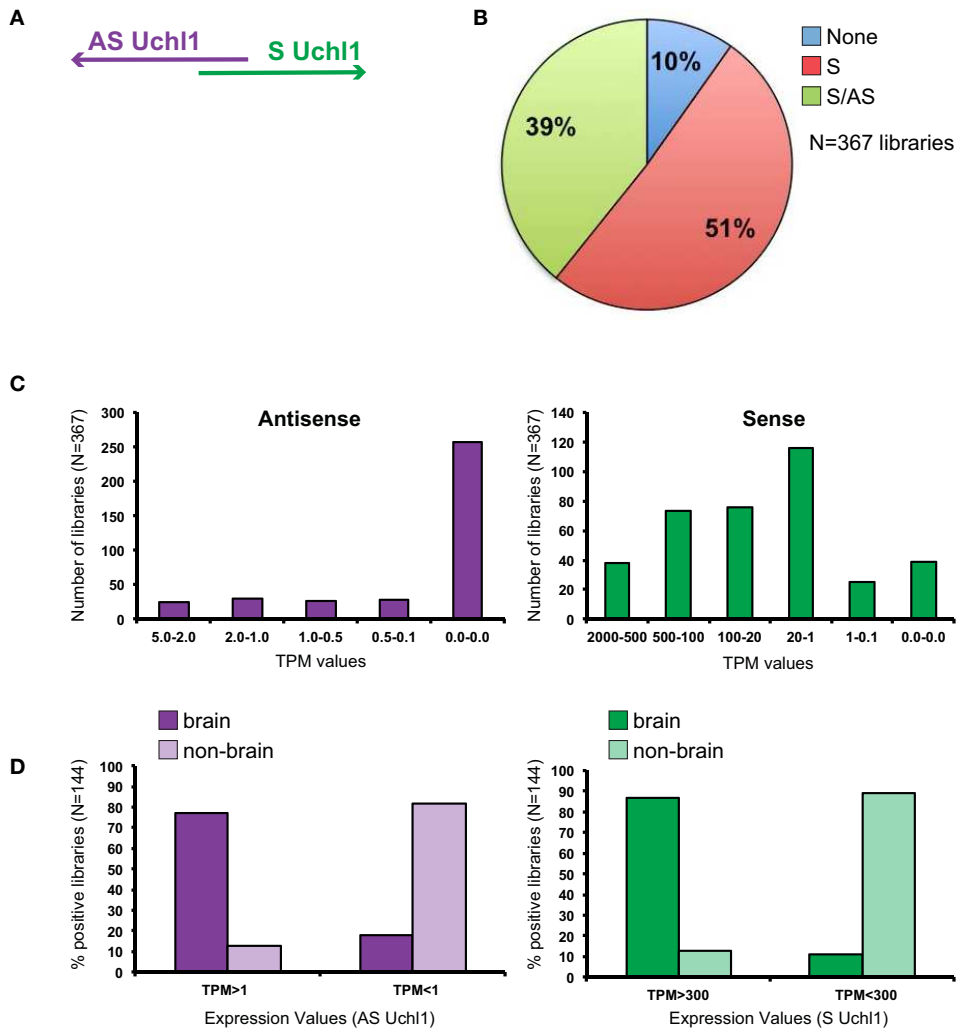

FIGURE 1 | General features of S/AS Uchl1 expression in mouse FANTOM5 datasets. (A) Schematic view of S/AS Uchl1 pair with 5′ head-to-head divergent anatomy. **(B)** FANTOM5 collection of 367 libraries from mouse cell lines, primary cells and tissues were interrogated for expression of *Uchl1* gene in sense and antisense orientation. Pie-chart representation of S/AS expression in FANTOM5 dataset. **(C)** Distribution of S/AS levels in mouse libraries. Transcript levels are expressed as tag per millions (TPM). **(D)** Higher levels of S and AS Uchl1 are enriched in the brain. Data were obtained from $N = 144$ FANTOM5 libraries that express both transcripts. Low and high levels of S and AS Uchl1 were defined based on average expression values for each transcript.

lymphoid and myeloid progenitor cells, megakaryocyte precursor cells) and tissues related to immune system (spleen and thymus). Very low expression is detected for AS Uchl1 (0.2 TPM) in only one/two of the replicas from naïve CD4 T cells, c-Kit+ stem cells and common myeloid progenitors (data not shown).

To assess whether transcription in opposite orientation is co-regulated, we analyzed FANTOM5 libraries containing tags for both RNAs ($N = 144$ libraries). Since Spearman Correlation analysis could not be applied, we calculated average TPM levels for each transcript and set this value to 1. We then normalized expression values in all libraries to the reference and monitored variation from the average. We found that the majority of libraries ($N = 122$) displays concordant variation of S/AS transcription from the average, with both positive and negative co-regulation. When expression of S/AS pair is not co-regulated ($N = 22$), variation from average values goes in both directions and is almost equally present in non-brain ($N = 9$) and brain ($N = 13$) libraries.

Since FANTOM5 collection comprises a set of brain regions for which neonatal and adult tissues are available, we monitored S/AS transcription in brain development. As previously found, sense and antisense levels are positively co-regulated with values in neonatal tissues often higher than in adult samples of the same area (**Figure 2A**). High levels of AS Uchl1 RNA are detected in neonatal corpus striatum, cortex, hippocampus and medulla oblungata.

As each brain region is composed of several cellular types of neuronal and non-neuronal origin, we monitored S/AS expression in primary brain cells as well as in neurospheres. Again, we found that the expression of S and AS Uchl1 RNAs is co-regulated. Both transcripts are highly expressed in neurons as compared to non-neuronal cells, with those from the Striatum, the *Substantia Nigra* and Raphe being the highest (**Figure 2B**). Interestingly, values in neurospheres prepared from cells isolated from different compartments show reduced quantities as compared to primary neurons. Non-neuronal cells have very low (Schwann cells) to undetectable (astrocytes) levels of Uchl1 S/AS pair.

Therefore, expression of S and AS Uchl1 is co-regulated in the developing and adult brain.

Analysis of TSS Usage in Mouse Uchl1 Gene Locus

To monitor promoter usage in mouse Uchl1 locus, we visualized single TSSs using the FANTOM5 Zenbu Genome Browser, a publicly available web resource tool (http://fantom.gsc.riken.jp/zenbu/) (Severin et al., 2014). To identify promoters across the genome the FANTOM5 consortium has developed a method based on tags proximity and signal decomposition (Forrest et al., 2014). To enrich for TSSs, 3 tags/library thresholds were applied and promoter subsets were defined and classified according to their robustness. For our analysis, we interrogated all hCAGE libraries pooled together and then we focused our attention on those with stronger evidences of TSS usages, as testis, cortex and primary neurons of the *Substantia Nigra*. As shown in **Figure 3**, three different TSSs can be identified using decomposition-based identification method and referred to as p1, p2 and p3, from 5′ to 3′ according to the sense of transcription. These drive expression of alternative variants of AS Uchl1 RNA. TSS usage is under tissue- and cell-specific regulation, as documented by different TPM values in the selected libraries. Interestingly, expression of AS Uchl1 reference sequence is validated by TSSs in testis and *Substantia Nigra* neurons, but this promoter does not pass the bioinformatics cutoffs. p2 and p1 are the most used promoters,

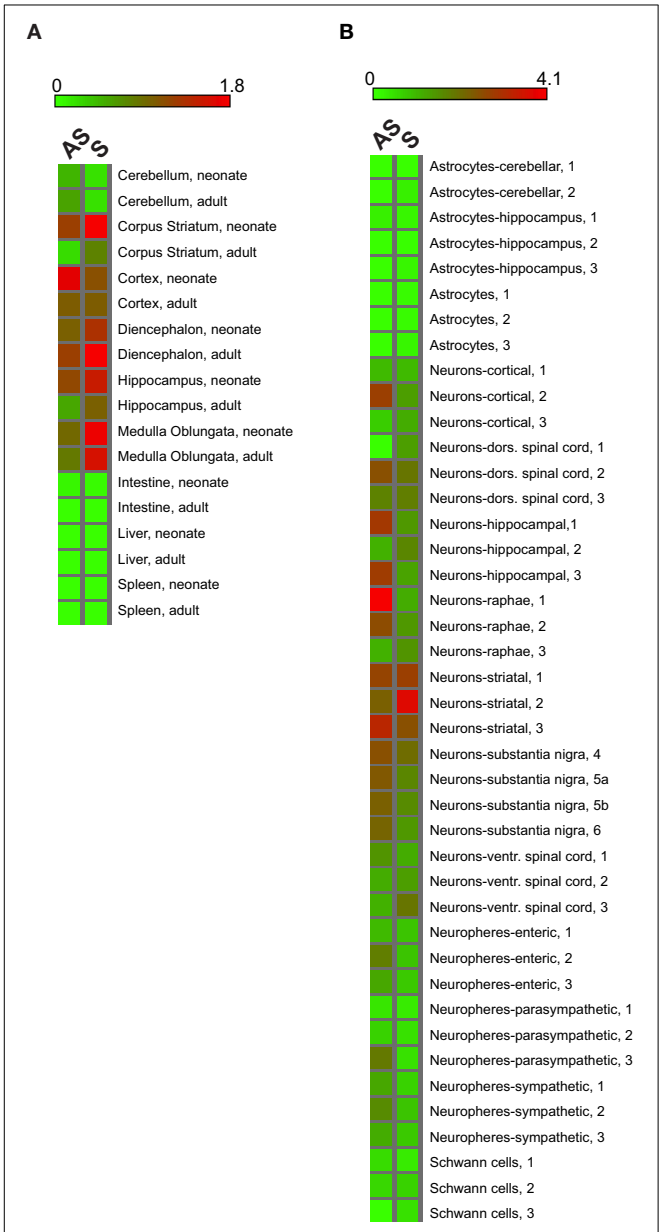

FIGURE 2 | S/AS Uchl1 are regulated during development and enriched in neurons. (A) Heat map graphical representation of S/AS expression in neonatal and adult tissues from brain and other organs. Expression values of S/AS transcripts in cortex were set to 1. **(B)** Heat map of S/AS Uchl1 in brain-derived primary cells and in neurospheres. Values in cortical neurons were set to 1.

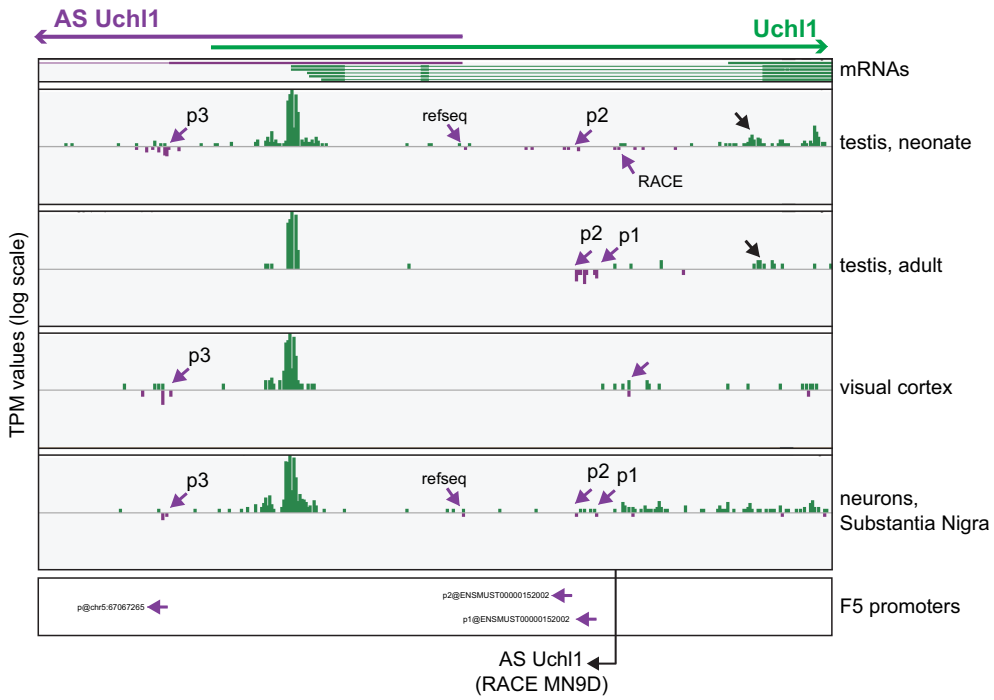

FIGURE 3 | Analysis of TSS usage in mouse S/AS Uchl1. Zenbu Genome Browser view of mouse Uchl1 locus. Sense transcripts are in green, antisense in purple. hCAGE data are presented for those libraries (testis, cortex and *Substantia Nigra* neurons), where antisense expression is highest. Purple arrows highlight antisense TSSs identified by FANTOM5 data analysis (p1, p2, and p3, 5′ to 3′ orientation relative to AS Uchl1) and by RACE experiments in MN9D cells. TSS supporting the expression of RefSeq AS Uchl1 transcript in testis and *Substantia Nigra* is also indicated. Promoters identified with FANTOM5 decomposition-based peak identification method are shown at the bottom (original promoter nomenclature is given). Black arrow indicated an alternative transcript variant of sense Uchl1. The 5′ end of AS Uchl1 cDNA cloned from mouse dopaminergic MN9D cell line is shown (Carrieri et al., 2012).

giving rise to transcript variants with a longer 5′ end. It is of note that 5′ end of AS Uchl1, as identified by RACE in murine dopaminergic MN9D cell line (Carrieri et al., 2012), is positioned upstream to p1 (**Figure 3**). Finally, an additional site of transcription initiation can be observed at the 3′ end of AS Uchl1 (p3), thus identifying a yet not-annotated variant with a shorter first exon. p3 seems to be under control of a bidirectional promoter.

The protein-coding Uchl1 mRNA starts almost exclusively from an internal portion of the 5′ UTR of the annotated transcript. It is shorter and independently validated by mm9 mRNAs (**Figure 3**). Interestingly, an alternative promoter for Uchl1 is also located around exon 3 and drives expression in testis (**Figure 3**, black arrow). This TSS identifies an annotated but yet uncharacterized transcript (AK170728), which potentially encodes for a shorter Uchl1 protein, lacking a canonical initiation methionine. Whether such protein is indeed functional remains to be established.

Nurr1 Activity Regulates AS Uchl1 Expression

The genomic region −1500/+1000 around the annotated AS Uchl1 TSS was then scanned for the presence of Transcription Factor Binding Sites (TFBSs). A NGFI-B element was identified in position −1230/−1222 to the AS Uchl1 TSS as defined by RACE (**Figure 4A**). This TFBS is the target of the Nurr subfamily of nuclear receptors including Nurr1, a key dopaminergic transcription factor required for late-dopaminergic differentiation and crucial for the expression of several dopaminergic-specific genes like VMAT2, AADC, DAT, and TH (Castro et al., 2001).

To study the role of this transcription factor in AS Uchl1 RNA transcription, we took advantage of mouse dopaminergic MN9D cell line overexpressing Nurr1 under a doxycicline inducible promoter (iMN9D cells) (Hermanson et al., 2003). In steady-state conditions, levels of Nurr1 protein are almost undetectable and AS Uchl1 is expressed at low level. Upon drug treatment, Nurr1 expression increases and Nurr1 target genes are induced (Hermanson et al., 2003). As a consequence, differentiation of DA neurons occurs (**Figure 4B**).

We then carried out chromatin immunoprecipitation (ChIP) experiments. iMN9D cells were treated with doxocycline for 12 h to achieve high levels of Nurr1 protein. Chromatin-protein complexes were then immunoprecipitated with anti-Nurr1 or control (IgG) antibodies and bound genomic DNA was quantified by qPCR using primers for Nurr1 response elements in AS Uchl1, Osteocalcin and Vescicular Monoamine Transporter 2 gene promoters. PCR reactions generated only the expected specific amplicon, as detected by gel electrophoresis and melting curve analysis (data not shown). As shown in **Figure 4C**, Nurr1 binding was significantly enriched relative to IgG control in AS Uchl1 promoter region.

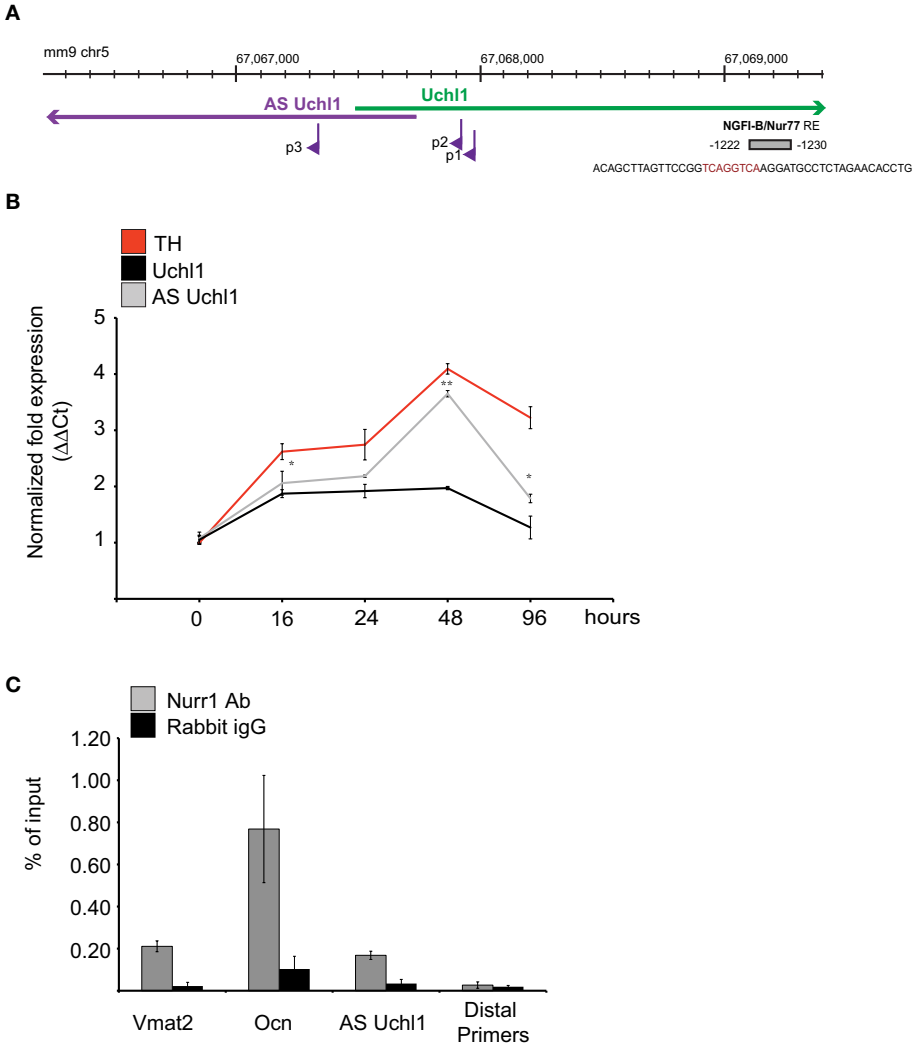

FIGURE 4 | AS Uchl1 is primary target of Nurr1 transcription factor. **(A)** Scheme of Nurr1 TFBS upstream AS Uchl1. Genomic coordinates (top), S/AS Uchl1 transcript anatomy (middle), and AS Uchl1 promoters (bottom) are shown. NGFI-B/Nur77 response element (NBRE) is indicated in gray and positioned to its genomic coordinates and relative to AS Uchl1 TSSs. **(B)** AS Uchl1 expression is up-regulated during differentiation. iMN9D cells were treated with doxocyclin at the indicated times. AS and S Uchl1 expression were monitored by qRT-PCR. Expression of TH was used as positive control for dopaminergic differentiation. $^{*}p < 0.05$; $^{**}p < 0.01$. **(C)** Immunoprecipitation of Nurr1-bound chromatin was performed from murine dopaminergic iMN9D cells treated with doxocyclin for 12 h to induce Nurr1 expression. Rabbit IgGs and distal primers targeting an unrelated region were used as controls. Chromatin immunoprecipitation of VMAT2 and OCN promoter regions were included as positive controls of Nurr1 binding.

Nurr1 induction led to a rapid up-regulation of AS Uchl1 RNA levels starting from 12 h, with kinetics comparable to the one observed for VMAT2, a well-known primary target of Nurr1. Within 48 h, both AS Uchl1 and VMAT2 mRNAs reached their peak of induction, while decreasing at later time points. Under these conditions, Nur77, another member of nuclear receptors family targeting NGFI-B motif, is undetectable (**Supplementary Figure 1**).

Interestingly, we found that transcription of sense protein-coding Uchl1 mRNA was poorly linked to Nurr1 activity, as the kinetics and strength of Uchl1 mRNA up-regulation was different from that of VMAT2 and AS Uchl1 (**Figure 4B**).

Expression of Mouse AS Uchl1 is Down-Regulated in Neurochemical Models of PD *In Vitro* and *In Vivo*

We then investigated the behavior of Uchl1 S/AS pair in neurochemical models of PD. First, we analyzed the effects of intoxication on S/AS Uchl1 levels *in vitro*. When iMN9D cells were exposed to 100 μM 1-methyl-4-phenylpyridinium (MPP^{+}) for 16 h, we observed that expression of both transcripts was altered and a statistically significant co-reduction could be measured. While AS Uchl1 RNA was reduced to only 25% of its physiological level, the impact on Uchl1 mRNA was less pronounced, with only 30% reduction (**Figure 5A**).

To test whether S/AS transcription is regulated during DA neuron intoxication *in vivo*, we injected mice with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), following a sub-acute protocol (Jackson-Lewis and Przedborski, 2007). To uncouple transcriptional control of S/AS expression from down-regulation due to cell loss, we resolved to laser capture microdissection of fluorescently labeled DA neurons from TH-GFP transgenic mice to purify equal numbers of cells from untreated and treated animals. Under these conditions, surviving DA neurons showed an almost complete loss of AS Uchl1 transcript but a modest down-regulation of Uchl1 mRNA (**Figure 5B**) recapitulating what we had observed *in vitro* in iMN9D cells treated with MPP^+.

Altogether, these data show that AS Uchl1 is a component of the Nurr1-dependent dopaminergic gene network and is down-regulated in neurotoxicity.

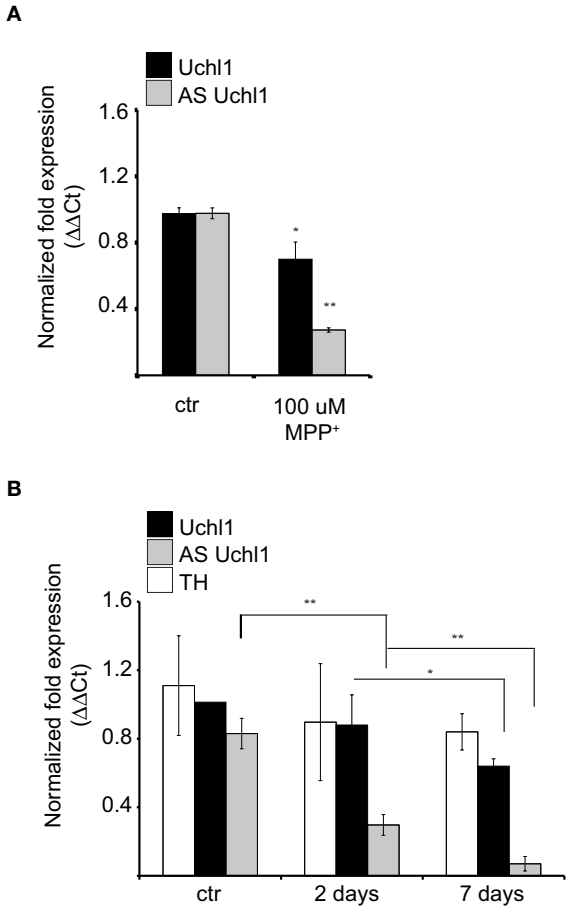

FIGURE 5 | S/AS Uchl1 pair is co-downregulated in PD neurochemical models *in vitro* and *in vivo*. (A) qRT-PCR of S and AS Uchl1 transcript levels in iMN9D cells treated with 100 μM MPP^+ for 16 h. $^*p < 0.05$; $^{**}p < 0.01$. **(B)** TH-GFP mice were treated with MPTP or saline control (ctrl). Animals were sacrificed at the indicated times. RNA was extracted from LCM-purified DA neurons and used for qRT-PCR. Data indicate mean ± s.d., $n = 8$. $^*p < 0.05$; $^{**}p < 0.01$.

Discussion

One of the key features of genomes' organization is that the large majority of genes share their genomic region with another gene on the opposite filament forming S/AS pair (Carninci et al., 2005; Derrien et al., 2012). Massive antisense transcription seems to be a common feature of cells ranging from bacteria to human (Katayama et al., 2005; Van Dijk et al., 2011). Despite some provocative examples we poorly understand how each locus decides between repressive and activating responses to antisense transcription suggesting we are still missing the basic principles of regulatory choices and molecular switches. This is particularly relevant when we consider that lncRNAs and antisense transcription may be exploited in RNA therapeutics.

The S/AS pair at the Uchl1 locus is organized according to the configuration where a protein-encoding gene presents a lncRNA on the opposite strand, overlapping "head-to-head" at their 5′ ends. AS Uchl1 is the representative member of SINEUPs, a new functional class of natural and synthetic antisense lncRNAs that activate translation. Their activity depends on the combination of two RNA elements: the overlapping region is indicated as the Binding Doman (BD) while the embedded inverted SINEB2 element is the Effector Domain (ED). The ED is required for the increase of translation of the protein-coding sense mRNA and the BD is targeting the activity to the sense transcript.

UchL1 is one of the most abundant proteins in the brain where it is expressed in selected neuronal cell types. We have previously shown that in iMND9 cells, the very same cell line used in this study, its mRNA is associated to light polysomes for basal translation. In these conditions AS Uchl1 is mainly restricted to the nucleus where it exerts an unknown function. S/AS transcripts are also co-expressed in mesencephalic DA cells *in vivo* where they retain their differential subcellular localization. Upon inhibition of mTOR activity by rapamycin, AS Uchl1 shuttles to the citoplasm where it facilitates Uchl1 mRNA loading to heavy polisomes for a more efficient translation and increase in cellular UchL1 protein levels. Both rapamycin treatment and Uchl1 over-expression are considered neuroprotective suggesting that antisense-mediated translation may be part of a pro-survival cellular response to stress.

A first step toward a better understanding of antisense transcription consists in studying how its expression is regulated. Increasing information is accumulating over the role of DNA methylation and selected TFs such as Sox2, Oct4, and Nanog on the control of long intergenic non-coding RNAs (lincRNAs) (Guttman et al., 2009) whereas very little is known about the transcriptional regulation of the lncRNAs component of S/AS pairs. Recent genome-wide studies on human cell lines evidence epigenetic regulation of AS lncRNAs expression, with accumulation of histone marks and RNA polymerase II occupancy (Conley and Jordan, 2012). Interestingly, TSSs in antisense orientation frequently associate with transposable elements, suggesting that repetitive sequences might contribute to regulation of AS transcription (Conley et al., 2008; Kapusta et al., 2013). Few gene-specific examples exist in which transcriptional control of a natural AS transcript has been investigated. This is the case, for example, of S/AS Msx1 in which the promoter region of AS RNA

contains binding sites for Msx1 transcription factor, thus generating an auto-regulatory loop of S/AS regulation (Petit et al., 2009; Babajko et al., 2011). In prostate cancer cell line, the expression of CTBP1-AS is regulated by binding of androgen receptor at the 5′ of its TSS. Upon androgen-dependent activation of CTBP1-AS, the overlapping protein-coding gene (CTBP1) is repressed (Takayama et al., 2013).

Here we find that almost all the FANTOM5 libraries present evidences of Uchl1 transcription while AS Uchl1 is expressed only in 40% of them. This data raise the question about how UchL1 protein synthesis is regulated in physiological conditions and upon stress in the absence of antisense transcription. It will be interesting to assess whether Uchl1 mRNA is normally associated to heavy polisomes and whether mTOR inhibition or stress are able to increase UchL1 protein levels according to an antisense–independent mechanism. This is particularly relevant considering that UchL1 is target of oxidative stress inactivation and is down-regulated in *post-mortem* brains of neurodegenerative diseases. In summary these data may raise the interesting possibility of differences in the anti-stress control and function of Uchl1 expression dependent on the presence of AS Uchl1 RNA.

We did not find any example of AS Uchl1 expression in the absence of Uchl1. Therefore, it is not surprising that when both S/AS pair transcripts are present they show a concordant pattern of expression. The highest levels of AS Uchl1 have been found in *Substantia Nigra*, striatum and diencephalon. This analysis confirms AS Uchl1 is present in selected regions of the brain as shown in Carrieri et al. (2012).

Interestingly, neonatal neurons express larger quantity of AS Uchl1 RNA than adult tissues. This is in line with recent genome-wide and gene-specific studies that indicate spatiotemporal regulation of AS lncRNAs expression during cerebral corticogenesis (Ling et al., 2011; Lipovich et al., 2013) and aging (Pardo et al., 2013) (Francescatto et al., FANTOM5 satellite, submitted).

Even when AS Uchl1 is highly expressed, its levels are lower than Uchl1 mRNA. However, we know that AS Uchl1 is able to increase the amount of UchL1 protein through its activity on Uchl1 mRNA. The quantity of UchL1 protein *in vivo* is the sum of several molecular events ranging from transcription to mRNA localization, rate of protein synthesis and degradation. It is unclear what is the life cycle of a single Uchl1 mRNA from transcription to its association to ribosomes or stress granules and P bodies. Therefore, it remains crucial to determine which fraction of total cellular Uchl1 mRNA is associated to light polisomes in physiological conditions and how much is available for RNA-RNA interaction and association to heavy polisomes in an antisense-dependent fashion under stress.

By decomposition-based identification method three different promoters can be defined while evidences for additional TSSs are shown. While p1 and p2 extend the overlapping region at genomic level respect to the annotated sequence of reference, a previously reported cDNA clone obtained with RACE starts 5′ to p1. p3 identifies a non-overlapping transcript probably involved in a bidirectional promoter. Interestingly, a potential internal alternative TSS for Uchl1 is evidenced in testis and *Substantia Nigra* suggesting the possibility of a N-terminal truncated protein.

At 1230 nt from the TSS of AS Uchl1 identified with RACE, a DNA binding site for Nurr1 has been bioinformatically discovered and experimentally validated. Nurr1 is a member of the nuclear receptor superfamily, whose expression is crucial for DA neurons differentiation and maintenance in the adult (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Kadkhodaei et al., 2009, 2013). Importantly, reduced quantities of Nurr1 in adult mice increase DA cells vulnerability to neurotoxic insults (Le et al., 1999). In humans, Nurr1 polymorphisms have been associated to sporadic PD (Xu et al., 2002; Grimes et al., 2006) and reduced Nurr1 levels have been measured in peripheral blood and brain of PD patients (Chu et al., 2002; Liu et al., 2012). In summary, Nurr1 controls the major transcriptional axis for differentiation of DA neurons and maintenance of their integrity.

This work strongly suggests that AS Uchl1 is a component of Nurr1-dependent transcriptional network. This functional interaction adds a new layer to the complexity of circuitry that may contribute to DA cells' homeostasis.

We then prove that neurochemical intoxication of DA cells down-regulates S/AS expression *in vitro* and *in vivo* with a kinetics that impact more strongly AS Uchl1 than its overlapping sense mRNA. This is different from the consequences of pharmacological inhibition of mTOR where no effects on transcription were observed (Carrieri et al., 2012). Further studies are needed to understand whether survival or apoptotic outocomes of different treatments reside, at least in part, on the ability to regulate antisense RNAs at transcriptional or post-transcriptional levels.

In summary, antisense transcription imposes an additional level of regulatory networks to control the activity of protein-coding genes. Determining the repertory of transcription factors controlling the expression of AS RNAs under physiological and pathological conditions will further contribute to our understanding on the biological functions of AS lncRNAs in health and disease. Finally, since increasing evidences suggest Uchl1 over-expression could be beneficial in neurodegenerative diseases, the use of AS Uchl1 as RNA-based drug may represent a new therapeutic strategy.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2015.00114/abstract

Supplementary Figure 1 | Nur77 expression in iMN9D cells. iMN9D cells were treated with doxycyclin for 48 h. Expression of Nur77 was monitored by qRT-PCR with specific primers. Expression of VMAT2 mRNA was used as positive control for Nurr1-mediated activation. Data were analyzed using the $2^{-\Delta Ct}$ method. Data indicate mean ± SD. Nurr1 expression was verified by western blotting.

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Conflict of Interest Statement: SG, PC, CC, AF, and SZ are named inventors in patent issued in the US Patent and Trademark Office on SINEUPs and licensed to TransSINE Technologies. SG and PC are co-founders of PARKscreen, an Italian SME aimed to use and develop therapeutic SINEUPs.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
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SINEUPs are modular antisense long non-coding RNAs that increase synthesis of target proteins in cells

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Despite recent efforts in discovering novel long non-coding RNAs (lncRNAs) and unveiling their functions in a wide range of biological processes their applications as biotechnological or therapeutic tools are still at their infancy. We have recently shown that AS Uchl1, a natural lncRNA antisense to the Parkinson's disease-associated gene Ubiquitin carboxyl-terminal esterase L1 (Uchl1), is able to increase UchL1 protein synthesis at post-transcriptional level. Its activity requires two RNA elements: an embedded inverted SINEB2 sequence to increase translation and the overlapping region to target its sense mRNA. This functional organization is shared with several mouse lncRNAs antisense to protein coding genes. The potential use of AS Uchl1-derived lncRNAs as enhancers of target mRNA translation remains unexplored. Here we define AS Uchl1 as the representative member of a new functional class of natural and synthetic antisense lncRNAs that activate translation. We named this class of RNAs SINEUPs for their requirement of the inverted SINEB2 sequence to UP-regulate translation in a gene-specific manner. The overlapping region is indicated as the Binding Doman (BD) while the embedded inverted SINEB2 element is the Effector Domain (ED). By swapping BD, synthetic SINEUPs are designed targeting mRNAs of interest. SINEUPs function in an array of cell lines and can be efficiently directed toward N-terminally tagged proteins. Their biological activity is retained in a miniaturized version within the range of small RNAs length. Its modular structure was exploited to successfully design synthetic SINEUPs targeting endogenous Parkinson's disease-associated DJ-1 and proved to be active in different neuronal cell lines. In summary, SINEUPs represent the first scalable tool to increase synthesis of proteins of interest. We propose SINEUPs as reagents for molecular biology experiments, in protein manufacturing as well as in therapy of haploinsufficiencies.

Keywords: SINEUP, long non-coding RNA, antisense, protein expression, cell lines

Introduction

Large genomic projects such as ENCODE (Djebali et al., 2012) and FANTOM (Forrest et al., 2014) have shown that the majority of the mammalian genome is transcribed, thus generating a previously underestimated complexity in gene regulatory networks. Protein encoding genes present a large repertory of alternative Transcription Start Sites (TSSs) that may drive transcription in a cell type-specific manner (Valen et al., 2009). Different 5′UTRs may contain information for mRNA sorting to neuronal compartments as well as for stimulus-dependent translation. Furthermore, in addition to 25000 genes encoding for proteins, at least an equal number of long non-coding RNA (lncRNA) genes have been identified so far. These generate >200 base pairs long transcripts that do not encode for proteins. About one third of annotated lncRNAs overlaps with protein-coding genes (Derrien et al., 2012). Many of these are transcribed from the opposite strand forming sense/antisense (S/AS) pairs (Katayama et al., 2005; Derrien et al., 2012).

The nervous system appears as a privileged site for lncRNA expression, as the vast majority of these transcripts is brain-enriched and regulates neuronal development and functions (Qureshi and Mehler, 2012). Furthermore, a complex network of natural S/AS pairs may participate in brain development and homeostasis in physiological conditions. Interestingly, an increasing number of lncRNAs are associated with brain dysfunction and extensive AS transcription has been measured in *loci* associated to hereditary neurodegenerative diseases (Zucchelli et al., submitted).

Manipulating RNA expression *in vivo* has been proposed as new strategy for molecular therapy. Special attention has been devoted to small antisense oligonucleotides (ASOs) and siRNAs as tools to decrease gene expression of pathological target genes such as, for example, mutant huntingtin in Huntington's disease (Kordasiewicz et al., 2012; Yu et al., 2012).

An equally challenging large group of diseases would strongly benefit from the discovery of RNAs that can increase protein levels of genes for which low expression is pathogenic. Several hereditary intellectual and cognitive disabilities are haploinsufficiencies where only a single functional copy of a gene is unable to produce sufficient protein to maintain a physiological condition (Van Bokhoven, 2011). Therefore an RNA-based drug that can restore physiological amounts of the target protein can in principle be curative. Unfortunately, no molecules that can increase protein levels of a specific mRNA type *in vivo* have been found to date.

In search for AS transcripts that may regulate the expression of Parkinson's disease (PD)-associated genes, we recently discovered AS Uchl1, a natural lncRNA antisense to Ubiquitin carboxyl-terminal esterase L1 (Uchl1/PARK5) (Carrieri et al., 2012). AS Uchl1 is a nuclear-enriched transcript, that is expressed in dopaminergic neurons in the *Substantia Nigra*, the target of PD neurodegeneration, and is down-regulated upon PD-mimicking intoxication *in vitro* and *in vivo* (Carrieri et al., 2015). AS Uchl1 activity increases UchL1 protein synthesis at the post-transcriptional level. Upon stressful insults, AS Uchl1 shuttles from the nucleus to the cytoplasm, where it induces Uchl1 mRNA association to heavy polysomes to increase its translation (Carrieri et al., 2012). AS Uchl1 activity depends on two functional domains: the overlapping region that defines target specificity and the inverted SINE element of B2 subclass (invSINEB2) that confers protein synthesis activation. This functional organization is shared with other lncRNAs part of S/AS pairs in the mouse genome. Finally, by substituting the overlapping region with a sequence antisense to the Green Fluorescent Protein (GFP) mRNA, this synthetic RNA was able to increase GFP protein synthesis with a post-transcriptional mechanism (Carrieri et al., 2012).

The potential scalability of AS Uchl1-derived synthetic lncRNAs to a platform of mRNA-specific translation enhancers remained to be addressed.

Here we propose that AS Uchl1 is the representative member of a new functional class of natural and synthetic RNAs that increase protein synthesis. We name these RNAs as SINEUPs for their activity requires an invSINEB2 element (SINE) to UP-regulate translation of partially overlapping sense mRNAs. The overlapping region is indicated as the Binding Doman (BD) while the embedded inverted SINEB2 element is the Effector Domain (ED). By swapping BD, synthetic SINEUPs are designed targeting mRNAs of interest.

This work shows that synthetic SINEUPs are a versatile tool to increase synthesis of target proteins of interest paving the way for future applications of SINEUPs as molecular biology reagents and for manufacturing of recombinant proteins. Most importantly, SINEUPs may be directed to selected mRNA species *in vivo* representing a new type of RNA-based drug for molecular therapy.

Materials and Methods

Constructs

Plasmids expressing target proteins were previously described. In particular, for this study we used pEGFP-C2 (Carrieri et al., 2012), pcDNA3-2XFLAG-DJ-1 (Herrera et al., 2007; Zucchelli et al., 2009), pcDNA3-2XFLAG-TTRAP (Zucchelli et al., 2009; Vilotti et al., 2012), pcDNA3-2XFLAG-Hba (Biagioli et al., 2009) (Codrich et al., manuscript in preparation) and pcDNA3-2XFLAG-TRAF6 (Zucchelli et al., 2010, 2011).

Target specific SINEUPs were constructed using pcDNA3-Δ5′-ASUchl1 as backbone (Carrieri et al., 2012). SINEUP-backbone lacks the region of overlap (BD) to Uchl1 and retains AS Uchl1 ED with inverted SINEB2, Alu sequence and 3′ tail. SINEUP target-specific BDs were designed, in antisense orientation, around the ATG of protein-coding sequence with a −40/+32 anatomy.

SINEUP targeting EGFP (AS-GFP, here named SINEUP-GFP) has been described in Carrieri et al. (2012). SINEUP targeting FLAG-tagged proteins (SINEUP-FLAG) was cloned with the following primers (5′ to 3′ orientation):

Abbreviations: lncRNA, long non-coding RNA; AS, antisense; SINE, short interspersed nuclear element; invSINEB2, inverted SINE of B2 subfamily; PD, Parkinson's disease; TRAF6, tumor necrosis factor receptor associated factor 6; TTRAP/TDP2, TRAF and TNF receptor associated protein/tyrosyl-DNA phosphodiesterase 2; Hba, hemoglobin alpha chain.

FWD AS 2xFLAG:
ATATCTCGAGAATTCCTTGTCATCGTCGTCCTTGTAGTCCATCAATTCCAGCACACTGGCGGCCGT
REV AS 2xFLAG:
GAGAGATATCCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTGATGGACTACAAGGACG

Primers were annealed, elongated by PCR, digested and ligated into XhoI-EcoRV sites of SINEUP-backbone.

Short SINEUP targeting GFP (miniSINEUP-GFP) was generated combining BD of SINEUP-GFP and ED of AS Uchl1. Briefly, inverted SINEB2 was PCR amplified and cloned into EcoRI and HindIII sites of pcDNA3.1(-). SINEUP-GFP BD was subsequently added to the inverted SINEB2-containing plasmid at XhoI and EcoRI sites to obtain miniSINEUP-GFP. The following primers were used:

FWD EcoRI InvSINEB2: TATAGAATTCCAGTGCTAGAGGAGG
REV HindIII InvSINEB2: GAGAAAGCTTAAGAGACTGGAGC
FWD ApaI all O/L: TATAGGGCCCTCTAGACTCGAG
REV EcoRI O/L GFP20: GAGAGAATTCCAGCACAGTGGCGGCCGC

SINEUPs targeting DJ-1 were generated by annealing and PCR-based method (−40/+32) or with annealing and ligation of phosphorylated oligonucleotides (−40/+4), using the following primers:

SINEUP-DJ-1 (−40/+32) FWD:
ATATCTCGAGGCCAGGATGACCAGAGCTCTTTTGGAAGCCATTTTTATGTTATATGTTT
SINEUP-DJ-1 (−40/+32) REV:
GAGAGATATCTTTCAGCCTGGTGTGGGGCTTGTAAACATATAACATAAAAATGGCTT
SINEUP-DJ-1 (−40/+4) FWD:
TCGAGCCATTTTTATGTTATATGTTTACAAGCCCCACACCAGGCTGAAA
SINEUP-DJ-1 (−40/+4) REV:
TTTCAGCCTGGTGTGGGGCTTGTAAACATATAACATAAAAATGGC

All constructs were verified by sequencing.

Cell Lines and Transfection

HEK 293T/17 cells were obtained from ATCC (Cat. No. ATCC-CRL-11268 293T/17) and maintained in culture with Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% FBS (SIGMA) and 1% antibiotics (penicillin/streptomycin), as suggested by the vendor. HepG2 cells were kindly provided by Professor Collavin L. from the University of Trieste, Italy (Lunardi et al., 2009). HepG2 and SK-N-SH were cultured in Eagle's minimal essential medium (SIGMA) supplemented with 10% FBS, 1% antibiotics, 1% GlutaMAX and 1% non-essential aminoacids. HeLa cells were grown with DMEM supplemented with 10% FBS and 1% antibiotics as previously described (Angelini et al., 2007). SH-SY5Y cells were maintained in culture as previously described (Zucchelli et al., 2009). BE(2)M17 were grown in 1:1 MEM-Glutamax (GIBCO)/F12 (GIBCO) supplemented with 10% FBS, 1% antibiotics, and 1% non-essential aminoacids.

When required, HEK 293T/17 cells were treated with rapamycin (SIGMA) at 1 μM for 1 h or at 100 nM for 16 h. DNA damage was induced by doxorubicin (SIGMA), at 1 μM for 1 h or 500 nM for 16 h.

HEK 293T/17 cells were transfected with Fugene HD (Roche) and Lipofectamine 2000 (Life Technologies), following manufacture's instruction. HepG2, HeLa, SH-SY5Y, BE(2)M17 and SK-N-SH cells were transfected with Lipofectamine 2000. All cells were transfected with a 1:6 ratio between sense and SINEUP encoding plasmids, maintaining the conditions described for S/AS Uchl1 (Carrieri et al., 2012). In detail, cells were plated in 6-well plates the day before transfection at 60% (for Fugene HD protocol) or 80–90% (for Lipofectamine protocol) confluency. For transfection with Fugene 0.3 μg pEGFP and 1.8 μg SINEUP plasmid were used; for Lipofectamine 2000 0.6 μg pEGFP and 3.4 μg SINEUP. Cells were collected at 24 h (HeLa cells) or 48 h (HEK 293T/17 and HepG2 cells) after transfection and split in two samples for RNA extraction and Western Blot analysis.

For SINEUPs targeting endogenous mRNAs, SINEUP-encoding plasmid was transfected at the highest dose (4 μg) following manufacture's instructions.

Data of RNA and protein levels were obtained from the same transfection in each replica.

Western Blot

For Western blot analysis, cell pellets were directly resuspended in Laemli sample buffer, briefly sonicated, boiled and loaded on poly-acrilamide gels.

Primary antibodies used in this study include anti-GFP rabbit polyclonal antibody (Life Techologies, Cat. No. A6445), used 1:1000, anti-FLAG M2 (SIGMA, Cat. No. 3165), 1:1000, anti-β-actin (SIGMA), 1:5000, and anti-TRAF6 (Abnova), used 1:500. To detect endogenous DJ-1 protein an antibody produced in our laboratory was used (Zucchelli et al., 2009; Foti et al., 2010). For the detection, anti-mouse-HRP or anti-rabbit-HRP (Dako) in combination with ECL (GE Healthcare) was used. Image detection was performed with Alliance LD2-77WL system (Uvitec, Cambridge). Image quantification was done using Adobe Photoshop CS5.

RNA Isolation, Reverse Transcription and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells using RNeasy Mini Kit (QIAGEN) following manufacturer's instructions. RNA was treated with on-column DNase I (QIAGEN) followed by a second DNase I digestion in solution (Ambion). Two rounds of DNase digestion were required to avoid plasmid DNA contamination in this experimental setting. Single strand cDNA was prepared from 1 μg of purified RNA using the iSCRIPT™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions. qRT-PCR reaction was performed on diluted cDNA (1:20) using SYBR-Green PCR Master Mix (Applied Biosystem) and an iCycler IQ Real time PCR System (Bio-Rad). Relative expression was calculated with the ΔΔCt method (Schmittgen and Livak, 2008). Oligonucleotide sequences of primers used in this study for GFP and GAPDH (Carrieri et al., 2012), DJ-1 (Foti et al., 2010),

TTRAP (Zucchelli et al., 2009), TRAF6 (Zucchelli et al., 2010) and Hba (Biagioli et al., 2009) were previously described.

SINEUP-GFP, SINEUP-FLAG and SINEUP-DJ-1 were detected with primers designed on the 3′ end of AS-Uchl1 (mAS Uchl1 FWD and REV, Primers 3′) (Carrieri et al., 2012). MiniSINEUP-GFP was quantified using the following primers: pTSinvB2 FWD-RT (CAGTGCTAGAGGAGGTCAGAAGA) and pTSinvB2 REV-RT (GGAGCTAAAGAGATGGCTCAG CACTT).

Cellular Fractionation

For fractionation experiments, GFP/SINEUP-GFP were transfected in 10 cm plates at 1:6 ratio using Lipofectamine 2000. Nucleo cytoplasmic fractionation was performed as previously described (Wang et al., 2006). Nucleus and cytoplasmic RNAs were extracted using Trizol reagent (Invitrogen) following manufacturer's instruction. RNA was eluted and treated with DNase I. The purity of the cytoplasmic fractions was confirmed by qRT-PCR on pre-ribosomal RNA using the following primers (Murayama et al., 2008):

FWD 5′-GAACGGTGGTGTGTCGTTC-3′
REV 5′-GCGTCTCGTCTCGTCTCACT-3′

Statistical Analysis

All data are expressed as mean ± standard deviation on $n \geq 3$ replicas. Statistical analysis was performed using Excel software. Statistically significant differences were assessed by Student's *t*-test. Differences with $p < 0.05$ were considered significant.

Results

SINEUPs: Definition and Design

As shown in Carrieri et al. (2012), AS Uchl1 stimulates translation of partially overlapping sense protein-coding mRNAs with no effects on RNA levels.

Here we propose AS Uchl1 as the representative member of a new functional class of natural and synthetic antisense lncRNAs that activate translation. We named these lncRNAs as SINEUPs since they take advantage of an embedded invSINEB2 element to UP-regulate translation. Therefore SINEUPs can be considered the first example of gene-specific inducers of protein synthesis.

SINEUPs display a modular architecture (**Figure 1A**). In the 5′ region, SINEUPs contain the sequence that overlaps, in antisense orientation, to the sense protein-coding mRNA. We named this sequence as SINEUP's Binding Domain (BD) since it provides target selection and SINEUP specificity by RNA-RNA base pairing. In AS Uchl1, BD is 73 base pair long, centered across the ATG with a −40/+32 configuration, spanning part of Uchl1 5′UTR and a portion of its CDS (Carrieri et al., 2012). The remaining SINEUP sequence presents the embedded invSINEB2 element in the non-overlapping part of the transcript. Since this region has been proven essential for protein synthesis up-regulation, we defined it as Effector Domain (ED) (**Figure 1A**).

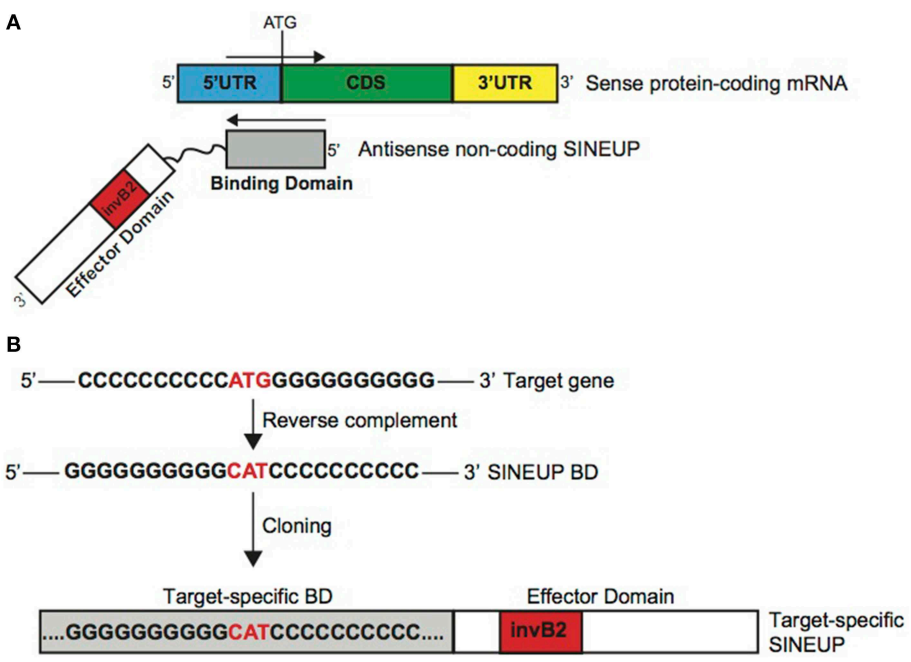

FIGURE 1 | Schematic representation of SINEUPs. (A) SINEUP modular structure. SINEUP binding domain (gray): SINEUP sequence that overlaps, in antisense orientation, to the sense protein-coding mRNA. SINEUP effector domain (red): non-overlapping portion of SINEUPs (white), containing the inverted SINEB2 element (invB2) that confers activation of protein synthesis. 5′ to 3′ orientation of sense and antisense RNA molecules is indicated. Structural elements of protein-coding mRNA are shown: 5′ untranslated region (5′UTR, blue), coding sequence (CDS, green) and 3′ untranslated region (3′UTR, yellow). **(B)** SINEUP design strategy. Schematic representation of the cloning strategy to generate target-specific SINEUPs. An artificial target gene sequence is indicated as example. SINEUP domains are represented as in **A**.

In Carrieri et al., we showed that this modular structure is shared with several natural lncRNAs component of S/AS pair in the mouse genome. For AS Uxt we proved it is able to increase UXT protein synthesis with no effects on Uxt mRNA levels. Therefore these define the first entries in the list of natural SINEUPs.

Given their modular structure, target-specific synthetic SINEUPs can be designed at will by manipulating AS Uchl1 sequence (**Figure 1B**). In Carrieri et al., we have designed the first synthetic SINEUP directing its activity to GFP mRNA by swapping BDs. However, any invSINEB2 sequence from other natural SINEUPs can in principle sustain activity and be considered potential ED.

Provided with the exact TSS for the target gene, sequence of interest is extrapolated centered across the initiating ATG. After reverse-complement manipulation, gene-specific BD is generated by annealing and PCR amplification of specific oligonucleotides. Target-specific SINEUP is then obtained by cloning specific BDs upstream to the SINEUP effector domain. For expression in mammalian cells, SINEUPs are cloned into pcDNA3.1 plasmid. Vectors for retroviral and lentiviral packaging can also be efficiently used (data not shown).

SINEUPs Work *in vitro* in Different Cell Lines

Mammalian cell cultures *in vitro* are routinely used as model systems to study the molecular mechanisms of gene functions as well as cell factories to produce therapeutic proteins. Considering the flexibility of SINEUP technology and its potential applications in molecular biology experiments, protein manufacturing and therapeutics, we investigated the efficacy and reproducibility of SINEUPs in different cell lines *in vitro*. To this purpose we selected hepatocellular carcinoma HepG2 cells, for their use as a model system by large multicenter consortia, epithelial carcinoma HeLa cells for their wide use in cell biology and in therapeutic protein production as well as HEK 293T/17 cells as positive control of SINEUP activity. As a representative synthetic SINEUP, we took advantage of SINEUP-GFP to increase GFP protein levels in transient overexpression experiments.

We estimated SINEUP activity as fold changes in protein levels encoded by targeted mRNAs in the presence/absence of SINEUP with mRNA amounts kept constant ($p > 0.05$).

Similar fold-changes were observed for SINEUP activity with GFP target at 24 and 48 h after transfection (data not shown). However timing for optimal activity was cell-line dependent, as best conditions were found at 24 h in HeLa and 48 h in HEK 293T/17 and HepG2 cells (**Figures 2A–C**).

In HEK 293T/17 cells we obtained an average 2.4 fold change (**Figure 2A**), confirming previously published data (Carrieri et al., 2012) on an independent batch of cells and on a larger cohort of transfections. SINEUP activity ranged from a minimum of 60% induction to a maximum of 400% (**Figure 2D**). SINEUP effect in transfected HEK 293T/17 cells was not enhanced upon stressful stimuli such as rapamycin and DNA-damage inducing drug doxorubicin (**Supplementary Figure 1**). HepG2 cells and HeLa cells proved to support SINEUP activity (**Figures 2B,C**), with an average induction of 1.65 and 1.82-fold, respectively. Minimal values were 20% in HepG2 and 40% in HeLa cells, and top effect was 250 and 220% (**Figure 2D**). No statistical differences could be measured in SINEUP activity between the three cell lines ($p > 0.05$), albeit HEK 293T/17 cells tended to be more effective (**Figure 2D**).

We observed that SINEUP activity is maintained independently of the reagent used for transfection. In HEK 293T/17 cells an average of 2.4 fold change could be measured with Lipofectamine ($n = 5$ experiments) and 2.3 with Fugene HD ($n = 6$ experiments) (data not shown). Under these experimental conditions, RNA from transfected SINEUP-GFP was detected in the cytoplasmic fraction although a prominent accumulation in the nucleus was evident (**Supplementary Figure 2**). Interestingly, sense GFP mRNA was equally distributed between cytoplasmic and nuclear compartments. Altogether these data indicate that SINEUPs can be used *in vitro* in different cell systems to up-regulate proteins of interest.

SINEUPs can be Designed to Increase Production of Target Proteins of Interest

The modular structure of SINEUPs predicts that by swapping the BD with an appropriate sequence it should be possible to redirect SINEUP activity to target mRNA of interest.

To test the flexibility of BD design, we generated a SINEUP molecule targeting the commonly used FLAG tag sequence. FLAG-specific SINEUP would be able to act at a post-transcriptional level increasing the quantities of proteins expressed in frame with an N-terminal FLAG tag. SINEUP targeting FLAG-tagged proteins (SINEUP-FLAG) was designed to mimic the molecular anatomy of SINEUP-GFP. In particular, SINEUP-FLAG BD encompasses −40 nucleotides (in pcDNA3 plasmid backbone) before FLAG-initiating Met and +32 bases covering the first FLAG tag sequence (**Figure 3A**).

We took advantage of a series of protein-coding genes available in the laboratory to test SINEUP-FLAG activity. We used human TRAF6 (RefSeq NM_004620) (Zucchelli et al., 2010, 2011), human TTRAP (RefSeq NM_016614) (Zucchelli et al., 2009; Vilotti et al., 2012), human DJ-1 (RefSeq NM_001123377) (Herrera et al., 2007; Zucchelli et al., 2009) and mouse Hba-a1 (RefSeq NM_008218) (Biagioli et al., 2009) (Codrich et al., manuscript in preparation) cloned in pcDNA3-2XFLAG. HEK 293T/17 cells were transfected with plasmids for FLAG-tagged targets in combination with SINEUP-FLAG (+SINEUP). Cells transfected with an empty vector were used as control (-SINEUP). SINEUP activity was measured quantifying protein levels by Western blot and RNA amounts by qRT-PCR. We found that the quantity of three of four FLAG-tagged proteins that we tested was modulated by co-expression of SINEUP-FLAG (**Figure 3B**). SINEUP effect was different toward the three targets, ranging from 1.5 to 3.0 fold changes. FLAG-TRAF6 showed the strongest SINEUP-mediated activation with a protein induction consistently in the range of 2.6 to 3.0 fold (**Figure 3B** and data not shown). No effect on TRAF6 mRNA was present, as expected ($p = 0.46$). Interestingly, increased TRAF6 levels could

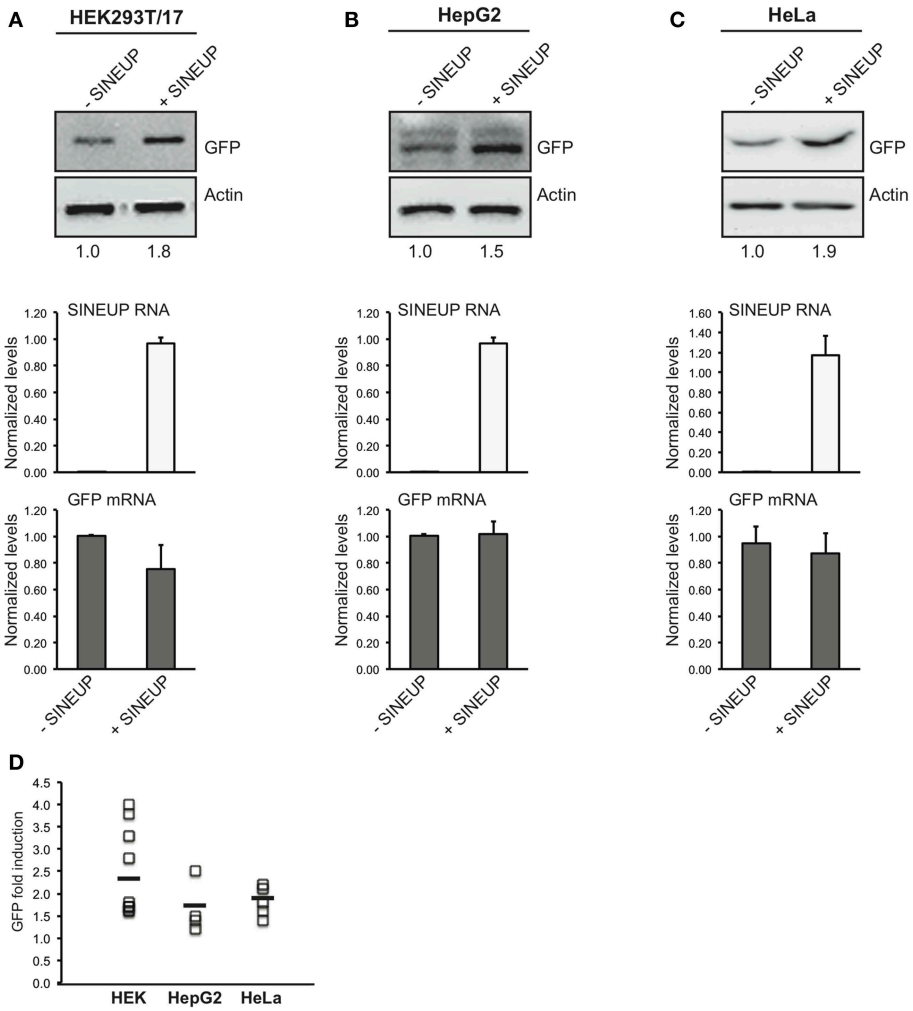

FIGURE 2 | SINEUP activity in cell lines. HEK 293T/17 **(A)**, HepG2 **(B)** and HeLa **(C)** cells were transfected with pEGFP-C2 and SINEUP-GFP at 1:6 ratio (+SINEUP). Control cells were transfected with pEGFP-C2 and an empty control plasmid (-SINEUP). 24 h (HeLa) or 48 h (HEK 293T/17 and HepG2) after transfection, cells were lysed and processed for protein (top) and RNA (bottom) levels. Western blot was performed with anti-GFP antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples. Expression of SINEUP-GFP (white bars) and quantity of GFP mRNA (gray bars) were monitored by qRT-PCR using specific primers. Data indicate mean ± standard deviation. Data are representative of >3 independent replicas. **(D)** Graphical representation of SINEUP-mediated GFP up-regulation in HEK ($n = 10$), HepG2 ($n = 4$) and HeLa ($n = 5$) cells. No statistical difference was present between the three cell lines ($p > 0.05$).

be measured when probing lysates with anti-TRAF6 specific antibody (**Supplementary Figure 3**).

A 50% up-regulation was measured with FLAG-DJ-1 and 90% with FLAG-Hba. In both cases, sense mRNA in -SINEUP and +SINEUP transfections was not statistically different ($p = 0.36$ in FLAG-DJ-1 samples and $p = 0.38$ in FLAG-Hba).

A modest activation was observed with FLAG-TTRAP where a 10–20% increase was typically observed by Western blot. However, this was consistently accompanied by a similar modulation of TTRAP mRNA levels (**Supplementary Figure 4**), thus excluding this effect from SINEUP definition.

In summary synthetic SINEUPs can be designed to commonly used tag sequences and the same SINEUP can dictate translation of different tagged proteins of interest.

MiniSINEUPs Containing Exclusively BD and ED are Active

A major limitation in the use of naked RNA for *in vitro* and *in vivo* applications is the instability and low cellular permeability of long molecules. Chemical modifications can bypass such limits, but with specific constraints in RNA length. Synthetic SINEUPs derived from natural AS Uchl1 are about 1200 nucleotides (nt) long with BD of 72 base pairs, ED of 170 base pairs in addition to intervening sequences, a partial Alu element (73 base pairs) and a 3′ tail. This length is suitable for delivery systems such as viral vectors, but incompatible with the use of SINEUPs as naked RNA therapeutic molecules. Therefore, we aimed at synthesizing the shortest functional SINEUP that retains its translation enhancement activity. MiniSINEUP-GFP

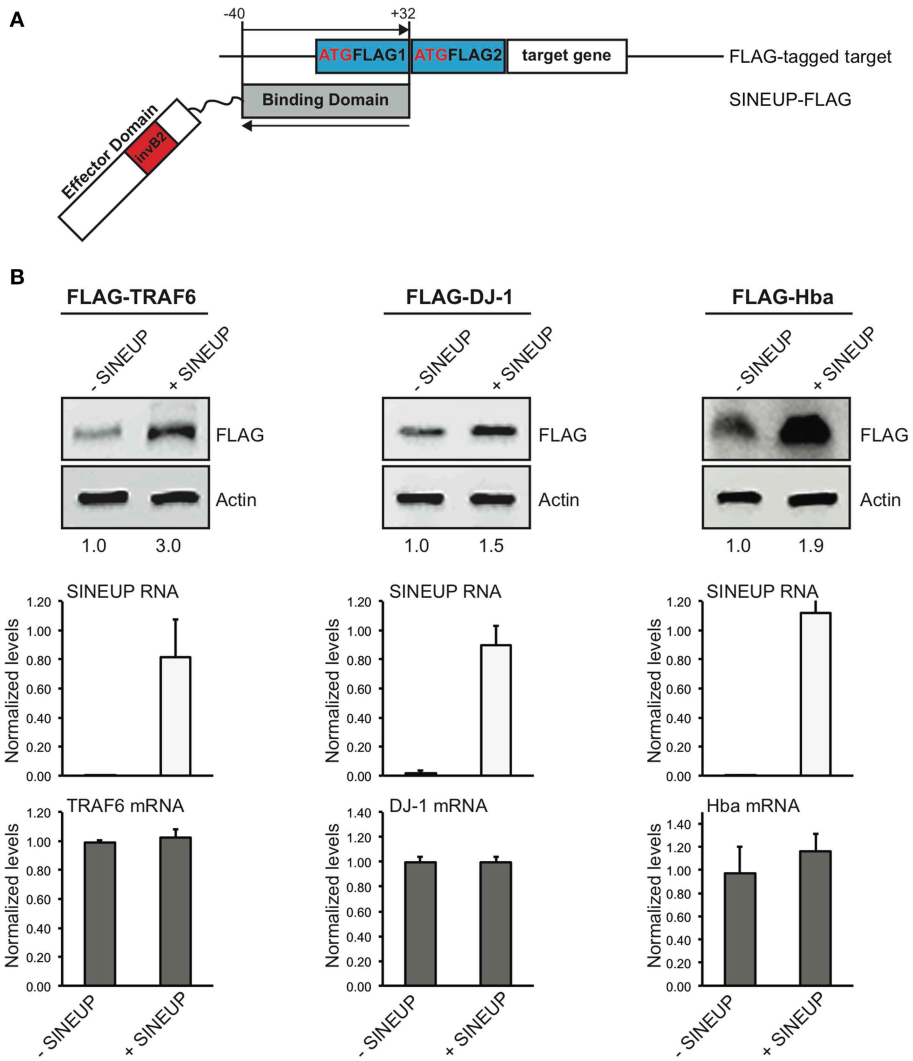

FIGURE 3 | Examples of target-specific SINEUPs. (A) Scheme of FLAG tag-specific SINEUP. SINEUP-FLAG was designed to target the −40/+32 region around the ATG of 2XFLAG expression plasmid (pcDNA3-2XFLAG). **(B)** Activity of SINEUP-FLAG was tested in HEK 293T/17 cells transfected with pcDNA3-2XFLAG-TRAF6, DJ-1 and Hba, as indicated. Protein (top) and RNA (bottom) levels were analyzed by Western blot with anti-FLAG antibody and by qRT-PCR with SINEUP (white) and target-specific (gray) primers, respectively. SINEUP activity was calculated as increase in protein levels relative to empty control samples (fold changes are shown). In all conditions, sense RNA quantities were stable ($p > 0.05$). Data indicate mean ± standard deviation and are representative of three independent experiments.

was obtained combining SINEUP-GFP BD and ED from AS Uchl1 (**Figure 4A**) giving rise to a ≈250 nt long transcript. When transfected in HEK 293T/17 cells miniSINEUP-GFP promoted a 2.5 fold increase in GFP protein levels with unaffected mRNA quantities ($p = 0.11$) (**Figure 4B**). Interestingly, the activity of miniSINEUP was comparable to that obtained with SINEUP-GFP (1.6 fold in this experiment, 2.4 average increase in HEK 293T/17 cells, **Figure 2D**). Under these conditions, we observed a 10-fold excess of miniSINEUP-GFP RNA relative to canonical SINEUP-GFP, as expected from its reduced size. Despite elevated RNA quantities, no impact was observed on GFP mRNA, proving that miniSINEUP retains the very same post-transcriptional mechanism of its full-length counterpart.

SINEUPs can be Targeted to Endogenous mRNAs of Interest

The use of SINEUPs as a versatile tool to increase protein synthesis is strictly dependent on their ability to act on endogenous, cellular mRNAs transcribed from a genomic locus that does not present a natural SINEUP antisense gene.

To prove this crucial point we designed synthetic SINEUPs targeted to endogenous DJ-1 mRNA, a gene involved in recessive familial Parkinson's Disease (PD). We generated two SINEUP-DJ-1 constructs with two different BD elements: −40/+32 (long, L), from −40 nucleotides (in annotated 5′ untranslated region) before DJ-1 translation start site to +32 bases in the coding sequence, as well as −40/+4 (short, S), ending at the

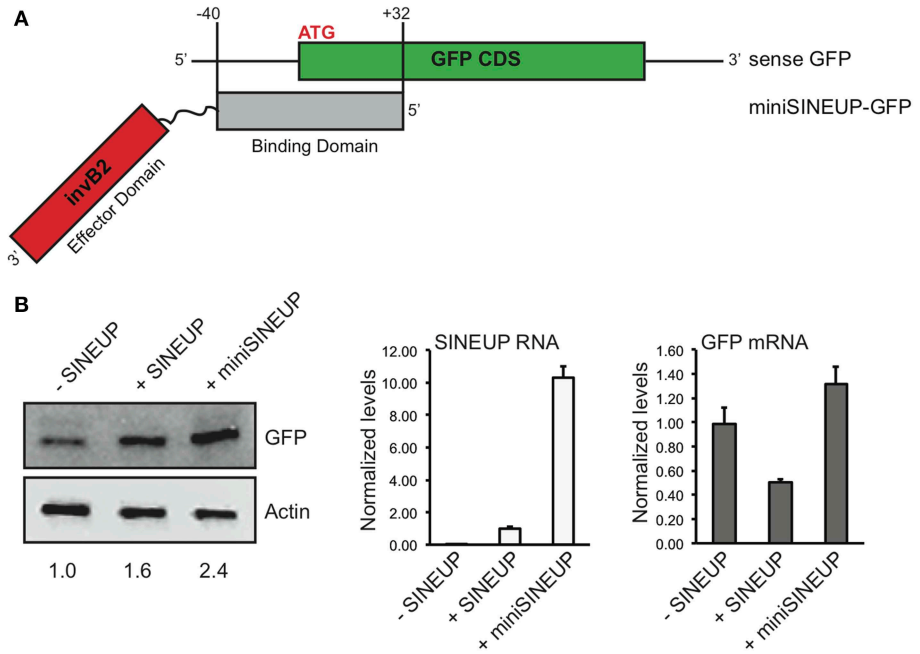

FIGURE 4 | Short SINEUPs are active. (A) Domain organization of miniSINEUP-GFP. Binding (gray) and effector (invB2, red) domains are indicated. **(B)** HEK 293T/17 cells were transfected with pEGFP-C2 and miniSINEUP-GFP (+miniSINEUP). SINEUP-GFP was included as positive control (+SINEUP). Control cells received pEGFP-C2 and pcDNA3.1(-) empty plasmid (-SINEUP). Total proteins (left) and RNA (right) were extracted and tested for GFP expression and GFP and SINEUP RNA quantities, respectively. Data are representative of three independent experiments and indicate mean ± standard deviation.

first nucleotide at 3′ of ATG (**Figure 5A**). Together with HEK 293T/17, we also carried out experiments in three human neuronal cell lines [SH-SY5Y, BE(2)-M17 and SK-N-SH cells] as representative of those frequently used as *in vitro* system to study the function of PD-associated genes. Cells transfected with an empty vector were used as control (-SINEUP).

As shown in **Figure 5B**, SINEUP activity on endogenous DJ-1 ranged from 1.7 to 2 fold with no changes in endogenous DJ-1 mRNA levels. SINEUP-DJ-1 activity was confirmed in all three human neuronal cell lines, proving the versatility of the technology (**Figures 5C–E**). Interestingly, the highest induction of DJ-1 protein levels was measured in SK-N-SH cells (3 fold).

In summary synthetic SINEUPs can be designed to mRNAs of interest that do not present a natural SINEUP in their genomic locus opening up the scalable use of SINEUPs to target endogenous protein coding transcripts of mammalian cells.

Discussion

To our knowledge SINEUPs are the only tool available so far that uses lncRNAs to enhance translation of target proteins. Their modular structure allows synthetic design of an overlapping region (BD) to target proteins of interest, without changing the overall structure of the original lncRNA. Indeed, by extracting solely the target-selecting BD and the ED from the original lncRNA, SINEUPs can be resized to miniSINEUPs retaining their activity.

Over competing technologies, SINEUPs have two major advantages: (1) they modulate translation of target mRNAs without introducing stable genomic changes into target cells; (2) their induction of selected protein is typically in a more physiological range (2-fold) than most conventional gene replacement strategies. These features render SINEUPs (and miniSINEUPs) a potentially interesting tool for a number of applications. First, SINEUPs may be used as reagents for molecular biology. As siRNAs have become an invaluable instrument to inhibit gene expression, many cases exist in which increasing the amount of a specific protein is required. SINEUPs may be designed to a single gene of interest or to a tag that is common to several targets and achieve translation enhancement, thus formally becoming the opposite counterpart of siRNAs. This is especially relevant in the nervous system where regulation of translation has an enormous impact on synaptic plasticity and memory formation. The synthesis of specific proteins requires a fine tuned control at single synapse and spines involving the translation of selective subtypes of mRNAs. The special challenges posed by the anatomo-functional organization of the brain require cellular machinery for controlling mRNAs localization according to the morphology and connectivity of single neuronal cell types. This is achieved at least in part by the use of different TSSs leading to the synthesis of mRNA isoforms with specific 5′UTR containing information for subcellular localization and translation (Baj et al., 2011). SINEUPs can thus induce translation from the mRNA isoform expressed in a defined

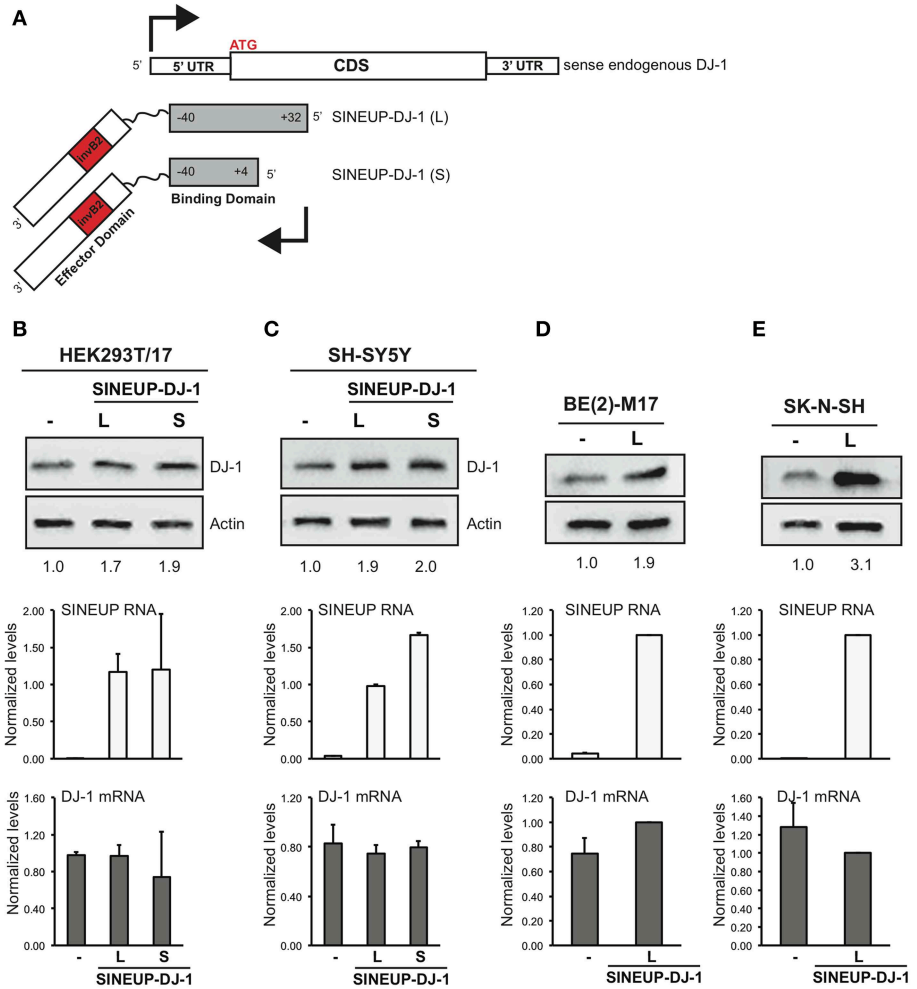

FIGURE 5 | SINEUP is active on endogenous mRNA. (A) Scheme of SINEUP-DJ-1. Two different SINEUPs were designed to target the −40/+32 (L) and −40/+4 (S) regions around the ATG of human DJ-1 gene. Activity of SINEUP-DJ-1 was tested in non-neuronal HEK 293T/17 **(B)**, and in neuronal SH-SY5Y **(C)**, BE(2)-M17 **(D)** and SK-N-SH **(E)** cells. Controls were transfected with empty pcDNA3. Protein (top) and RNA (bottom) levels were analyzed by Western blot with anti-DJ-1 antibody and by qRT-PCR with SINEUP (white) and target-specific (gray) primers, respectively. SINEUP activity was calculated as increase in protein levels relative to empty control samples (fold changes are shown). In all conditions, DJ-1 mRNA quantities were stable ($p > 0.05$). Data indicate mean ± standard deviation and are representative of three independent experiments.

cellular compartment of a selected neuronal cell type increasing specificity.

Here we show that a synthetic SINEUP against the endogenous mRNA for DJ-1, a gene involved in familial PD, is able to increase its protein synthesis. This is important since, to our knowledge, the mammalian genomic DJ-1 locus does not present a natural SINEUP. This experiment thus proves SINEUPs can potentially act on protein coding transcripts of mammalian cells whether or not they are under an endogenous SINEUP-mediated translational control.

Second, considering their effect on translation, SINEUPs may find applications in protein manufacturing. More than 130 therapeutic proteins are currently in use and many more are under development, including antibodies (Leader et al., 2008). Most production strategies have concentrated efforts in optimizing culture conditions and transcription of recombinant genes leaving room for improvement at post-transcriptional level. Recently, large-scale manufacturing platforms have been developed using transiently transfected cells, mainly CHO and HEK293 (Bandaranayake and Almo, 2014). The data presented here and elsewhere (Cotella et al., submitted) support the feasibility of SINEUPs and their potential to be integrated in existing platforms.

Several aspects regulating SINEUP efficacy with selected targets have still to be elucidated. Here we found that, despite identical sequence in the overlapping region, SINEUP-FLAG failed to up-regulate FLAG-TTRAP, whilst being the most effective with FLAG-TRAF6. Furthermore, the very same BD directed to a single mRNA species can increase protein levels with different efficacies according to the host cellular type. Finally, BD of different lengths can act unlike. In this context the role of the secondary structure of the target mRNAs around AUG remains unclear, although different levels of protein increase may be also accounted by turnover rates specific for each protein

or cellular context. While testing synthetic SINEUPs against a large repertory of endogenous mRNAs, we have found that a major cause of apparent lack of activity is due to the wrongful assumption that cells express the Refseq mRNA isoform of the gene. As shown by genome-wide analysis of TSS usage in mammalian cells, the complexity of alternative 5′ ends of mRNAs is staggering. Therefore, when available, we routinely interrogate the FANTOM5 dataset of CAGE libraries (Forrest et al., 2014) of the very same cells used in the experiments taking advantage of the online tool Zenbu (http://fantom.gsc.riken.jp/5/) to identify the correct AUG-surrounding region of the mRNA of interest. Importantly, we observed that high number of cell passages negatively influence SINEUP activity (data not shown). Global CAP-dependent translation is maintained through mTOR activity (Laplante and Sabatini, 2012) and is reduced in the majority of stress conditions (Holcik and Sonenberg, 2005; Sonenberg and Hinnebusch, 2009). We have previously showed that inhibition of mTOR with rapamycin induced an increase in UchL1 protein level dependent on the activity of the natural SINEUP AS Uchl1 (Carrieri et al., 2012). This occurs by triggering shuttling of AS Uchl1 RNA from the nucleus to the cytoplasm and the consequential increased association of Uchl1 mRNA to heavy polysomes for efficient translation. Here we showed that rapamycin and other stressors do not influence the amount of protein increase triggered by synthetic SINEUPs. We may hypothesize that by overexpression we saturate the cytoplasmic content of SINEUP RNA and/or the quantity of target mRNAs that can be associated to heavy polysomes. Further dissection of the cellular pathways that control SINEUP function and identification of SINEUP-binding proteins will provide fundamental insights to improve experimental design and answer these fundamental biological questions.

Finally, lncRNAs represent a new frontier in drug-development. The field of RNA therapeutics has emerged for its great potentials and is set to increase the number of targets beyond initial expectations (Kole et al., 2012). RNA-based drugs have been developed in the past two decades often relying on short non-coding molecules, ASOs or siRNAs, to degrade mRNA or miRNAs of interest. Most recently, lncRNAs appear as therapeutic targets of ASO technology (Modarresi et al., 2012), although the field is still at an early phase. The use of lncRNAs as tools to modulate gene expression is vastly unexplored. This suffers from the limited knowledge of lncRNAs' structure/function relationship and from major obstacles in delivering long RNA molecules. In this context, miniaturization of lncRNAs represents a prerequisite toward applicability in therapeutics. Here we demonstrate that SINEUPs' modular architecture can be employed to construct a miniSINEUP that maintains full-length activity with a length within the small RNA range. Knowing the ED tridimensional structure may provide further insights to help SINEUPs design and optimize activity with minimal length requirements.

In current medical practice there are several unmet therapeutic needs for increasing protein levels *in vivo*. Among them, haploinsufficiency is a condition that arises when the normal phenotype requires the protein product of both alleles, and reduction to 50% or less of gene function results in an abnormal phenotype. This is the cause of a wide spectrum of diseases including ataxias and intellectual and cognitive disabilities. An efficient SINEUP activity specific for the gene of interest would be in principle curative. Furthermore, in many complex and metabolic diseases the increase of pro-survival factors and dysregulated enzymes may impact the well being of patients. As an example, augmented production of neurotrophic factors has been proposed as therapeutic treatments for the majority of neurodegenerative diseases. Therefore SINEUP molecules specific for the transcripts selectively expressed at the site of injury may potentially slow or stop disease progression. Furthermore, it may avoid the unwanted side effects of unregulated expression in the brain that have halted many clinical trials in the past. Our ability to increase endogenous levels of the PD-associated DJ-1 protein represents the first formal prove that synthetic SINEUPs may be a new class of nucleic acid-based drugs.

We are conscious that any potential application of SINEUPs in therapy will strictly depend on their efficient delivery. To this purpose we are currently exploring the preservation of their activity with different delivery systems and chemical modifications.

In summary, here we show first evidences that synthetic SINEUPs may represent a scalable platform for manipulating gene expression of single mRNA species within their physiological range in an array of cell lines.

SINEUPs may thus become a new tool for laboratory experiments, for protein manufacturing and for potential therapeutic intervention *in vivo*.

Acknowledgments

We are indebted to all the members of the SG lab and to the SINEUP network (SISSA, University of Eastern Piedmont, RIKEN) for thought-provoking discussions. We thank Cristina Leonesi for technical support. We are grateful to SISSA technical and administrative staff, especially to J. Franzot, H. Krmac, Monica Sirk and Annalisa Sulli. This work was supported by the Italian Ministry of Education, University and Research (FIRB grant prot. RBAP11FRE9) to SG and FP.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2015.00174/abstract

Supplementary Figure 1 | Treatment with stressful stimuli does not increase SINEUP activity in transfected cells. HEK 293T/17 cells were transfected with pEGFP in combination with SINEUP-GFP (+SINEUP) or control plasmid (-SINEUP). After transfection, cells were treated with rapamycin or doxorubicin as indicated. Lysates were probed anti-GFP antibody. β-actin was used as loading control. Fold-induction was calculated on Western blot images normalized to β-actin and relative to empty control samples.

Supplementary Figure 2 | SINEUP RNA is detected in the cytoplasm of transfected cells. HEK 293T/17 cells were transfected with pEGFP in combination with SINEUP-GFP (+SINEUP). RNA was purified from separated

nuclear and cytoplasmic fractions. RNA was reverse transcribed and probed for SINEUP RNA and GFP mRNA, as indicated. Purity of nuclear and cytoplasmic fractions was monitored by qRT-PCR on precursor rRNA. Data were normalized to the level of GAPDH in each fraction and analyzed with the ΔΔ Ct method. RNA levels in the cytoplasm were set to 1. Data indicate mean ± standard deviation and are calculated on 3 independent replicas.

Supplementary Figure 3 | SINEUP-increased targets can be detected with target-specific antibodies. HEK 293T/17 cells were transfected with pcDNA3-2XFLAG-TRAF6 in combination with SINEUP-FLAG (+SINEUP) or control plasmid (-SINEUP). Lysates were probed anti-TRAF6 antibody.

Supplementary Figure 4 | SINEUP-FLAG does not increase FLAG-TTRAP protein levels. HEK 293T/17 cells were transfected with pcDNA3-2XFLAG-TTRAP in combination with SINEUP-FLAG (+SINEUP) or control plasmid (-SINEUP). Lysates were probed anti-FLAG antibody. SINEUP RNA and TTRAP mRNA were quantified by qRT-PCR with specific primers. Data indicate average ± stdev and are representative of $n = 3$ independent experiments.

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Involvement of ELAV RNA-binding proteins in the post-transcriptional regulation of HO-1

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Heme oxygenase-1 (HO-1) is an inducible rate-controlling enzyme of heme catabolism. The cytoprotective function of HO-1 activity has been verified in multiple studies, and together with its by-products is considered a key component of the cellular stress response. The transcriptional induction of HO-1 has been largely studied in response to multiple forms of stressful stimuli but our understanding of HO-1 post-transcriptional control mechanisms in neuronal cells is currently lacking. In the present report we show the involvement of the RNA-binding proteins (RBPs) embryonic lethal abnormal vision (ELAV) in the regulation of HO-1 gene expression. Our study demonstrates a specific binding between HO-1 messenger RNA (mRNA) and ELAV proteins, accompanied by an increased expression of HO-1 at protein level, in a human neuroblastoma cell line treated with hemin. Clarifying the induction of HO-1 expression at post-transcriptional level may open therapeutic perspectives for treatments associated with the modulation of HO-1 expression.

Keywords: heme oxygenase-1, ELAV, hemin, post-transcriptional regulation

INTRODUCTION

Heme oxygenase-1 (HO-1) is an intracellular enzyme that catalyzes the initial and rate-limiting step in the oxidative degradation of heme, and generates biliverdin, free iron (Fe^{2+}), and carbon monoxide (CO; Calabrese et al., 2006). HO-1 is a heat shock protein (HSP32) and it is recognized as one of the major stress-inducible protein in mammalian cells (Maines, 1997). HO-1 activity can increase several-fold in response to a wide variety of stimuli that cause changes in the cellular redox state (Ryter and Choi, 2002). The products of heme metabolism such as bilirubin, ferritin and CO, mediate many of the anti-inflammatory and antioxidant effects associated with the potent cytoprotection provided by HO-1 enzymatic system (Kirkby and Adin, 2006). HO-1 expression is induced ubiquitously in response to oxidative challenges but brain tissue is particularly susceptible to free radical damage (Poon et al., 2004). Indeed, there is an increasing support that HO-1 plays a crucial protective role in the central nervous system (CNS), primarily in astrocytes and microglia/macrophages, particularly during aging and in several disease states where oxidative stress is implicated, including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD; Calabrese et al., 2004). Although numerous published reports have demonstrated multiple beneficial effects of HO-1, its mechanism of action has not been completely elucidated. Expression of HO-1 is regulated essentially at transcriptional level, even though a post-transcriptional modulation of HO-1 messenger RNA (mRNA) in distinct cellular contexts has been described (Gozzelino et al., 2010). For instance, in human dermal fibroblasts hypoxia regulates HO-1 gene expression by a specific post-transcriptional mechanism: stabilization of mRNA (Kitamuro et al., 2003). Therefore, the regulation of HO-1 mRNA levels in response to cellular stress may be induced by both transcriptional and post-transcriptional events that act independently, and vary in function of the stress inducer (Leautaud and Demple, 2007). Although post-transcriptional control mechanisms are not yet fully understood, it is clear that they are key determinants in the regulation of mRNA stability and translation (Bolognani and Perrone-Bizzozero, 2008; Pascale et al., 2008; Keene, 2010). In particular, RNA-binding proteins (RBPs) regulate gene expression at post-transcriptional level and influence pre-mRNA processing as well as transport, localization, stability and translation of target mRNAs (Dreyfuss et al., 2002). RBPs are crucial in many aspects of cellular physiology and may play a direct role in the pathophysiology of neurodegenerative diseases (Pascale and Govoni, 2012; Perrone-Bizzozero and Bird, 2013; Romano and Buratti, 2013). Recent studies have identified hundreds of RBPs previously unknown (Castello et al., 2012) and a new and fascinating idea is that neurons have their own systems for regulating RNA metabolism, processing, localization, and expression (Darnell, 2013). In this context, embryonic lethal abnormal vision (ELAV) proteins are RBPs mostly expressed in neurons and post-transcriptional regulation in neuronal cells strongly depends on the control exerted by these RBPs (Pascale et al., 2008). In vertebrates, the ELAV (or Hu) family comprises the neuron-specific members HuB, HuC and HuD, and the ubiquitously

expressed HuR (Colombrita et al., 2013). Moreover, emerging insights into the regulation by which cells establish patterns of gene expression suggest that post-transcriptional events influence inducible genes and signaling cascades such as HSPs and oxidative stress-activated pathways (Abdelmohsen et al., 2008; Amadio et al., 2008). HO-1 gene product and its mRNA abundance is controlled at various stages and it was reported that HO-1 induction by nitric oxide (NO) is regulated by the HuR/ELAV. Interestingly, the HuR/ELAV was found to associate with HO-1 mRNA, and this interaction increased following NO treatment (Kuwano et al., 2009). The HO-1 chemical inducer hemin is a highly reactive compound exhibiting pro-oxidant properties in several biochemical reactions. In the present study, we investigated, in SH-SY5Y human neuroblastoma cells, the expression of HO-1 mRNA and protein following hemin exposure and whether in these conditions HO-1 mRNA may represent a target of ELAV RBPs.

MATERIALS AND METHODS

CELL CULTURES AND TREATMENTS

The SH-SY5Y human neuroblastoma cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, L-glutamine (2 mM), nonessential amino acids (1 mM), and sodium pyruvate (1 mM) at 37°C in an atmosphere of 5% CO_2 and 95% humidity, as previously described (Racchi et al., 2003). The cells were exposed to the solvent (PBS) or to hemin (50 or 100 μM as reported in figure legends) for 2 h.

REVERSE-TRANSCRIPTION AND REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Total RNA was extracted from cells using the RNeasy Micro Kit (Qiagen, Milan, Italy). The reverse transcription was performed following standard procedures. PCR amplifications were performed using the LightCycler instrument (Roche Molecular Biochemicals) in the presence of QuantiTect SYBR Green PCR mix (Qiagen, Milan, Italy), with primers designed by using the PRIMER3 software.[1] Primer sequences were as follows: HO-1, 5′-AGC AAC AAA GTG CAA GAT TCT GC-3′ (forward); 5′-CAG CAT GCC TGC ATT CAC ATG-3′ (reverse); product size: 161 bp; RPL6 (Ribosomal Protein L6), 5′- AGA TTA CGG AGC AGC GCA AGA TTG-3′ (forward), 5′-GCA AAC ACA GAT CGC AGG TAG CCC-3′ (reverse). RPL6 mRNA was chosen as the reference mRNA on which HO-1 was normalized because this RNA remained substantially stable during the treatments. Since RPL6 mRNA does not bear ARE sequences, it was also used as negative control in the immunoprecipitation experiments coupled with real-time quantitative polymerase chain reaction (qPCR).

IMMUNOPRECIPITATION

Following treatment, SH-SY5Y cells were harvested and homogenized in a buffer [containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, 0.32 mM sucrose, and a protease inhibitor cocktail] by using a teflon/glass homogenizer. Immunoprecipitation was performed on total homogenates according to a previously published protocol (Amadio et al., 2009). Briefly, immunoprecipitation was carried out at room temperature for 2 h using 1 μg of anti-ELAV antibody (Santa Cruz Biotech, CA, USA) per 50 μg of proteins diluted with an equal volume of 2× Immunoprecipitation Buffer [2% Triton X-100, 30 mM NaCl, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 0.4 mM sodium vanadate, protease inhibitor cocktail and a RNAase inhibitor] in presence of 50 μl of protein A/G plus agarose beads (Santa Cruz Biotech, CA, USA) previously blocked with 5% BSA in PBS. The samples were finally subjected to RNA extraction and reverse transcription. The negative control was obtained in the same conditions, but in presence of an irrelevant antibody with the same isotype of the specific immunoprecipitating antibody. For binding assay, 100 μl of the immunoprecipitation mixes were collected from each sample and used as "input signal" to normalize the real-time qPCR data. For both HO-1 and RPL6, the mRNA content present in the immunoprecipitated pellet has been normalized on the mRNA amount present in the relative "input signal".

WESTERN BLOTTING

Total lysate were diluted in 2× sodium dodecyl sulfate (SDS) protein gel loading solution, boiled for 5 min, separated by 12% SDS-polyacrylamide gel electrophoresis, and then processed following standard procedures. The antibodies anti-ELAV, anti-HO-1, and anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted in TBST buffer [10 mM Tris-HCl, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5] containing 5% milk. The nitrocellulose membranes were processed with Pierce ECL Plus from Thermo Scientific (Rockford, IL, USA). The experiments were performed at least on three different cell preparations using α-tubulin to normalize data. Densitometric analysis was performed using the NIH Image software.[2]

DATA ANALYSIS

For statistical analysis the GraphPad Instat statistical package (version 3.05 GraphPad software, San Diego, CA, USA) was used. The data were analyzed by analysis of variance (ANOVA) followed, when significant, by an appropriate *post hoc* comparison test, as indicated in figure legends. Differences were considered statistically significant when p values ≤ 0.05.

RESULTS

HEMIN INCREASES HO-1 mRNA LEVELS IN SH-SY5Y CELLS

To determine whether hemin treatment affects HO-1 expression in human neuroblastoma SH-SY5Y cells, we first measured HO-1 mRNA levels in total homogenates of cells treated with hemin (100 μM) for 2 h + 4 h recovery. Real-time qPCR data demonstrated a marked increase of HO-1 mRNA level in 2 h hemin-treated samples followed by 4 h recovery as compared to the other samples (**Figure 1**). RPL6 mRNA level remained substantially stable during the experiments (data not shown). These data reveal that in SH-SY5Y cells hemin treatment up-regulates the expression of HO-1 mRNA.

[1]http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm

[2]http://rsb.info.nih.gov/nih-image

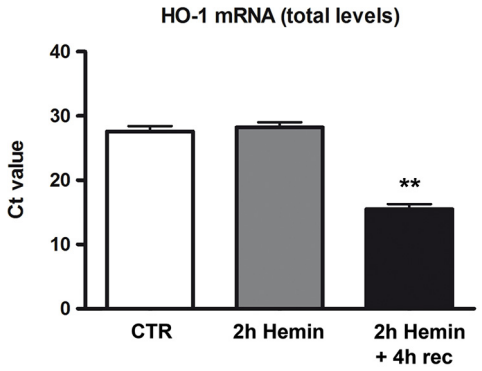

FIGURE 1 | Effect of hemin exposure on HO-1 mRNA levels. Determination of HO-1 mRNA levels by real-time qPCR in human neuroblastoma SH-SY5Y cells following treatments with 100 μM hemin for 2 h + 4 h recovery (rec). HO-1 mRNA contents are expressed as means of the cycle threshold (Ct) ± S.E.M. $^{**}p < 0.01$, Dunnett Multiple Comparisons Test, $n = 5$.

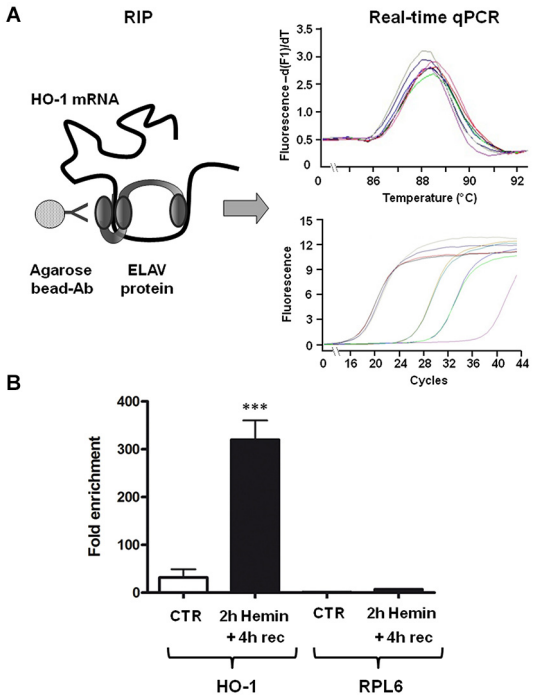

FIGURE 2 | ELAV proteins specifically bind to HO-1 mRNA. **(A)** Schematic representation of the procedure of the RNA-immunoprecipitation (RIP) coupled with the real-time qPCR to determine the interaction between ELAV proteins and HO-1 mRNA (left) and representative real time qPCR melting curves with the corresponding amplification plots (right). Ab: antibody **(B)** Fold enrichment detected by real-time qPCR of HO-1 and RPL6 (housekeeping) mRNAs in control (CTR) and 2 h hemin-treated + 4 h recovery (rec) SH-SY5Ycells following immunoprecipitation with the anti-ELAV antibody. $^{***}p < 0.001$, Tukey-Kramer Multiple comparisons test, $n = 3$.

ASSOCIATION OF HO-1 mRNA WITH ELAV RBPs

To test the hypothesis that ELAV RBPs interact with HO-1 mRNA following hemin treatment, we performed an immunoprecipitation assay on SH-SY5Y cells, which express all the four isoforms of ELAV proteins, followed by real-time qPCR. As shown in **Figure 2**, the association of ELAV RBPs with HO-1 mRNA occurs; indeed, we found that the amount of HO-1 transcript bound by ELAV proteins in the total homogenate is increased following 2 h hemin stimulus + 4 h recovery (**Figure 2**). Moreover, the housekeeping RPL6 mRNA was almost undetectable in the same immunoprecipitated pellets (**Figure 2**), confirming the existence of a specific binding between ELAV proteins and HO-1 mRNA.

HEMIN TREATMENT UPREGULATES HO-1 PROTEIN LEVELS IN A CONCENTRATION-DEPENDENT MANNER

We finally measured HO-1 protein levels by Western blotting, finding an increase after 2 h hemin exposure followed by 4 h recovery (**Figure 3**). Interestingly, in basal conditions, HO-1 protein content is almost undetectable and its increase is proportional to hemin concentration, reaching statistical significance only at 100 μM concentration (**Figure 3**). In the same samples we also measured ELAV proteins levels, finding they are not modified following hemin stimulus (not shown).

DISCUSSION

HO-1 is one of the main players of the endogenous antioxidant responses and since it provides cytoprotection against various neurotoxic insults, it is crucial to elucidate how HO-1 gene expression is regulated. A comprehensive knowledge regarding HO-1 expression in human neural cells is still lacking. Although most studies have focused on the molecular mechanisms responsible for the cytoprotective effects of HO-1, the importance of post-transcriptional processes in the modulation of HO-1 gene expression has been greatly underestimated. Furthermore, there is an emerging consensus that neuronal transcripts are differentially processed in the brain than in other tissues (Darnell, 2013). Interestingly, it was recently demonstrated that RBPs and non-coding RNAs are critical components underlying the post-transcriptional mechanisms for the coordinate regulation of mRNA expression in neuronal systems (Loya et al., 2010). Human ELAV RBPs are involved in the post-transcriptional control of several early-responsive genes such as MYC, FOS and cytokines (Keene, 2007; Papadopoulou et al., 2013). Since HO-1 system is a promising approach to treat specific disorders and its induction has significant consequences in the CNS, the present study explores the role of ELAV proteins in the regulation of HO-1 expression at both mRNA and protein levels. In particular, we treated human neuroblastoma SH-SY5Y cells with hemin, a pro-oxidant molecule. Although HO-1 is activated by various electrophilic compounds including polyphenols (Davinelli et al., 2013), hemin is one of the most effective stressors in terms of reactive oxygen species (ROS) production and it is widely used to study HO-1 function. Therefore, we chose hemin as a challenge for our experiments. However, it is essential to point out that at elevated concentrations hemin may contribute to cell injury by cytotoxic effects, although strengthening endogenous defense against oxidative stress, at nontoxic concentrations its therapeutic

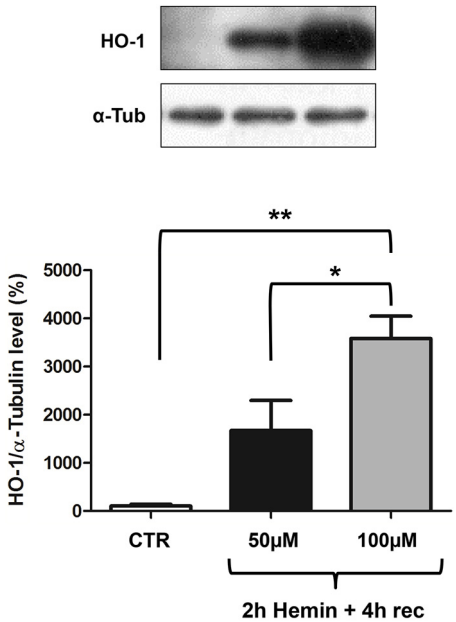

FIGURE 3 | Hemin treatment affects HO-1 protein total levels in SH-SY5Y human neuroblastoma. (Upper) Representative Western blotting of HO-1 and α-tubulin in control (CTR) and hemin-treated SHSY5Y cells. Cells were exposed to either 50 or 100 μM hemin for 2 h followed by 4 h recovery (rec). **(Lower)** Mean gray levels ratios (mean ± S.E.M.) expressed as percentages with respect to control (100%) of HO-1/α-tubulin immunoreactivities measured by Western blotting. $*p < 0.05$; $**p < 0.005$, Tukey-Kramer Multiple comparisons test, $n = 3$.

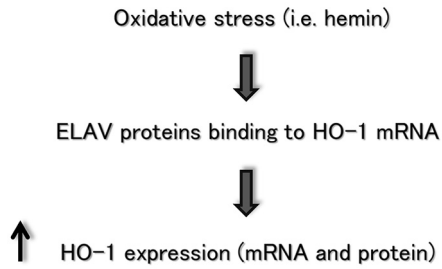

FIGURE 4 | Flow chart depicting the ELAV/HO-1 cascade, in which an oxidative stress stimulus favors ELAV proteins binding to HO-1 mRNA, producing an increase of HO-1 expression at post-transcriptional level. See the text for more details.

potential has been reported in multiple acute injury models, including those at brain level (Lu et al., 2014). In our study, we found that HO-1 expression is induced in 100 μM hemin-treated neuronal cells, as demonstrated by real time qPCR and Western blotting experiments (**Figures 1**, **3**). This finding is consistent with previous studies showing the activation of HO-1 by hemin in human neuroblastoma cells (Nakaso et al., 2003). Interestingly, and in accordance with previous evidence in rat hippocampal neurons (Scapagnini et al., 2004), we observed that, in human SH-SY5Y neuroblastoma cells, HO-1 protein expression is almost absent in basal conditions and it is strongly induced by hemin in a concentration-dependent manner (**Figure 3**). However, we cannot exclude that the sensitivity of Western blotting technique is not sufficient to detect very low amount of protein as in control cells. The hemin-induced increase of HO-1 protein may be due to a positive regulation of HO-1 at post-transcriptional level. Indeed, our results shows a specific association between ELAV and HO-1 mRNA (**Figure 2**), suggesting that this binding may have potential consequences for HO-1 protein expression. Our hypothesis is that HO-1 is present as mRNA in control cells so that, as many early response genes, it can be rapidly translated following a specific stress stimulus, such as hemin exposure, thus contributing to the physiological cellular response.

Although it was well-established that HO-1 is highly inducible by a large number of stressful stimuli such as heme or certain other metalloporphyrins, the regulation of gene expression and induction of HO-1 is complicated by the fact that its regulatory response is not restricted to heme or other physical and chemical factors. A purpose of this study was to bridge the significant gap in understanding the regulation of HO-1 induction in neural cells. Previous evidence from our group showed that the ELAV RBPs are involved in regulating the post-transcriptional fate of HSP70 and SOD-1 mRNA following H_2O_2-mediated oxidative stress in SH-SY5Y cells (Amadio et al., 2008; Milani et al., 2013). HSP70, SOD-1 and HO-1 modulate crucial defensive mechanisms for neurons exposed to an oxidant challenge. Consistently with the data reported for the other genes, the results described in this report suggest a novel role for ELAV RBPs in regulating, post-transcriptionally, HO-1 expression in SH-SY5Y neuronal cells, as schematically represented in **Figure 4**. Future studies are needed to identify whether this interaction is a characteristic feature of HO-1 induction during the cellular stress response.

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RTP801/REDD1: a stress coping regulator that turns into a troublemaker in neurodegenerative disorders

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Mechanistic target of Rapamycin (mTOR) pathway regulates essential processes directed to preserve cellular homeostasis, such as cell growth, proliferation, survival, protein synthesis and autophagy. Importantly, mTOR pathway deregulation has been related to many diseases. Indeed, it has become a hallmark in neurodegenerative disorders, since a fine-tuned regulation of mTOR activities is crucial for neuron function and survival. RTP801/REDD1/Dig2 has become one of the most puzzling regulators of mTOR. Although the mechanism is not completely understood, RTP801 inactivates mTOR and Akt via the tuberous sclerosis complex (TSC1/TSC2) in many cellular contexts. Intriguingly, RTP801 protects dividing cells from hypoxia or H_2O_2-induced apoptosis, while it sensitizes differentiated cells to stress. Based on experimental models of Parkinson's disease (PD), it has been proposed that at early stages of the disease, stress-induced RTP801 upregulation contributes to mTOR repression, in an attempt to maintain cell function and viability. However, if RTP801 elevation is sustained, it leads to neuron cell death by a sequential inhibition of mTOR and Akt. Here, we will review RTP801 deregulation of mTOR in a context of PD and other neurodegenerative disorders.

Keywords: RTP801, REDD1, mTOR, Akt, stress, neurodegeneration, neuron, Parkinson's disease

RTP801 OVERVIEW

RTP801 (also known as REDD1 or Dig2) is a protein encoded by the stress responsive gene DNA-damage-inducible transcript 4 (*DDIT4*). It was initially identified and cloned in 2002 by two different groups simultaneously.

Shoshani et al. (2002) screened for hypoxia-regulated genes in rat C6 glioma cells and they identified a highly up-regulated gene responsive to HIF-1, involved in the regulation of cellular reactive oxygen species (ROS). It was designated *RTP801* (Shoshani et al., 2002).

Concurrently, Ellisen et al. (2002) cloned a gene induced after DNA damage and during embryogenesis in a p53 and p63 dependent manner. This gene was involved in the regulation of ROS and was alternatively named *REDD1*, for regulated in development and DNA damage responses one (Ellisen et al., 2002).

Later in 2003, Wang et al. discovered Dig2 (for dexamethasone-induced gene 2), the mouse homolog of *RTP801/REDD1* in an oligonucleotide microarray analysis from dexamethasone-treated murine lymphoma T cells (Wang et al., 2003).

Further studies displayed *DDIT4* as a rapidly upregulated gene under multiple cellular stresses, such as heat shock (Wang et al., 2003), ionizing radiation (Ellisen et al., 2002), hypoxia (Shoshani et al., 2002; Brugarolas et al., 2004) and energy depletion (Sofer et al., 2005). Moreover other chemical molecules also upregulated *DDIT4* expression, such as dopaminergic neurotoxins 6-hydroxydopamine, MPTP/MPP+ and rotenone (Malagelada et al., 2006), endoplasmatic reticulum (ER) stress inducers tunicamycin and thapsigargin (Wang et al., 2003; Whitney et al., 2009), DNA damage agent etoposide (Wang et al., 2003) and arsenite (Lin et al., 2005b).

RTP801, as a 232 aminoacids protein, is ubiquitously expressed at low levels in numerous human adult tissues (Shoshani et al., 2002). RTP801 localizes in the cytoplasm, the nucleus (Ellisen et al., 2002; Lin et al., 2005b; Michel et al., 2014) and in the membranes (DeYoung et al., 2008; Michel et al., 2014). Besides, a small fraction of RTP801 was detected in the mitochondria in HEK293T cells (Horak et al., 2010) and in *RGC-5* retinal ganglion cell line (del Olmo-Aguado et al., 2013).

There is a related human transcript called RTP801L (RTP801-like) or REDD2 that displays ∼50% sequence identity to RTP801 and has similar functions (Ellisen et al., 2002; Corradetti et al., 2005).

Besides humans, RTP801 is also present in other organisms such as rat, mouse and *Xenopus*. In *Drosophila* it has two related orthologs called Scylla and Charybdis (Reiling and Hafen, 2004).

No functional motifs or structural domains could be identified from RTP801 amino acid sequence analysis and, to date, the entire crystal structure has not been solved. Indeed, only one group crystallized a segment containing aminoacids 89–226 with a deletion of the hydrophobic region ^{200}FLPGF^{204} of the human RTP801 protein (Vega-Rubin-de-Celis et al., 2010). Their work has given new insights into RTP801 structure. They found

that RTP801 presents a unique topology characterized by a two-layered α/β sandwich with a psi-loop motif. Furthermore, a surface patch formed by highly conserved residues was found to be critical for its function. It is formed by two separated regions, ^{138}EPCG^{141} and ^{218}KKKLYSSE^{225}, that are contiguous in the three-dimensional structure (Vega-Rubin-de-Celis et al., 2010). Importantly, the stretch of three lysines ^{218}KKK^{220} is necessary for RTP801 to localize in both mitochondria (Horak et al., 2010) and plasma membrane (Michel et al., 2014).

The key function of RTP801 is its ability to inactivate mTOR (Brugarolas et al., 2004; Corradetti et al., 2005), a master regulator kinase that integrates extracellular signals with intracellular responses to nutrients, growth factors or stress. Indeed, mTOR regulation has a crucial role in development, cancer or in neural survival and plasticity (Hoeffer and Klann, 2010; Laplante and Sabatini, 2012).

RTP801 has a dual role depending on the cellular context, meaning that in proliferating non differentiated cells, RTP801 is anti-apoptotic, and in non-dividing differentiated cells like neurons, RTP801 is pro-apoptotic (Shoshani et al., 2002; Malagelada et al., 2006). This dual function could be nicely observed in a study of rat cortical neurogenesis, where RTP801 controlled neuroprogenitors proliferation and neuronal differentiation. In cortical neuroprogenitors RTP801 was elevated without being toxic. On the contrary, newborn and mature neurons showed lower levels of RTP801. Indeed, if RTP801 elevation was sustained in these differentiating neurons it became pro-apoptotic (Malagelada et al., 2011). How RTP801 can trigger these dual actions based on the cell context is not completely understood and requires further investigation.

In the last decade the role of mTOR in neural cells has become very relevant. Indeed, in the nervous system mTOR controls crucial processes such as protein translation, long-lasting synaptic plasticity and survival via Akt (Tang et al., 2002; Cammalleri et al., 2003; Malagelada et al., 2008). Evidence suggests that mTOR deregulation is involved in neurodegeneration, and therefore the role of RTP801 has emerged as an object of study. In this sense, it will be crucial to understand the fine balance between RTP801 as a stress-coping protein and RTP801 as a pro-apoptotic effector in neurological disorders. Understanding these complex mechanisms will help to design successful therapeutic strategies to halt or, at least, delay neurodegeneration.

HOW IS RTP801 UPREGULATED?

Previous studies suggest that RTP801 toxicity in neurons is proportional to its protein levels (Malagelada et al., 2010; Ota et al., 2014). RTP801 protein increase can be the end point of two different processes: (1) as a result of gene activation by cellular stress (Ryu et al., 2005; Malagelada et al., 2006); and (2) a defective RTP801 degradation (Romaní-Aumedes et al., 2014). Here we will describe the transcription factors responsible for *DDIT4* gene induction, the microRNAs (miRNAs) that regulate its translation and the post-translational events in charge of regulating RTP801 protein levels.

RTP801 TRANSCRIPTIONAL REGULATION

The variety of transcription factors able to induce *DDIT4* gene expression in response to different stressors illustrates the complexity of its regulation. In fact, one feature of the regulation of RTP801/*DDIT4* is its rapidity, crucial to activate the coping mechanisms of the cell in response to the hostile environment. For example, hypoxia upregulates RTP801 expression via HIF-1, since the *DDIT4* gene contains a HRE (hypoxia-response element) in the promoter (Shoshani et al., 2002; Brugarolas et al., 2004). Another hypoxia-mimetic agent, cobalt chloride ($CoCl_2$), needs co-activation of HIF-1 and Sp1 to induce RTP801 (Jin et al., 2007).

Deoxyribonucleic acid (DNA) damaging agents, including ionizing radiation and the DNA alkylating agent methyl methane sulfonate (MMS) also boosted RTP801 expression (Ellisen et al., 2002; Lin et al., 2005a). Ionizing radiation induced RTP801 in a p53-dependent manner in mouse embryonic fibroblasts (MEFs; Ellisen et al., 2002). DNA-damage-inducible transcript 4 transcription was also enhanced by MMS in human keratinocytes via Elk-1 and CCAAT/enhancer-binding protein (C/EBP) in a p53-independent manner (Lin et al., 2005a). Furthermore, RTP801 has also been identified as a transcription target of Elk-1 and C/EBP in response to arsenic-induced ROS (Lin et al., 2005b).

Endoplasmatic reticulum (ER) stress caused by tunicamycin or thapsigargin upregulated RTP801 via activating transcriptional factor 4 (ATF4; Jin et al., 2009; Whitney et al., 2009). ATF4 was also identified as a transcription factor for RTP801 in response to oxidative stress induced by hydrogen peroxide (Jin et al., 2009). Interestingly, ATF4 has a protective role in cellular models of Parkinson's disease (PD) by modulating the levels of the E3 ligase parkin (Sun et al., 2013).

Other transcription factors have been described for *DDIT4* gene regulation like the nuclear factor of activated T-cell c3 (NFAT c3; Zhou et al., 2012) or PLZF in spermatogonial progenitors (Hobbs et al., 2010).

It is noteworthy that all these stressors, via different transcription factors, elevate RTP801 with a common objective to inactivate mTOR. This common response to stressors corroborates the complexity of the integration of the stress signals to modulate mTOR effectively.

Many other stress responsive genes with pro-apoptotic functions are also upregulated in parallel with DDIT4. However, due to space limitations, they will not be reviewed in this text (reviewed in Fulda et al., 2010).

RTP801 TRANSLATIONAL REGULATION

MicroRNA are negative regulators of gene expression and can function as tumor suppressors or oncogenes. To date, at least three miRNAs have been described as regulators of RTP801/REDD1 expression in a context of tumorigenesis. MiR-495 regulates breast cancer stem cells proliferation and hypoxia resistance by regulating RTP801 expression (Hwang-Verslues et al., 2011). Another miRNA, the miR-221, stimulates hepatocarcinogenesis by down-regulating RTP801 expression (Pineau et al., 2010). Furthermore, Micro-RNA30c down-regulates REDD1 expression in human hematopoietic and osteoblast cells after gamma-irradiation (Li et al., 2012).

To our knowledge, no miRNA that modulates RTP801 expression in a context of neurodegeneration has been described. In a near future, miRNAs along with the long non-coding RNAs may have a relevant impact in the regulation of RTP801 levels and function.

RTP801 POST-TRANSLATIONAL REGULATION

Post-translational modifications like phosphorylation, acetylation, ubiquitination or myristoylation, have an important impact in protein stability, function and cellular localization. Indeed, cellular stresses can also affect turnover of many proteins, including RTP801.

Apart from its rapid gene induction under stress, RTP801 proteostasis will also determine its stability, and therefore, its regulatory function towards mTOR.

Related to that, RTP801 mRNA (Wang et al., 2003) and RTP801 protein (Kimball et al., 2008; Katiyar et al., 2009; Malagelada et al., 2010) half-lives are significantly short, revealing that RTP801 is an extremely unstable protein with a fine-tuned post-translational regulation.

One of the modifications that will lead to a rapid protein turnover is ubiquitination. In fact, RTP801 is poly-ubiquitinated and targeted for the ubiquitin-proteasome system (UPS; Katiyar et al., 2009; Romaní-Aumedes et al., 2014).

To date, only three E3 ring ligases have been identified to poly-ubiquitinate RTP801. The first one described was CUL4A-DDB1-ROC1-β-TRCP E3 ligase complex. The complex ubiquitinated RTP801 and targeted it for proteasomal degradation, in a GSK3β-phosphorylation-dependent manner (Katiyar et al., 2009).

The second E3 ubiquitin ligase for RTP801 was HUWE1/MULE that modulated RTP801 protein levels although this regulation seemed to be UPS-independent (Tan and Hagen, 2013).

So far, the role of both ligases in regulating RTP801 in neurodegenerative disorders has not been elucidated.

In our recent work, we found that parkin RING E3 ligase poly-ubiquitinates RTP801 to mediate its UPS degradation. Based on the results obtained in cellular and animal models and in samples from human parkin mutant carriers, we proposed that RTP801 elevation due to parkin loss-of-function in both parkin mutants and in idiopathic PD might contribute importantly to neurodegeneration (Romaní-Aumedes et al., 2014).

In a near future, other ligases will eventually be proved to ubiquitinate RTP801, due to its central role in regulating mTOR.

RTP801 INACTIVATES mTOR VIA TUBEROUS SCLEROSIS COMPLEX

Under stress conditions downregulation of mTOR activity caused by hypoxia, energy stress or exposure to dopaminergic neurotoxins required the expression of RTP801 and an intact TSC1/TSC2 tumor suppressor complex (Brugarolas et al., 2004; Sofer et al., 2005; Malagelada et al., 2006). Interestingly, despite the clear necessity of the intact TSC1/2 complex for RTP801 to downregulate mTOR, RTP801 does not seem to interact physically with either TSC1 or TSC2 (Vega-Rubin-de-Celis et al., 2010).

Tuberous sclerosis complex (TSC) is a heterodimer formed by two proteins, the tuberous sclerosis tumor suppressors TSC1 and TSC2. TSC2 has a catalytic function as a GTPase activating protein (GAP), and it acts toward the small GTPase Rheb, an upstream positive mTOR regulator (Inoki et al., 2003; Tee et al., 2003). TSC2 can be phosphorylated by several kinases in response to upstream signals, and they will modify its regulation towards mTORC1 (reviewed in Ma and Blenis, 2009). Akt can phosphorylate TSC2 in response to growth factors (Inoki et al., 2002). This event is thought to favor TSC2 binding to protein 14-3-3, instead to TSC1, leading to TSC2 inhibition, to finally activate mTORC1 (Cai et al., 2006).

Regarding the modulatory role of 14-3-3 towards TSC2, DeYoung et al. (2008) proposed a molecular mechanism by which RTP801 regulates TSC1/2-mTOR signaling involving 14-3-3. Other studies also reported interaction between RTP801 and 14-3-3 proteins by co-immunoprecipitation experiments (Favier et al., 2010; Hernández et al., 2011; Pieri et al., 2014).

However, this RTP801 direct binding to 14-3-3 has been questioned by others (Vega-Rubin-de-Celis et al., 2010). The supposed 14-3-3 binding motif in RTP801 (^{133}RLAYSEP^{139}) is not conserved within species and the crystallized RTP801 structure analysis does not reveal any established mode for 14-3-3 binding. Thus, the inhibitory mechanisms of RTP801 towards mTOR need to be further investigated.

RTP801 UPREGULATION IN NEURODEGENERATION

The etiology of PD and many other neurodegenerative disorders involves both environmental factors and genetic predisposition. Indeed, exposure to several environmental toxins such as pesticides and metals has been implicated in its pathogenesis (Migliore and Coppede, 2009; Cannon and Greenamyre, 2011; Baltazar et al., 2014).

Arsenic is a heavy metal that has been linked to neurotoxicity and carcinogenesis in humans by a mechanism involving ROS production (reviewed in Qian et al., 2003). Interestingly, arsenite induced RTP801 transcription (Lin et al., 2005b).

Moreover, extensively used dithiocarbamate pesticides Maneb (MB) and Mancozeb (MZ) induced DNA damage and elevated RTP801 mRNA and protein expression (Cheng et al., 2014). The toxicity mechanism of these pesticides has been linked to NF-κB activation, revealing a cross-talk between RTP801 and NF-κB (Cheng et al., 2014).

In line with this, RTP801 was also identified as an amyloid-β-peptide (Aβ) responsive gene. Amyloid-beta is a neurotoxic molecule and the main component of senile plaques in Alzheimer's disease (AD; Kim et al., 2003).

Dopaminergic neurotoxins also upregulated RTP801 in cellular and animal models. Specifically, RTP801 was upregulated at both transcriptional and protein level in neuronal PC12 cells treated with PD mimetic toxins 6-OHDA, MPP^+ and rotenone, and it was also induced in neurons of MPTP-treated mice and in Substantia Nigra pars compacta (SNpc) degenerating neurons of PD patients. Furthermore, RTP801 knockdown with short hairpin RNAs (shRNAs) protected the cultures from PD mimetic toxins (Malagelada et al., 2006).

Ubiquitin-proteasome system deregulation has been implicated in neurodegenerative disorders (Keller et al., 2000; Jana et al., 2001; McNaught et al., 2003). Accumulations of misfolded or aggregated proteins may hamper cellular functions and eventually lead to neuronal death (reviewed in Tanaka and Matsuda, 2014). RTP801 brief protein half-life suggests that UPS malfunction would affect RTP801 protein levels. Therefore it is logical to suggest that a proper RTP801 degradation might be crucial for neuron function and survival.

Thus, chronic and progressive RTP801 elevation could become a hallmark of neurodegenerative disorders due to a combination of stress-induced gene activation and UPS malfunction.

THE ROLE OF RTP801 IN NEURODEGENERATIVE DISORDERS

RTP801 behaves like many other stress-induced genes, where a small increase is beneficial but a chronic and sustained increase is detrimental for the neuron. Interestingly, since RTP801 is able to inactivate protein translation, via mTOR, and survival, via Akt (Malagelada et al., 2006, 2008, 2010), its pro-apoptotic role may be relevant to other neurodegenerative diseases.

In PD, RTP801 elevation was initially identified in cellular and animal models of the disease. These results were further confirmed in nigral neurons from both idiopathic PD and mutant parkin human brains, meaning that RTP801 may have a relevant role in the disease itself (Malagelada et al., 2006).

Since RTP801 elevation is necessary and sufficient to trigger neuronal death, it is logical to think that the upregulation in the SN from PD human brains might be detrimental for the nigral neurons. Interestingly RTP801 induces cell death through a mechanism involving TSC1/2 and mTOR repression (Malagelada et al., 2006). Indeed, RTP801 repression of mTOR led also to the suppression of the phosphorylation of the neuronal survival kinase Akt (Malagelada et al., 2008), reviewed in (Greene et al., 2011). This negative regulation of Akt was also observed in nigral neurons from PD human brains.

Based on these results, we proposed a mechanism to explain RTP801 contribution to neurodegeneration in PD, where low levels of RTP801 would help neurons to cope with stress, but if this inactivation were sustained in time, it would impair neuronal function and survival. At the latest stage, and when the critical threshold of mTOR/Akt inactivation is surpassed, neuronal death will occur (Malagelada et al., 2006, 2008; see **Figure 1**). This proposed mechanism is supported by the observation of elevated RTP801 and diminished Akt phosphorylation in nigral neurons of PD brains (Malagelada et al., 2006; Romaní-Aumedes et al., 2014).

To extend this working model, one possibility would be that at early stages of many neurodegenerative disorders, RTP801 induction by cellular stresses would contribute to mTOR depression in an attempt to preserve neuron function and viability. However, at more advanced disease stages, when RTP801 upregulation is prolonged in time, it can eventually promote neurodegeneration and neuronal death through mTOR and pro-survival kinase Akt inactivation, as it has been proposed in the PD studies.

In AD an elevation of both RTP801/REDD1 gene and protein was observed in lymphocytes from AD patients compared to age-matched controls. Damjanac et al. (2009) showed that Double-stranded RNA-dependent protein kinase (PKR), a cognitive decline biomarker with an impairing protein translation function, by phosphorylating p53, activated REDD1 gene transcription. It could be interesting to explore whether this mechanism can be observed also in hippocampal neurons or in AD murine models.

Recently, RT801 has been determined crucial for stress-mediated synaptic loss and depressive behavior. Chronic unpredictable stress increased RTP801 mRNA and protein levels in rat prefrontal cortex (PFC). This RTP801 upregulation was coincident with a reduced phosphorylation of mTORC1/2 signaling targets S6K, 4EBP1, and Akt. In agreement, RTP801 was elevated in postmortem PFC of patients with major depressive disorder compared to psychiatrically healthy controls. RTP801 knockout mice were resilient to the synaptic and behavior deficits caused by stress, while RTP801 overexpression in rat PFC was sufficient to promote neuronal atrophy and depressive behavior (Ota et al., 2014).

Further investigations are needed to elucidate the role of RTP801 in many other neurological disorders. It will be interesting to explore whether RTP801 levels are upregulated and whether they correlate with the degree of mTOR/Akt repression. Also, it will be important to determine whether RTP801 is promoting neuron death through the same mechanism proposed as in PD models.

RTP801 AS A POTENTIAL THERAPEUTIC TARGET IN NEUROLOGICAL DISORDERS

Neurodegenerative disorders are characterized by neuronal death of specific subpopulations, such as loss of SN dopaminergic neurons in PD (reviewed in Dauer and Przedborski, 2003). However, current PD therapies ameliorate symptoms but do not prevent neuronal death. Thus, it is essential to investigate the mechanisms that underlie neuronal death and identify targets involved in the pathophysiology (reviewed in Levy et al., 2009). Based on several studies, RTP801 could become one such potential therapeutic target (Brafman et al., 2004; Malagelada et al., 2010; Tarazi et al., 2014).

RTP801 promotes cell death by sequentially inactivating mTOR and Akt (Malagelada et al., 2006, 2008). Thus, compounds that can restrain RTP801 expression or modulate the mTOR/Akt pathway may become therapeutic and delay neurodegeneration and neuronal cell death in PD (reviewed in Tarazi et al., 2014).

One such compound is 8-methyl-6-phenoxy-2-(tetrahydropyran-4-ylamino)pyrido[2,3-d]pyrimidin-7-one (FLZ), a synthetic squamoside derivative from a Chinese herb that protected dopaminergic neurons from apoptosis triggered by MPP^+ and 6-OHDA (Zhang et al., 2007a,b). Interestingly, FLZ neuroprotective actions in PD involved activation of Akt/mTOR signaling pathway and inhibition of RTP801 expression (Bao et al., 2012, 2014).

Another promising compound is rapamycin, an allosteric inhibitor of some but not all mTOR activities. Rapamycin conferred neuroprotection in both cellular and animal models of PD (Tain et al., 2009; Dehay et al., 2010; Malagelada et al., 2010). The results obtained support the hypothesis in which rapamycin

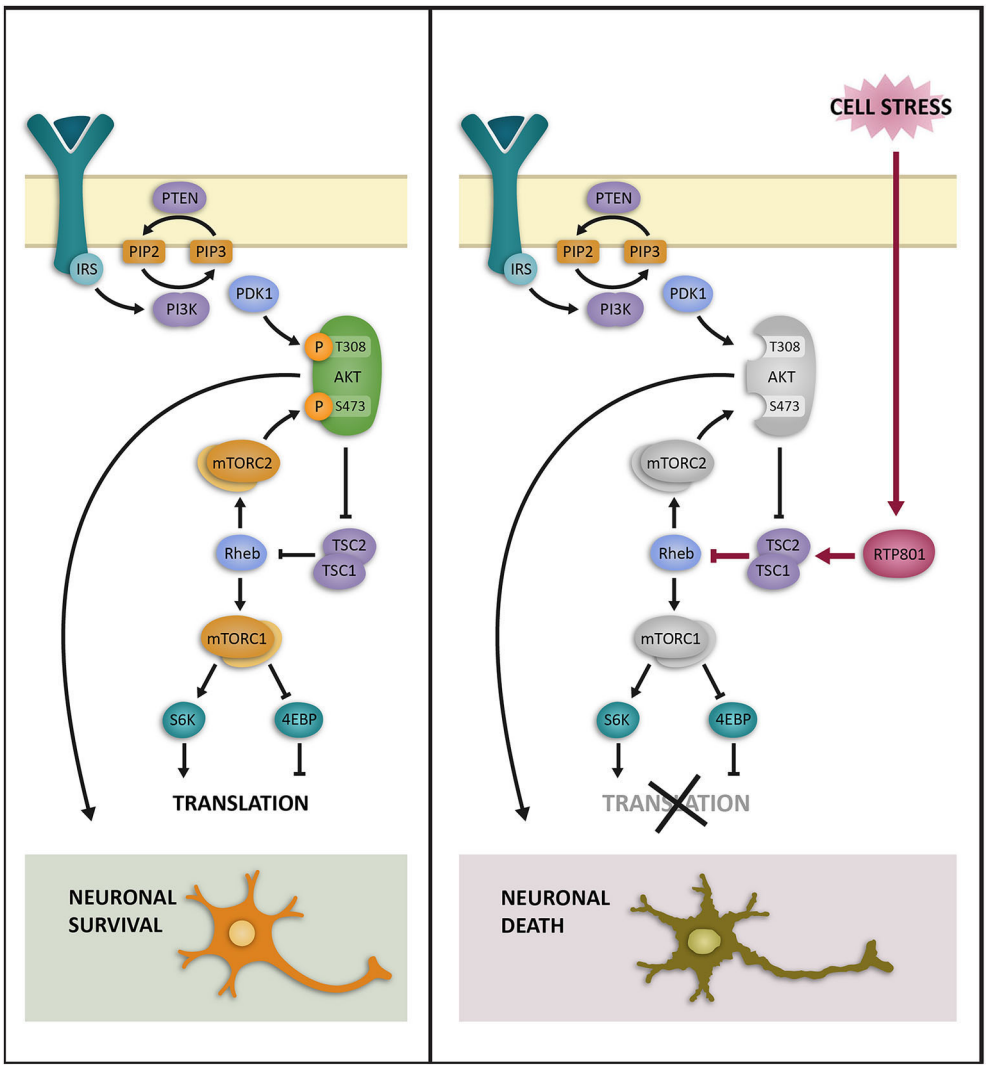

FIGURE 1 | Schematic representation of the hypothesized regulation of mTOR/Akt by RTP801 in neurons. In physiological conditions the gene and the protein levels of RTP801 are low; mTOR is active and promotes protein translation (mTORC1) and Akt phosphorylation at Ser473 residue (mTORC2). These signals mediate neuronal survival (left panel). However, when neurons are under stress, RTP801 is induced at gene and protein levels, and promotes mTORC1 and mTORC2 inhibition through TSC1-TSC2 complex and Rheb protein. These events result in protein translation inhibition and prevent Akt phosphorylation at residues Ser473 and, consequently, at Thr308. If this mTOR/Akt repression is sustained over time neuron function is impaired and leads to neuron death (right panel). *Illustration by Olivares-Boldú L.*

blocks RTP801 translation and, as a consequence, it mitigates mTOR repression leading to Akt phosphorylation maintenance at a site critical for its pro-survival activity (Malagelada et al., 2010). In contrast, Torin 1, an inhibitor of all mTOR actions since it blocks the ATP-binding site, was not protective and promoted Akt dephosphorylation and neuron death. So, rapamycin protection derives from its partial suppression of certain mTOR actions due to its allosteric properties (Malagelada et al., 2010).

Nonetheless, further investigations are required to evaluate the potential therapeutic role of these two agents in PD.

In animal and cellular models of cerebral ischemia, Ligustilide, a major active agent of Radix Angelicae Sinensis, the root of a chinese herb called Danngui, is neuroprotective by inhibiting RTP801 expression, in addition to promote Erythropoietin transcription via extracellular-signal-regulated kinases (ERK) signaling pathway (Wu et al., 2011).

The most advanced example of RTP801 as a therapeutic target is found in retinopathies. RTP801 has an important role in the pathogenesis of retinopathies, since the absence of RTP801 expression in a mouse model attenuated the development of the disease (Brafman et al., 2004). PF-04523655, a 19-ribonucleotide siRNA designated to inhibit RTP801 transcription is currently in clinical trials for retinopathy treatment (Lee et al., 2012; Nguyen et al., 2012a,b; Rittenhouse et al., 2014).

In summary, RTP801 is one clear example of a protein that is upregulated to cope with cellular stress although its sustained

progressive elevation leads to neuron degeneration and death. RTP801 progressive elevation and its inhibitory function towards pro-survival kinases mTOR and Akt could explain its role in several neurodegenerative diseases.

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Legal but lethal: functional protein aggregation at the verge of toxicity

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Many neurodegenerative disorders are linked to irreversible protein aggregation, a process that usually comes along with toxicity and serious cellular damage. However, it is emerging that protein aggregation can also serve for physiological purposes, as impressively shown for prions. While the aggregation of this protein family was initially considered exclusively toxic in mammalians organisms, it is now almost clear that many other proteins adopt prion-like attributes to rationally polymerize into higher order complexes with organized physiologic roles. This implies that cells can tolerate at least in some measure the accumulation of inherently dangerous protein aggregates for functional profit. This review summarizes currently known strategies that living organisms adopt to preserve beneficial aggregation, and to prevent the catastrophic accumulation of toxic aggregates that frequently accompany neurodegeneration.

Keywords: amyloids, prions, proteotoxicity, neurodegenerative diseases, proteostasis regulators

INTRODUCTION

Low structural complexity is at the basis of highly diversified molecular recognition, whereby one flexible protein region can bind to various heterogeneous ligands by conformational adaptation. Proteins can functionally benefit from binding promiscuity for key regulatory processes such as signal transduction, transcription, RNA processing and translation. Proteins situated on intersecting hubs of different pathways can undergo multifunctional interactions, functioning as molecular switches by means of conformational variability. However, the benefits of conformational freedom come along with the menace of protein misfolding and multifunctional failure. Although each living organism invests conspicuous amounts of energy for the rescue or elimination of misfolded polypeptides, a possible inability of the cell to cope with misfolded polypeptides inevitably leads to a massive functional destabilization, whereby proteins can either lose their original function (loss-of-function), or they acquire an improper, and therefore mostly lethal function (gain-of-function), eventually aggregating after the complete collapse of folding and clearance pathways.

The central nervous system (CNS) is particularly susceptible to protein misfolding, but the reasons of such selective neuronal vulnerability are still elusive (Saxena and Caroni, 2011). Although healthy adult neurons can manage proteostasis by standard folding and degradation routines (see Section Molecular catchers in the amyloid rye: heat shock proteins and proteolytic pathways), macroscopic protein misfolding manifestations prevail with senescence, as attested by the occurrence of ageing-associated neurodegenerative disorders such as Parkinson Disease (PD), Alzheimer Disease (AD), spongiphorm encephalopathies, or amyotrophic lateral sclerosis (ALS), all of them displaying unsoluble protein inclusions as a signature.

Abbreviations: CNS, central nervous system; PD, Parkinson Disease; AD, Alzheimer Disease; ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; HD, Huntington Disease; aSyn, alpha-synuclein; Abeta, beta-amyloid; prp, major prion protein; htt, huntingtin; PLR, prion-like region; SG, stress granules; hsp, heat shock protein; UPS, ubiquitin-proteasome system; CMA, chaperone–mediated autophagy; MA, macroautophagy; GAG, glycosaminoglycan; HSPG, heparan suphate proteoglycans.

AMYLOID AGGREGATION

STRUCTURAL PROPERTIES OF AMYLOIDS

Irrespectively of the protein structure and the exact anatomic localization, one type of proteinaceous inclusions appearing in the CNS features some unifying histologic and biophysical hallmarks classified under the term "amyloid", consisting of (A) a fibrous, non-branched morphology, (B) the ability to alter the spectral properties of the dyes Congo red and thioflavin T, and (C) X-ray diffraction patterns typical of cross-beta structure. The term was coined by German pathologist Rudolf Virchow during the characterization of masses in human brains described as "corpora amylacea". Virchow perceived the relationship between amyloids and disease, when he addressed the problem of "amyloid degeneration" (Virchow, 1855). Today, it is well established that the appearance of amyloids is associated with a chronic tissue degeneration of the brain, and although not restricted to this organ, our current understanding of misfolding diseases is invariably linked to amyloid-associated neurological brain disorders (For a detailed list of known amyloid diseases, we refer to the classification of the International Society of Amyloidosis Sipe et al., 2014).

Amyloid aggregation is stereotypically linked to conformational flexibility, which allows for structurally diverse polypeptides to fold from a native into an alternative structure of the same

chemical composition, but with high beta-sheet content, a remarkable resistance against proteolytic and denaturing agents, and the ability to self-associate into fibers of typical cross-beta structure (Eisenberg and Jucker, 2012). The pathway of this association is dynamically variegated, highly perturbable, and sensitive to a repertoire of factors such as genetic mutations, small ligands, changes of the physico-chemical environment, or post-translational side chain modifications (Eichner and Radford, 2011). The resulting heterogeneous multiplicity of conformational intermediates differing in size, shape and stability poses an obstacle in isolating and unambiguously categorizing amyloid aggregates, especially these conformers among them which are believed to be proteotoxic (Eisenberg and Jucker, 2012), and which likely accumulate in low amounts somewhere between native polypeptide and mature amyloid. Improved biophysical techniques can in part overcome these limitations, and they are beginning to provide useful structural insights of amyloid folding intermediates. By combining rapid fluorescence techniques with NMR spectroscopy, Sarkar et al. measured conformational fluctuations of a short-lived, low-abundance AD-associated beta-amyloid (Abeta) oligomer (Sarkar et al., 2014). They could pinpoint the dynamic structure of this oligomer to a patchwork of amino acid segments which fold locally before the transition into highly ordered amyloid filaments. Likewise, Röthlein et al. employed time resolved fluorescence and computational calculations to obtain structural views of an extremely unstable huntingtin (htt) amyloid filament (Röthlein et al., 2014).

MOLECULAR PATHWAYS OF AMYLOID PROPAGATION

Amyloid folding has been coupled early to prion diseases (Prusiner et al., 1983), a type of devastating neurodegenerative disorders that are transmitted by direct and self-sustained intercellular propagation of toxic agents, which constitutes an outstanding and devastating strategy to bypass genetic routes of infection. While the infectious prion particle known as PrP^{Sc} is typically amyloid (Prusiner et al., 1983), the cellular conformer PrP^{c} is a non-amyloid, non-infectious globular protein of still undefined function. This striking difference led to suppose that prion proteins must access an amyloid state to acquire transmissible properties.

A growing amount of studies suggests that this type of cell-to-cell transmission is apparently common to amyloids, as it has been observed also for other amyloidogenic proteins with well-known implication in neurodegenerative diseases (**Table 1**), suggesting that the amyloid fold is a structural prerequisite of cell-to-cell propagation (Eisenberg and Jucker, 2012). Indeed, it was discovered, that amyloidogenic proteins such as alpha-Synunclein (aSyn), tau, or Abeta can also propagate between cells, and that these transmissible particles share structural properties closely similar to those of the archetypic infectious particle PrP^{sc}: they have a predominant beta-sheet structure, a remarkable resistance against proteolytic and denaturing agents, and a self-templating ability (Prusiner et al., 1983; Luk et al., 2012; Iba et al., 2013; Sanders et al., 2014; Stöhr et al., 2014; Watts et al., 2014). Similar rearrangements are likely to occur for endogenous tau in mice brains upon the stereotactic delivery of toxic tau seeds, which is sufficient to initiate spreading of AD-like neurofibrillary tangles along a spatially defined trajectory (Iba et al., 2013). In close analogy, the injection of aSyn seeds into brains of healthy mice triggers the self-perpetuating polymerization of endogenous aSyn and the development of clinical symptoms of PD (Luk et al., 2012). In both cases, the sequential diffusion of proteotoxicity along interconnected brain regions substantiates the theory of a staged evolution of PD and AD (Braak et al., 2006). With respect to original prions, all these proteins have thus been attributed "prion-like" or "prionoid" propagation properties. However, complicating a pathologic interpretation is the occurrence of different conformationally variable toxic species from the same protein, with separate propagation behavior and distinct phenotypic manifestations of the same condition. Sanders et al. demonstrated that tau, the signature protein of tauopathies, forms biochemically and morphologically distinct oligomers accounting for different disease phenotypes, with AD inclusions showing the most homogenous composition (Sanders et al., 2014). Similar has been shown for Abeta, the major component of AD amyloid plaques. Different patient-derived as well as synthetic species of Abeta display an individual transmission behavior, accumulation pattern, aggregate morphology, and chemical stability (Stöhr et al., 2014; Watts et al., 2014). Bousset et al., also reported of two differently toxic aSyn conformers with separate propagation properties (Bousset et al., 2013). Additional complexity rises from the ability of particular amyloid aggregates to act as cross-nucleation seeds for other structurally unrelated proteins. Alpha-Synunclein (aSyn), the major constituent of PD associated Lewy Inclusions, can initiate the deposition of tau in primary neurons and transgenic mice (Guo et al., 2013), while TDP-43 amyloids can seed for Abeta fibril growth (Fang et al., 2014), which might contribute to co-morbidities observed for some types of amyloid pathologies.

Exosomes

We have only a slight clue on the mechanisms of intercellular prion spreading. Recent investigations point to exosomes (**Table 1**), a type of membrane-enclosed vesicle sized 30–100 nm, with the ability of transporting a remarkably diverse cargo ranging from active proteins to different RNA particles for cellular exchange (Kowal et al., 2014). Exosomes have been associated with prion transmission since the isolation of PrP^{sc} from exosome preparations after cellular infection with sheep prions (Fevrier et al., 2004), and PrP-loaded exosomes could be successively isolated from brain fluids of infected animals (Vella et al., 2008).

It seems that the exosome-mediated secretion of amyloid-associated proteins is an organized process, as it can be triggered by determinate signaling events, such as calcium release (Emmanouilidou et al., 2010), or platelet activation (Robertson et al., 2006). Of fundamental importance, PrP^{sc} or aSyn oligomers isolated from exosomes retain full toxicity, suggesting that these organelles can indeed serve as infectious vehicles (Fevrier et al., 2004; Vella et al., 2007; Danzer et al., 2012). This leads to hypothesize that the exosome-mediated release

Table 1 | Summary of representative proteins handled within this review, with respect to pathology, and prion/prion-like properties.

Protein name	Most relevant associated diseases	Amyloid polymerization	Cell-to-cell transmission	Exosomal secretion	HSPG binding
aSyn	PD; Dementia with Lewy Bodies	Yes[a]	Yes (Luk et al., 2012)	Yes (Alvarez-Erviti et al., 2011)	Yes (Holmes et al., 2013)
Abeta	AD	Yes[a]	Yes (Stöhr et al., 2014; Watts et al., 2014)	Yes (Rajendran et al., 2006)	Yes (Holmes et al., 2013)
PrP	spongiphorm encephalopathies	Yes[a]	Yes[a]	Yes (Fevrier et al., 2004)	Yes (Horonchik et al., 2005)
tau	tauopathies	Yes[b] (Iba et al., 2013; Morozova et al., 2013)	Yes (Iba et al., 2013)	Yes (Saman et al., 2012)	Yes (Holmes et al., 2013)
FUS	ALS; FTD	Yes (Schwartz et al., 2013 (in the presence of RNA); Nomura et al., 2014 (G156E variant); Han et al., 2012; Kato et al., 2012; Kwon et al., 2013 (FUS low complexity region)).	?	?	?
TDP-43	ALS; FTD	Yes (Guo et al., 2011; Fang et al., 2014 (A315T variant); Chen et al., 2010; Furukawa et al., 2011 (G294A variant))	Yes (Nonaka et al., 2013)	Probable (Nonaka et al., 2013)	?
Cu/Zn SOD	ALS	Yes (DiDonato et al., 2003(ALS-associated mutants))	Yes (Münch et al., 2011; Grad et al., 2014)	Yes (Grad et al., 2014)	Yes (Inoue et al., 1991)
hnRNPA	multiple system proteinopathy	Yes (Kato et al., 2012; Kim et al., 2013)	Yes (Kato et al., 2012; Kim et al., 2013)	?	?
TIA-1	Welander distal myopathy	Yes (Furukawa et al., 2009; Li et al., 2014)	Yes (Li et al., 2014)	?	?
polyQ-huntingtin (htt)	HD	Yes (Lotz et al., 2010; Falsone et al., 2012)	?[c]	?	No (Holmes et al., 2013)
CPEB[d]	-	Yes (Raveendra et al., 2013)	Yes (Si et al., 2003)	?	?
Sup35[e]	-	Yes[a]	Yes[a]	?	-

[a] *prototypic representatives*

[b] *in the presence of heparin*

[c] *prion-like propagation with synthetic polyQ fibrils (Ren et al., 2009)*

[d] *from Aplysia californica*

[e] *from Saccharomyces cerevisiae.*

of noxious agents might originally represent a rational strategy to relieve cells from toxicity. Such a hypothesis is supported by studies showing that when the intracellular clearance of aSyn is compromised, an elevated deployment of exosomes carrying this protein as a cargo becomes observable (Alvarez-Erviti et al., 2011; Danzer et al., 2012). With this respect, an intact exosomal secretion pathway appears essential for neuroprotection. Upon the manipulation of PARK9/ATP13A2, a component of the exosome biogenesis machinery, cells can less efficiently counteract intraneuronal aSyn toxicity. Accordingly, surviving neurons from PD patients display increased levels of PARK9/ATP13A2 (Kong et al., 2014), whereas affected neurons show decreased levels (Murphy et al., 2013). In a purely speculative way, deregulated cells that are no longer able to efficiently handle the clearance of proteotoxic species might pass over their own toxic burden to functioning cells for disposal.

Interestingly, *in vitro* generated aSyn, tau, Abeta, PrP or polyQ aggregates can be efficiently internalized when directly applied to growing cells or animals, apparently without an exosomal delivery (Horonchik et al., 2005; Ren et al., 2009; Luk et al., 2012; Holmes et al., 2013; Iba et al., 2013; Aulić et al., 2014; Volpicelli-Daley et al., 2014), suggesting the existence of additional pathways of transmission. As shown for ALS-associated Cu/Zn-superoxide dismutase (SOD), parallel exosome dependent and independent mechanisms have been

postulated, whereby an active exosome-associated secretion can be backed by a passive, carrier-free diffusion of toxic particles, probably upon their release from necrotizing cells (Grad et al., 2014).

Proteoglycans

A recent study identifies heparan sulphate proteoglycans (HSPGs) as further key players of intercellular transmission (Holmes et al., 2013; **Table 1**). HSPGs are a class of membrane proteins conjugated to the heavily sulphated glycosaminoglycan (GAG) heparan sulphate, which is a constituent of the extracellular matrix (Xu and Esko, 2014). Although HSPGs are traditionally coupled to cell attraction and migration, a pathologic link with amyloid diseases has been postulated ever since the identification of GAGs in various types of amyloid deposits from affected brains (Snow et al., 1988, 1990; Spillantini et al., 1999).

HSPGs are direct targets of pathologic PrP, aSyn, Abeta and tau (Horonchik et al., 2005; Holmes et al., 2013). All of them become actively internalized after HSPG binding, consequently propagate within neuronal cells, and sustain the development of a pathologic condition. The physico-chemical integrity of the GAG-chains appears essential for binding, as the subsequent internalization becomes affected upon chemical or genetic alteration of the GAG residues (Holmes et al., 2013). Indeed, GAGs were attributed amyloid modifying properties *in vitro* depending on the size (Vieira et al., 2014) and charge (Lawson et al., 2010) of the GAG chain. Given that the proteoglycan expression and GAG-composition are cell-specific and vary with brain development and senescence (Rykova et al., 2011), it can be speculated that ageing-related changes in cell-surface proteoglycan patterns will influence HSPG-mediated prion-like propagation.

HSPGs might represent a converging hub for exosome-dependent and independent prion propagation, owing to some potential overlaps between both pathways. HSPGs can regulate also exosome internalization (Christianson et al., 2013), and GAG modifications affect both amyloid internalization and exosomal uptake (Christianson et al., 2013; Holmes et al., 2013). Both processes further initiate macropinocytosis, a mechanism whereby macromolecules are taken up by actin-membrane ruffles (Fitzner et al., 2011; Holmes et al., 2013). Finally, exosomes and proteopathic seeds activate identical neuroinflammatory pathways (Thellung et al., 2007; Tomasi, 2010; Christianson et al., 2013). Collectively, these considerations suggest an exosome/HSPG unifying route for prion entry.

AMYLOID FUNCTION

PHYSIOLOGIC SIGNIFICANCE OF AMYLOID POLYMERIZATION

While in humans the concept of amyloid has been traditionally interpreted in terms of lethality, observations from various evolutionary distinct organisms strongly support a role that extends well beyond toxicity. In prokaryotes, cell-surface amyloid polymerization is quite diffuse (Dueholm et al., 2013), and the biogenesis of curli filaments from enterobacteria is an example of how unicellular organisms use amyloidogenesis for physiological processes such as biofilm formation, host adhesion, and cellular clustering. CsgA protein, the principal component of curli in *E. coli*, polymerizes into amyloids after the secretion across the outer membrane. *In vivo*, this process is strictly regulated by CsgB, an accessory protein whose gene is localized to the same operon as the *CsgA* gene, and that serves as an obligatory nucleation seed for CsgA polymerization (Shu et al., 2012). CsgB rapidly assembles into beta-sheet rich oligomers that massively catalyze the transition of inherently unstructured CsgA monomers into amyloid. Interestingly, *in vitro* isolated CsgA can separately polymerize into amyloid fibrils also in the absence of CsgB. This difference suggests that curli amyloidogenesis in living cells is under the stringent control of a dedicated trigger which actually restricts the assembly of amyloids to an immediate physiological request.

In mammalian organisms, a functionally controlled amyloid growth process has been proposed for melanosomes, a type of organelles used for the synthesis and storage of melanin body pigments. The transmembrane protein Pmel17 was identified as a major component of amyloid fibers that organize in linear arrays through the length of the organelle (Raposo et al., 2001), and functionally serve as melanin deposits. Pmel17 is a multidomain protein that undergoes multiple sequential posttranslatory processing and protease cleavage steps on its way to becoming functionally incorporated into amyloid fibers on the membrane of mature melanosomes (Leonhardt et al., 2013). One critical point is the late cleavage and extralumenal release of the N-terminally located regions RPT (an imperfect repeat region), PKD (polycstic kidney disease domain), and NTR (N-terminal region). RPT and PKD represent soluble core amyloidogenic fragments that can assemble into fibers once cleaved, while NTR is not amyloidogenic, and seems to serve as a specific stabilizer of RPT and PKD during fibril growth. When this property of NTR is impaired, the fibrillogenic fragments are degraded and amyloid polymerization of RPT and PKD cannot occur.

Both mechanisms described above highlight a possible safeguard strategy by which the integrated generation of specific amyloid promoting factors simultaneously to amyloidogenic particles drives polymerization towards mature amyloid fibers instead of potentially harmful misfolded intermediates. This provides a means to exploiting stringently controlled amyloid polymerization for functional purposes.

One further example for function-associated amyloidogenesis comes from mammalian secretory granules of pituitary glands, where a set of peptide hormones accumulate in form of aggregates with amyloid properties, being reactive to amyloid-specific immunodetection, ThioS and Congo Red staining, and with a typical cross-beta x-ray diffraction pattern (Maji et al., 2009). Interestingly, a variety of isolated peptide hormones is capable of amyloid fiber formation at moderately acidic pH and in the presence of glycosaminogylcans, two conditions similar to those within secretory granules, while partially resolubilising when exposed to extragranular conditions (pH 7.4). This behavior leads to suggest that secretory granules can store hormones in form of tightly packed amyloids, releasing monomeric hormone units under determinate stimuli, which would obviate the

need of a repeated *de novo* synthesis without affecting the immediate availability of the substance. The further confinement of hormone polymers within a coating membrane provides a means for cells to store amyloid under relatively innocuous conditions.

In spite of the strong pathologic link to disease, it appears that under certain conditions cells are also able to exploit the amyloid properties of prions for functional profit. Major mechanistic insights on prion function derive from the yeast *Saccharomyces cerevisiae*, an organism that ingeniously turns conformational variability underlying prionogenesis into selective advantage (Chernova et al., 2014). In yeast, the insurgence and propagation of new phenotypic traits is sometimes coupled to the ability of determinate proteins to convert from a native into an alternative conformation with a substantially altered original function. This type of conformational rearrangement occurs in a fashion typical of prions, in that the prion conformer (A) displays structural and physical features of amyloids, (B) catalyzes its own template-driven conversion, and (C) can be non-genetically transmitted. As prototypically shown for the yeast protein Sup35 (Tuite and Cox, 2006), a transition from a native [PSI$^-$] into a self-perpetuating prion conformation [PSI+] is accompanied by a remarkable acquisition of novel cellular phenotypes, which can be ascribed to a functional loss when soluble Sup35, which is a release factor that controls the fidelity of ribosome translation termination, converts into unsoluble amyloid. Under selective pressure, the functional loss of Sup35 can turn into an advantage, as it improves the prevalence of cryptic phenotypes capable of coping with an altered environment (Halfmann et al., 2012). Even more intriguing, also prokaryotes appear to sustain prion inheritance, being able to propagate the [PSI+] phenotype over several generations under conditions that do not permit *de novo* prion formation (Yuan et al., 2014). This finding points to prion transmission as an ancient mechanism of inheritance.

Although such an impressive strategy shows that prions can be tolerated in principle, bargaining of phenotypic homoeostasis is hardly conceivable in neurons. First, the outspoken toxicity of prions in mammalian neurons overrides any possible adaptation to selective pressure within an evolutionary observable time scale. Second, mammalian cells lack essential regulators of yeast prion inheritance (e.g., the molecular chaperone hsp104).

With this in mind, it might sound heretical to speculate about prion-associated physiological benefits for mammalians. However, also in higher eukaryotes, polypeptides with *bona fide* prion properties exist more frequently than it might sound reasonable for a purely toxic agent. Algorithms trained to identify possible prion signatures could pinpoint one prevailing structural pattern to amino acid stretches of low structural complexity abounding in glutamine and/or asparagine residues. The validity of these predictions could be experimentally verified case-by-case for proteins from yeast as well as from higher eukaryotes (Alberti et al., 2009; Toombs et al., 2010; Couthouis et al., 2011). Interestingly, the isolated region (referred to as prion-like region PLR) is sufficient to confer self-perpetuating traits typical of yeast prions even when artificially fused to unrelated proteins, when interchanged between proteins, or when heterologuosly expressed in yeast (Sondheimer and Lindquist, 2000; Gilks et al., 2004; Li et al., 2014; Udan-Johns et al., 2014).

Such a typically modular architecture of prion-like regions comes along with an autonomous ability to inherently fold into amyloid-like conformations (Kato et al., 2012). Given that protein modules serve for a precise functional purpose, it can be expected that amyloid folding provides more than the structural blueprint of proteotoxicity. For the neuronal isoform of cytoplasmic polyadenylation element-binding protein (CPEB) from the aquatic snail *Aplysia californica*, the aggregation into an active polymer is essential to regulate the simultaneous processing of multiple RNA molecules in a spatially organized and highly coordinated fashion (Raveendra et al., 2013). Amyloid aggregation guides the local and reversible assembly of CPEB monomers at the synaptic ends of neurons, where this protein operates for long-term memory purposes. The resulting supramolecule is functionally active, showing a much higher RNA binding affinity than the monomeric units. Biostructural analyses have confirmed that functional CPEB aggregates display a typical amyloid conformation, consisting of a high beta-sheet content and an x-ray diffraction pattern at 4.7 and 10.7 A. Importantly, the RNA-binding domain does not incorporate into amyloid fibers and remains solvent exposed, substantiating the hypothesis that only the prion-like region functions like an autonomous and specialized structural module.

In analogy, prion-like assembly is a functional aspect of RNA-binding protein FUS, a 53 kDa protein with an aggregation propensity that is typically linked to the occurrence of unsoluble protein inclusions in amyotrophic lateral clerosis (ALS) and frontotemporal dementia (FTD; Shelkovnikova, 2013). In fundamental contrast to such a type of irreversible aggregation, some recent studies point to FUS self-assembly as a reversible, regulated, and explicitly functional process. Schwartz et al. describe one type of RNA-induced FUS polymers as characteristic amyloid beta-zipper structures with a significantly increased affinity for RNA polymerase II (RNAPolII) as compared to monomeric FUS (Schwartz et al., 2013). In a separate study, Kwon et al. suggest a biologically regulated nature of this interaction, showing that amyloid-like hydrogels (see Section Prion-like aggregation as an organizing principle of intracellular granule formation) obtained from isolated FUS-PLR bind to RNAPolII in a mode that can be reversed by phosphorylation (Kwon et al., 2013). Further, by using a FUS-PLR/GAL4 gene reporter construct, the authors highlight how mutations of PLR amino acid repeats [G/S]Y[G/S], which are critical for beta-zipper formation, compromise amyloid-like association and transcriptional activation to the same extent, demonstrating that FUS amyloid polymerization is tightly coupled to function. Moreover, a recent study identifies PLR-mediated self-assembly of FUS as an essential process for chromatin binding and transcriptional activity (Yang et al., 2014). Although the authors do not specify the physical properties of the assemblies, the regulatory character of this biochemical process and mechanistic analogies to the

RNAPolII complex legitimate the hypothesis of an amyloid-like polymerization. Consistent with such an assumption, the chromatin-associated oligomerisation step requires the presence of RNA, which might trigger amyloidogenesis as a functional prerequisite.

PRION-LIKE AGGREGATION AS AN ORGANIZING PRINCIPLE OF INTRACELLULAR GRANULE FORMATION

Intriguingly, both CPEB and FUS are representative RNA-binding proteins, a category of polypeptides ranking unsuspectedly high among predicted prion-like candidates. This finding is significant, because proteins such as FUS are constituents of RNA granules, a species of remarkably dynamic organelles that controls major key steps of RNA metabolism from synthesis to splicing, processing, stabilization and degradation (Thomas et al., 2011). While some of them are constitutive, other can generate *de novo* upon specific needs and disassemble afterwards. Such a remarkable plasticity is favored by the lack of a confining membrane, which allows for an uncomplicated interchange of molecules with the intracellular environment. Underlying this dynamics is the property of RNA-binding proteins to shuttle between separate subcellular compartments, to repartition between various types of RNA granules, and to dissociate from them in probable adaptation to selective cellular demand. Systems approaches on neuronal RNA granules suggest that these types of organelles share only little core protein similarities, supporting the fact that the majority of protein components associates transiently and specific to immediate functional requirements. (Fritzsche et al., 2013).

The frequency by which prion-like protein candidates seem to actively influence function, size, and cell number of RNA granules has led to the assumption that their PLRs might serve as rational protein-protein interfaces that naturally control condensation, growth, integrity and dynamic reorganization of these organelles by means of amyloid polymerization. Strong support comes from the observation that some isolated, recombinant PLRs from RNA granule components can spontaneously condensate *in vitro* into hydrogel-like particles with a fibrous morphology and amyloid-like structural properties (Kato et al., 2012). Unlike mature amyloid fibers from unsoluble inclusions, however, these cell-free assemblies are relatively fragile, and reversibly decompose into monomers already under semi-denaturing conditions. Yet, they are sufficiently stable to *in vitro* emulate morphogenesis and steady-state dynamics of RNA granules with staggering simplicity (Han et al., 2012; Weber and Brangwynne, 2012; Kwon et al., 2013): (A) they originate *via* self-assembly either upon concentration-dependent or template-driven nucleation, (B) they undergo demixing phase separations typical of membrane-free organelles, (C) they are able to heterotypically incorporate or exchange additional RNA granule-associated protein components, (D) they tend to associate preferentially with extended 3'-UTR mRNAs sequences, and (E) they respond to post-translatory modifications in a way that reflects physiologic regulation of RNA granules. These findings highlight the potential of PLR-containing proteins to reproduce major hallmarks of RNA granule biogenesis and function by accessing a polymer state which is morphologically close to amyloid, but completely reversible and therefore devoid of any stable higher order aggregates characteristic of pathologic prions. As these polymers unify basic physical properties of stable amyloids (self-organization, cross-beta structure) with reversibility and a minimal toxic hazard, they are potentially appealing for physiological use. First, the spontaneous polymerization of prion-like components can be initiated only upon an incisive conformational change, e.g., by molecules that act as nucleators. Second, polymerization comes along with a sharp phase transition from liquid to gel-like, which closely resembles physical processes of granule condensation *in vivo* (Dundr, 2012; Weber and Brangwynne, 2012). Third, by retaining the unique cross-beta amyloid structure, amyloid-like polymers allow for a tight polymer stacking, which can be useful when a locally restricted accumulation of proteins up to very high concentrations is biologically required. Fourth, their aggregation/deaggregation is susceptible to biochemical stimuli such as post-translatory modifications, allowing for a physiologically organized dynamics.

In principle, these attributes all comply with physical prerequisites of RNA granule assembly and disassembly, such as nucleation upon demand, steady-state association, and a precise spatial and temporal separation, suggesting mechanistic parallels between cell-free hydrogel aggregation and granule biogenesis *in vivo*. The analysis of stress granule (SG) dynamics provides particular support to this assumption. SG are one type of membraneless organelles that originate transiently in the cytosol upon different forms of cellular challenge, contributing to the arrest of mRNA translation in response to generic insults such as intoxication, UV-irradiation, oxidative stress and heat shock (Kedersha et al., 2013). When homeostasis is restored, these granules dissolve rapidly and completely.

The protein and RNA composition of SG is extremely heterogeneous and highly variable, with a growing number of proteins being identified as regulators. This dynamic constitution reflects a possible ability of SG to individually respond and adapt to various types of stimuli. Significantly, SG reversibly incorporate proteins from multiple stress signaling pathways, therefore becoming actively integrated into the circuitry of separate signaling cascades. Phosphorylation of eukaryotic initiation factor 2a (eIF2a), which represents an integrated response to different stress stimuli and is the best investigated trigger of SG assembly, abolishes translation initiation, causing polysome disruption and the passing over of untranslated mRNA to SG under persisting stress (Anderson and Kedersha, 2008). Upon a restored homeostasis, SG rapidly dissociate, thereby releasing bound mRNA for appropriate processing. Further SG components point to regulation of cell death during stress. The incorporation of the adaptor protein RACK1 into SG inhibits the activation of MTK1, a kinase that acts upstream of the p38/JNK apoptosis pathway (Arimoto et al., 2008). Similarly, upon binding to SG, ROCK1, a component of Rho GTPase signaling cascades, loses the ability to transduce apoptotic stimuli (Tsai and Wei, 2010). Finally, SG sequestration of TORC1, a component of the Target of Rapamycin (TOR) pathway, alters metabolism during nutrient deprivation (Wippich et al., 2013). All these effects can be reversed upon SG dissociation, in alignment with a regulatory

nature of these interactions, which leads to ask how the SG proteome can adapt its composition to selected stimuli.

One possible explanation comes from the observation that several SG-associated signaling proteins can couple SG initiation to concomitant pathway-specific protein-protein interactions. As shown for dual specificity tyrosine-phosphorylation-regulated kinase 3 (DYRK3), this enzyme nucleates SG in response to a signal-induced concentration increase *via* an N-terminal low complexity region, which is concurrently required for the sequestration and functional inactivation of TORC1 (Wippich et al., 2013). As overexpressing the isolated N-terminal region is sufficient to simultaneously evoke both processes, it is likely that DYRK3 induces the condensation of granules specifically tailored to fit TOR signaling demands.

Although it is unclear whether DYRK3-mediated SG assembly actually involves amyloid-associated polymerization, it is intriguing to note how the majority of established SG-nucleating proteins have a significantly high predicted amyloidogenic propensity (**Table 2**). One more explicit indication comes from the study of the two prototypic SG nucleators TIA-1 and TIA-1 related (TIAR). Both proteins possess three distinct RNA binding domains (RRM1-3), and under normal conditions, they absolve splicing-associated roles in the nucleus, where TIA-1 has been described to recognize poly-uridine sequences to facilitate 5' splice site recognition by U1 small nuclear ribonuclein (Izquierdo et al., 2005; Singh et al., 2011). Under stress conditions, these proteins can sense the increase of translationally stalled mRNA by reversibly binding to AU-rich elements in the 3'-UTR (Kedersha et al., 2000). They subsequently move to the cytosol and nucleate the assembly of SG, a process that requires their intact C-terminal Q/N-rich PLR region, a low structural complexity sequence with a high predicted prion score (Gilks et al., 2004; Couthouis et al., 2011). Accordingly, TIA-1 is capable of self-sustained transmission in yeast (Li et al., 2014), and it can associate into amyloid fibrils *in vitro* (Furukawa et al., 2009; Li et al., 2014). While the deletion of the PLR region completely abolishes SG assembly, its replacement with an extraneous PLR with similar aggregation properties (e.g., that of yeast prion Sup35) completely restores the ability (Gilks et al., 2004), which is a striking indication for prion-like aggregation as an inherent mechanism of SG self-organization. In close conjunction, aggregated TIA-1 acquires the ability of binding stalled ribosomes, suggesting that this protein mediates the efficient sequestration of abortive preinitiation complexes by nucleating SG assembly (Gilks et al., 2004). These data provide a further demonstration of how SG can be modeled in response to specific functional purposes, further suggesting that amyloids can serve as structural backbone and adapter modules of highly dynamic supramolecular machines.

Table 2 | Amyloidogenic propensity of SG nucleating proteins (adapted from Kedersha et al., 2013) calculated by the PASTA algorithm, which evaluates the stability of putative cross-beta pairings between different amino acid stretches (Walsh et al., 2014).

Protein	Lowest energy value
Ago2	−9.49
Ataxin2	−9.06
Caprin1	−6.49
CPEB	−7.07
DDX3	−8.21
DYRK3	−6.77
FASTK	−10.38
FMR1	−6.94
G3BP1	−6.94
MEX3B	−7.44
PARP1	−6.94
PKR	−6.37
PQBP1	−3.75
DAZAP2	−4.84
Pumilio	−7.70
DHX36	−13.24
Roquin	−10.42
SMAUG	−7.25
SMN	−6.67
TIA1	−6.12
TIAR	−6.13
TTP	−4.9

The list reports the best aggregation pairing energy values for the most aggregation prone peptides. Values below a threshold of −5 are considered confidential. Values of representative amyloidogenic peptides from PrP, aSyn and Abeta are −16.04; −7.24; and −8.86, respectively.

STRESS GRANULES MISASSEMBLY AS AN INHERENT RISK OF FUNCTIONAL PROTEIN AGGREGATION

Intriguingly, the overexpression of an isolated TIA-1 PLR lacking the three RNA binding domains generates deteriorated aggregates which are no longer reversible, and refractory to SDS denaturation and protease digestion (Gilks et al., 2004), a pattern typical of pathogenic prions (see Section Molecular pathways of amyloid propagation). This type of aggregates is not functional, being unable to recruit ribosomes, and insensitive towards conventional degradation (see Section Molecular catchers in the amyloid rye: heat shock proteins and proteolytic pathways), which leads to an unusual cytosolic persistence (several days instead of a few hours) and precipitation. This process anticipates the catastrophic consequences of an uncontrollable and irreversible aggregation, giving an impressive suggestion of why functional aggregation must imply reversibility. This observation is outstanding, considering that alterations in SG integrity, such as assembly/disassembly disequilibria or the improper incorporation of protein components correlate to the prevalence of organic disorders (Banfield et al., 2014). Indeed, in the cell, many regulatory networks are composed of abundant, but thermodynamically unstable (supersaturated) proteins, which are primarily vulnerable to the appearance of excessively stabilized aggregates (Ciryam et al., 2013). While soluble under homeostatic conditions, this type of proteins can easily tilt into unsoluble upon any unbuffered conformational perturbation (Xu et al., 2013). Olzscha et al. have emulated *in situ* the effects of amyloid overload (Olzscha et al., 2011). The exaggerated exposure of cells to artificially superstabilised amyloid particles caused the collapse of entire metastable protein pathways, affecting RNA metabolism, protein turnover, and mitochondrial integrity among others. All these activities are indeed gravely impaired in amyloid-associated neurodegenerative disorders, and significantly, supersaturated

proteins abound within neurodegenerative disease pathways (Ciryam et al., 2013).

This concept can be extended to SG, as these organelles must preserve reversibility as a prerequisite for physiological function (Kedersha et al., 2000). Processes that modify the assembly/disassembly, and thereby the steady-state integrity of SG, have been associated to misfolding, mislocalisation, or sequestration of various SG components, such as TDP-43, FUS, and hnRNPA isoforms, which are all outstanding modifiers of inclusion body neuropathologies (Iguchi et al., 2013; Kim et al., 2013; Shelkovnikova, 2013). TDP-43 is a 43 kDa protein originally identified as a transcriptional repressor of HIV-1 transactivation response element. It is aggregation prone (Johnson et al., 2009), and unsoluble intracellular inclusions containing this molecule can be found in patients suffering from FTD and ALS. This protein seems to absolve multiple RNA-devoted roles and it is predominantly nuclear, associating preferentially with splicing components. In the cytosol, endogenous TDP-43 interacts with components of the translation machinery, and it incorporates into SG upon various acute stress stimuli (arsenite intoxication, heat shock, proteasome inhibition). Although it is not a primary nucleator of SG, this protein influences size, morphology, and on/off kinetics of this organelle. Cells deprived of TDP-43 or mutations that decrease SG incorporation (R361S) lead to a significant delay in the appearance of SG, which are smaller in size and of less regular shape (Colombrita et al., 2009; Liu-Yesucevitz et al., 2010; McDonald et al., 2011).

While the localization of TDP-43 to SG is reversible and usually restricted to acute stress conditions, a forced cytosolic permanence of TDP-43, either upon persistent stress or due to genetic mutations, seems to potentiate the appearance of irreversible protein aggregates (Liu-Yesucevitz et al., 2010; Bentmann et al., 2012; Parker et al., 2012). In cells exposed to sustained stress, TDP-43 initially localizes to SG, but finally assembles into unsoluble cytoplasmic inclusions resistant to SG-disrupting chemicals such as cycloheximide (Parker et al., 2012). A similar effect was observed after the expression of disease-associated mutants G294A, A315T, Q331K, and Q343R (**Figure 1**), which all have an increased cytosolic/SG localization pattern (Liu-Yesucevitz et al., 2010) and a major aggregation propensity (Johnson et al., 2009; Chen et al., 2010; Guo et al., 2011). In the presence of these mutants, SG were more abundant upon induction, and in contrast to wildtype-TDP-43, they partially converted into unsoluble species. These findings suggest that under adverse conditions, the incorporation of TDP-43 into SG can favor their conversion from physiologically regulated reversible organelles into irreversible cytosolic aggregates.

Similarly to TDP-43, FUS is an RNA-binding protein with probable roles in transcriptional activation and gene splicing (Schwartz et al., 2012, 2013; Yang et al., 2014), and consistently, it resides predominantly in the nucleus. The association of wildtype FUS with SG seems to be much weaker and more stimulus selective than for TDP-43 (Acosta et al., 2014), but it becomes generally favored upon a forced cytosolic permanence, such as for the disease-associated variants R495X, R521C, R521L, all impairing nuclear localization of FUS (**Figure 1**). These mutants have an increased aggregation propensity, and readily incorporate into SG upon arsenite treatment (Bosco et al., 2010; Aulas et al., 2012; Bentmann et al., 2012; Baron et al., 2013; Acosta et al., 2014). In analogy to TDP-43, the interaction is still reversible, but with evident alterations in SG morphology and dynamics, as shown by ALS-linked FUS-R945X. This NLS lacking truncation mutant increases the number and size of SG upon persisting stress, although initially disfavoring the assembly of SG (Bosco et al., 2010). Intriguingly, natural or artificial FUS mutants (e.g., R522G) fail to incorporate into SG, accumulating as separate cytosolic inclusions with morphological and dynamic features distinct from SG, and more reminiscent of intracellular bodies associated to neuropatholgic disorders (Shelkovnikova et al., 2014). In contrast to SG, this type of FUS inclusions has a more irregular shape, a much higher kinetic stability than SG, the ability to fuse with each other, and it barely contains SG markers. These findings lead to speculate that SG might transiently incorporate mislocalised and aggregation-prone variants of FUS to prevent aggregation during acute stress, however at the increased risk of an own overload and subsequent precipitation when the stress stimuli become excessive.

Mislocalisation of FUS seems to have multiple pathologic overlaps, as its prolonged extranuclear permanence, while affecting SG dynamics, also prevents this protein from correctly absolving typically nuclear functions. Wildtype FUS can bind to transcriptionally active chromatin, acting as a possible transcriptional regulator (Yang et al., 2014). This property is challenged when nuclear localization is impaired, as for FUS-R495X, which is transcriptionally far less active than the wildytpe. Moreover, mislocalised FUS is no longer able to assist the biogenesis of gems, a class of nuclear RNA processing granules with major roles in spliceosome organization (Yamazaki et al., 2012), leading to a condition closely similar to the motoneuron disorder spinal muscular atrophy (SMA). This type of disease is otherwise linked to mutations in SMN, a protein which is essential for the structural and functional integrity of gems (Liu and Dreyfuss, 1996). It was shown that FUS can interact with SMN, contributing to the regulation of homeostatic gem assembly, a property which was no longer observable for the R495X truncation variant. Therefore, one defect might link the occurrence of cytoplasmic inclusions in ALS with the loss of nuclear gems in SMA, suggesting a converging mechanism for both diseases.

As TDP-43, FUS and hnRNPA are amyloidogenic (**Table 1**), it is relevant to ask how this property might influence their association with SG. Some more amyloidogenic protein variants exhibit indeed a significantly enhanced tendency to incorporate into SG (**Figure 1**), suggesting a causal link between both processes. Recently discovered ALS/multisystem proteinopathy-associated hnRNPA1 and hnRNPA2B1 variants (D262V and D290V, respectively) show an increased tendency to form steric zippers, the complementary beta-stranded backbone of amyloid fibers, in concomitance to a significantly more pronounced recruitment to SG (Kim et al., 2013; **Figure 1**). Moreover, the removal of multiple tyrosine residues at different positions of [G/S]Y[G/S] beta-zipper nucleating repeats of FUS-PLR abolishes

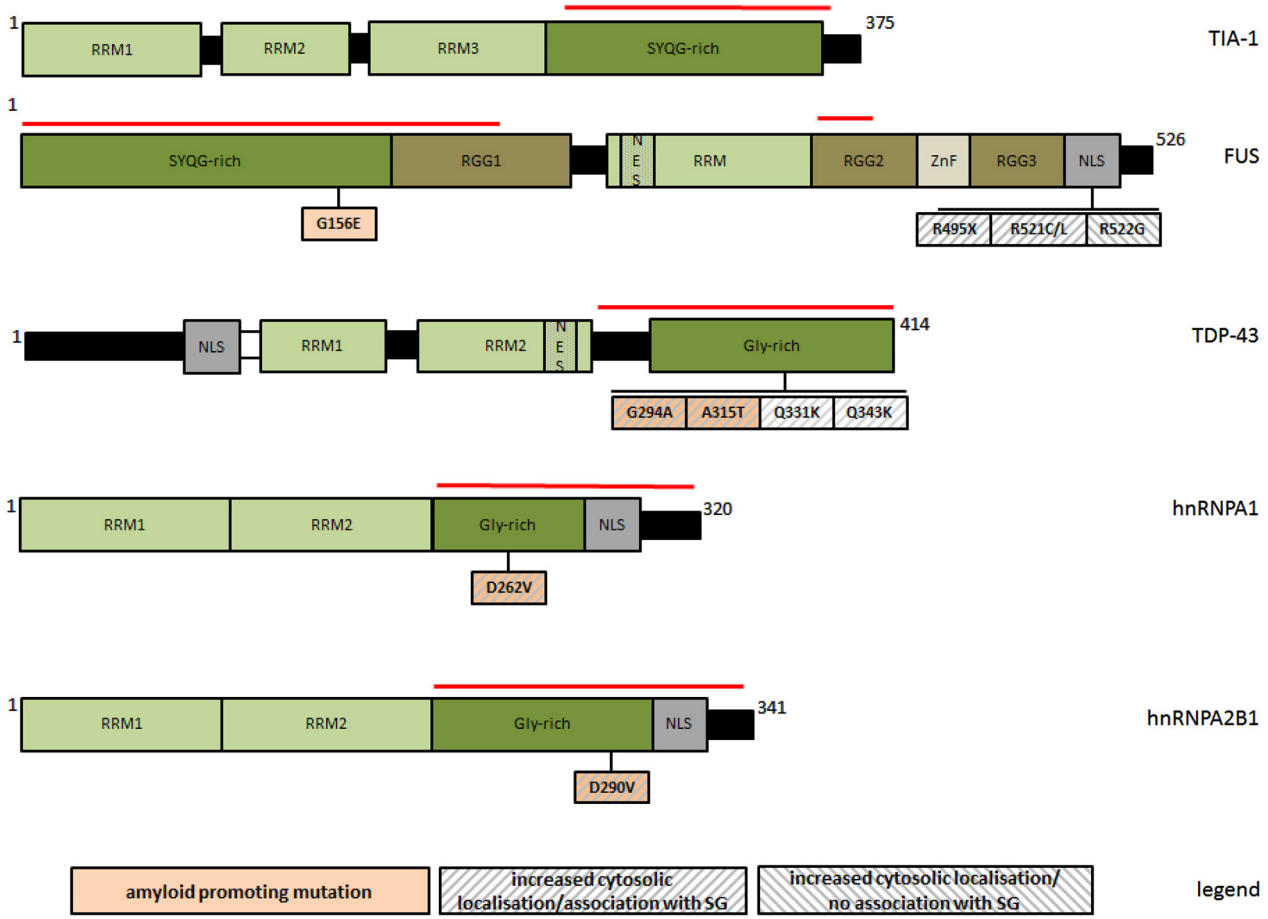

FIGURE 1 | Schematic representation of PLR-containing SG-associated proteins and of natural mutations discussed in this review with respect to their amyloid and SG-modifying properties. The red line marks respective prion-like regions. RRM: RNA recognition motif; RGG: glyine/arginine rich domain; NES: nuclear export signal; NLS: nuclear localization signal; ZnF: zinc finger domain; Gly-rich: glycine rich region; SYQG-rich: serine/tyrosine/glutamine/glycine rich region.

both amyloid-like hydrogel polymerization (see Section Prion-like aggregation as an organizing principle of intracellular granule formation) and the incorporation into SG (Kato et al., 2012). Such an explicit relationship has not been demonstrated for TDP-43, although two natural mutations (G294A and A315T) have been separately described as more amyloidogenic (Chen et al., 2010; Guo et al., 2011) and more prone to associate with SG (Liu-Yesucevitz et al., 2010; **Figure 1**).

SG dynamics is also challenged by amyloidogenic proteins without a "canonical" prion-like region, such as htt or tau. Huntington Disease (HD) key pathogen htt displays a highly variable polyglutamine (polyQ) sequence that develops amyloidogenic and pathogenic properties above a threshold length of around 40 glutamines. In a mouse model for HD, the intracellular aggregation of a 42Q htt variant caused a massive co-aggregation and functional inactivation of TIA-1 (Furukawa et al., 2009). This process is likely to involve cross-nucleation, as htt-derived amyloid fibers were able to seed for TIA-1 amyloids. Intriguingly, non-aggregating htt (25Q) seems to physiologically associate with components of RNA granules without compromising their activity (Savas et al., 2010), suggesting that htt evolves from granule-stabilizing into granule-destabilizing upon the acquisition of amyloidogenic traits. A similar disease-linked association likely occurs for hyperphosphorylated tau, which co-localizes with TIA-1 positive SGs in animal disease models (Vanderweyde et al., 2012). Interestingly, this interaction is dependent on the progression of the disease, being weak to moderate at the beginning, and increasing with disease severity.

Collectively, these data suggest that SG are extremely sensitive to processes affecting their transient nature, with an excessive stabilization leading them to evolve into irreversible toxic aggregates comparable to those of artificially stabilized amyloids (Olzscha et al., 2011). This might explain their rigorously time-, space- and stimulus-confined mode of action.

THE BOUNDARIES OF TOLERATED PROTEIN AGGREGATION

The previous section has capitalized how functional aggregation occurs incredibly close to catastrophe, and how the boundaries between function and toxicity are startlingly fragile. This leads

to wonder what kind of strategies cells adopt to physiologically benefit of a process that goes hand in hand with major system failure.

Gsponer and Babu propose that aggregation can indeed defy proteotoxicity as long as it is controllable, reversible, and temporally and spatially localized (Gsponer and Babu, 2012). They point to the existence of multilevel control mechanisms by which cells manage to keep the concentration of aggregation-prone proteins constitutively below a critical threshold. Aggregation can then be driven by a self-organized monomer-polymer transition following Le-Chatelier's principle basically by increasing protein abundance upon specific demand. Such a mechanism would apply to explain the dynamic consistency of SG and other transient cellular aggregates, which might form and disrupt upon precise fluctuations of their protein components.

In substance, the fates of aggregation-prone proteins are governed at nucleic acid and protein level. At nucleic acid level, a stringent control of transcripts aims at reducing the expression of aggregation-prone proteins to an essential minimum. The physical regulation of mRNAs encoding aggregation-prone proteins is fundamentally different from mRNAs of non-aggregating proteins. The former type of mRNA has a slower transcription rate, it is preferentially escorted by regulatory RNA-binding proteins, it has a higher tendency to form secondary structure retarding the initiation of translation, and it has mediocre translation efficiency due to a less optimal codon usage and a lower ribosome density per transcript (Gsponer and Babu, 2012).

At protein level, a scrupulous surveillance of protein function and folding quality aims at counteracting the accumulation of misfolded and dysfunctional polypeptides (Hipp et al., 2014). Cells are equipped with high-fidelity machineries that systematically minimize the accumulation of misfolded proteins by coupling protein quality control and clearance. These processes are kept in balance by an intensive cross-talk between molecular chaperones and different degradation machineries. Molecular chaperones are proteins capable of discriminating native from non-native and aggregation-prone protein conformations, selecting irreparably misfolded polypeptides for degradation. This decisional power is fundamental to neutralize adverse effects of irreversibly misfolded proteins which have been rated irrecoverable after failing chaperone quality screening. In this case, substrates are redirected from folding to degradation.

MOLECULAR CATCHERS IN THE AMYLOID RYE: HEAT SHOCK PROTEINS AND PROTEOLYTIC PATHWAYS

Heat shock proteins (hsps) are a class of representative and ubiquitously expressed, structurally unrelated molecular chaperones which bind to unfolded or partially folded polypeptides preventing them from aggregation. Hsps act in frequent combination with each other, thereby providing a powerful relay team, which is equipped with numerous accessory proteins accounting for fine-tuning and coordination.

Two potent modifiers of amyloid fiber assembly are hsp90 and hsp70. While hsp70 family chaperones assist generic protein folding processes (e.g., during polypeptide biosynthesis or membrane translocation), hsp90 acts especially to stabilize proteins in a near-native, yet unstable conformation until their full structural maturation. The rationale behind the stabilization of partially rather than entirely unfolded polypeptides is to locally protect unstable or destabilized regions, e.g., during rearrangements that functionally require the transient exposure of hydrophobic moieties (e.g., during the activation of kinases or steroid hormone receptors). Hsp90 thus prevents the collapse or the aggregation of metastable regions by keeping them in a stalled position (Eckl and Richter, 2013).

As hsp70 and hsp90 influence amyloid fiber assembly at substoichiometric amounts, it has been proposed that a transient interaction occurs already with low-abundance amyloid precursors in order to repartition them from toxic into non-toxic early during amyloidogenesis (Wacker et al., 2004; Evans et al., 2006; Falsone et al., 2009; Daturpalli et al., 2013).

Both chaperones require ATP for functioning, and although the sole presence of nucleotide-free hsp70 and hsp90 is sufficient to suppress amyloid fiber growth *in vitro*, it is only by consumption of ATP that these chaperones actively redirect oligomeric intermediates "on- pathway" for amyloid assembly (Falsone et al., 2009; Lotz et al., 2010). These findings underscore the importance of ATPase-modulating co-factors for a controlled processing of hazardous aggregates. Hsp70 requires a classic cooperation with co-chaperone hsp40 to efficiently neutralize toxic polyQ-htt in an energy-consuming fashion (Lotz et al., 2010). The underlying mechanism is the property of hsp40 to selectively recognize a specific subset of alternatively folded polyQ-htt aggregates originating during the initial amyloid growth lag phase, while leaving other aggregates unaffected. The targeted aggregates are antigenic for the conformational antibody A11, which is selective for "off-pathway" species. These are unable to template for fiber assembly, being most likely toxic. Hsp40 subsequently presents them to hsp70, and the resulting stimulation of the hsp70 ATPase leads to their active remodeling and "on-pathway" redirection for the assembly into less toxic mature fibers.

Several studies highlight the relationships between hsps and amyloid diseases. The deletion of hsp70 genes exacerbates pathogenesis of HD (Wacker et al., 2009), while the overexpression of hsp70 can reduce amyloid-related phenotypes (Klucken et al., 2004). Hsp90 was found in association with polyQ-repeat expansions, showing a high affinity for polyQ-expanded androgen receptor (AR), a pathogenic variant in spinal and bulbar muscular atrophy (Waza et al., 2005). Binding to wild type AR was more transient, and polyQ-AR was preferentially degraded after disrupting this interaction.

The expression of hsp70 and hsp90 is frequently perturbed in neurodegenerative disorders, and both proteins can be recovered from different cytoplasmic inclusions along with other types of chaperones (Hauser et al., 2005). This is consistent with the observation that a persistent exposure to aggregates causes the precipitation of major heat shock proteins, which is equal to a complete failure of protein folding pathways (Olzscha et al., 2011). With respect to SG, hsp90 and hsp70 supervise physiologic assembly of these granules by physically stabilizing separate components (Pare et al., 2009; Udan-Johns et al., 2014),

but they are apparently unable to counteract excessive on/off disequilibria, e.g., the accumulation of aberrantly stable TIA-1 aggregates. Unlike full-length TIA-1, fragments from the prion-like region of TIA-1 lead to more stable intracellular aggregates resistant to disaggregation (Gilks et al., 2004; see Section Stress Granules misassembly as an inherent risk of functional protein aggregation). Although the cell reacts by increasing the levels of some endogenous hsps, it is only the artificial overexpression of hsp70 that efficiently reverses the aggregation of TIA-1 prion-like region. Otherwise, the endogenous increase of hsps is not sufficient to impede the formation of intracellular inclusions and the concomitant co-precipitation of hsps. This suggests that chaperones can neutralize acute proteotoxic burden, but not a chronic overload.

In this context, it was found that hsp70 is more inclined to precipitate depending on the state of bound nucleotide (Roodveldt et al., 2009), implying that a functioning nucleotide turnover is essential to guarantee the stability of the cycle. This constitutes a far-reaching challenge, as any stalling of the hsp70 cycle might come along with chaperone deprivation, leading to a derangement of protein folding homeostasis. Generally, such a scenario might be evoked by any form of energetic misbalance, such as mitochondrial impairment, as frequently observed in neurodegeneration (Pathak et al., 2013).

In close relation, pathologically stabilized amyloid species appear to compete with physiological substrates for binding to hsps. As shown for aSyn, stable oligomers can inhibit hsp70, which is therefore no longer capable of functionally folding other substrates (Hinault et al., 2010). Similarly, pathologic tau displaces TDP-43 from cdc37, a dedicated hsp90 co-chaperone. Under normal conditions, hsp90 binds to and stabilizes TDP-43, and the pharmacologic inhibition of hsp90 primes TDP-43 for degradation (Falsone et al., 2007; Zhang et al., 2010). In cooperation, hsp90 and cdc37 regulate TDP-43 turnover and the autophagic clearance of cleaved TDP-43 (Jinwal et al., 2012). However, the inhibition of hsp90/cdc37 activity by hyperphosphorylated tau leads to the cytosolic accumulation of TDP-43 fragments, which eventually precipitate forming inclusions typical of ALS and FTD.

Hsp70 and hsp90 are closely interlaced with protein degradation, and both chaperones possess the ability to redirect toxic aggregates to degradation (Hipp et al., 2014). Consistently, degrading pathways take over misfolded polypeptides which have been sorted for proteolytic clearance. These are essentially the ubiquitin-proteasome system (UPS), chaperone–mediated autophagy (CMA), and macroautophagy (MA). The UPS is constituted of the core protease machinery called 26S proteasome, and a series of enzyme classes controlling substrate recognition, selection and targeting. For proteasome degradation, proteins have to be covalently marked by ubiquitin, a protein that is frequently found within protein inclusions (Dantuma and Bott, 2014). CMA consists in targeting polypeptides to the surface of lysosomes for their specific translocation through the membrane and the subsequent degradation. MA consists in the engulfment of a selected cargo into a double-membrane vesicle, with subsequent fusion to endosomes or directly to lysosomes. All these degradation mechanisms are directly involved in the clearance of amyloid aggregates at different stages. Partially redundant overlaps between proteolytic pathways additionally provide a compensatory advantage, since one malfunctioning complex can be usually backed by the mutual activation of at least another complex, keeping the toxic burden innocuously low. Such a fail-safe strategy is very effective at intransigently counteracting the risks arising from protein misfolding. Indeed, the entire amyloid folding landscape is rigorously subjected to this type of control. With functional aggregation occurring within supervised boundaries, pathogenic drifts towards misfolding can be timely suppressed and potentially toxic aggregates can be delivered to the most qualified clearance system. By these means, the cell can broadly manage the turnover of different physical species of protein aggregates in the most convenient way (Wang et al., 2009; Ebrahimi-Fakhari et al., 2011; Koga et al., 2011; Scotter et al., 2014). The UPS controls the homeostatic turnover of single monomeric proteins, whereas larger soluble or unsoluble aggregates require bulk autophagy. Therefore, proteins that aggregate in consequence to a single-molecule degradation failure can be removed by autophagy depending on size, morphology, and stability. Collectively, these systems provide a regulatory basis for functional aggregation, as they can reversibly shift monomer-oligomer transitions by regulating the turnover, and therefore the effective cellular concentration of each component. The aggregation of TDP-43, for example, can be kept in physiologic equilibrium by the interplay of UPS and autophagy, whereby monomeric TDP-43 can be degraded by the proteasome and soluble oligomers become targeted by MA (Scotter et al., 2014).

UPS degradation of soluble monomers seems to prevail under homeostatic conditions, and consistently, amyloidogenic proteins are substrates of various E3 ubiquitin ligases (Kumar et al., 2012). For aSyn, the degree of ubiquitylation affects amyloid aggregation in a way that reminds of active amyloid repartitioning preceding proteolytic clearance (Haj-Yahya et al., 2013), with ubiquitin chain length regulating stability, aggregation, phosphorylation, and clearance.

While ubiquitylation usually occurs on internal lysine residues, some disease-associated fragments from tau, aSyn, TDP-43 and Abeta can also undergo an unusual N-terminal ubiquitylation (Brower et al., 2013). This form of modification, known as N-end rule pathway, consists of polyubiquitylation of N-terminal arginine residues by specific E3 ligases called N-recognins. Upon their conjugation, each of these fragments undergoes rapid proteasomal degradation, which impedes an excessive accumulation of these highly aggregation-prone and proteopathic species. For Abeta fragments, an additional N-terminal argininylation configures these peptides for the subsequent ubiquitin conjugation step.

These findings highlight the predominant role of ubiquitin-mediated degradation in neuroprotection. Proteasome dysfunction seems central to neurodegeneration, and rather generic impairments of the UPS, whether pharmacologic, genetic, or physiologic, are sufficient for the unbiased appearance of diverse neurodegenerative phenotypes. The chemical UPS inhibition of dopaminergic cells favors the accumulation of Lewy-body like inclusions (Rideout et al., 2001). Likewise, the

stereotactic injection of proteasome inhibitors into mice brains causes symptoms closely related to PD, including dopaminergic neuronal death, decreased motor activities, and the accumulation of inclusions positive to aSyn and ubiquitin (Xie et al., 2010). Moreover, the deletion of single proteasomal (but not autophagic) subunits is sufficient to evoke ALS symptoms in knock-out mice, leading to mislocalisation and precipitation of TDP-43 and FUS in motor neurons (Tashiro et al., 2012).

The integrity of the proteasome can be further challenged by an uncontrolled overload of amyloid aggregates. For Abeta, it was shown that aggregates can overturn the function of the UPS by physically interacting with proteasome subunits. Zhao and Yang suggest that a decreased proteasome activity is not due to inhibition, but rather to the competition of natural proteasome substrates with increasing concentrations of amyloid aggregates, as shown for Abeta peptide (Zhao and Yang, 2010). Similarly, the intracellular accumulation of TDP-43 or aSyn aggregates comes along with massive UPS dysfunction (Nonaka et al., 2013; Tanik et al., 2013). These considerations underscore the importance of a compensatory cross-talk between each single proteolytic pathway, whereby stimulating one separate degradation machinery can efficiently rescue malfunctions of the other pathways (Xilouri et al., 2013). By these means, aggregates that become renitent to conventional disposal can be passed over for alternative clearance. aSyn, which is usually cleared by the UPS under normal conditions, can be passed over to MA during particular cellular challenges (Ebrahimi-Fakhari et al., 2011). In HD, a failed segregation of cytosolic cargo correlates with a marked increase of CMA components (Koga et al., 2011). An effect in the opposite direction is observed for disease-linked tau fragments, as the failure to process them via CMA leads to the activation of MA (Wang et al., 2009).

While the folding/degradation machineries presented above represent major aggregate modifying pathways, the recent discovery of the protein class MOAG-4/SERF suggests also the existence of more selective cellular mechanisms. MOAG-4/SERF possesses the unique ability to distinguish between amyloid and non-amyloid/amorphous aggregation, acting as a potent amyloid promoting factor (Falsone et al., 2012). One possible mechanistic interpretation of this property comes from the observation that the human homologue SERF1 binds to the C-terminal region of aSyn, which usually shields the central amyloidogenic region of this protein (Dedmon et al., 2005). The exposure of amyloidogenic core regions would thereby facilitate amyloid self-assembly. Intriguingly, the absence/presence of MOAG/SERF has pronounced effects on the proteotoxicity of intracellular htt, Abeta and aSyn aggregates in different model organisms. In *C. elegans*, aggregation and toxicity of polyQ expansions change in relation to the ageing-dependent expression levels of the orthologue MOAG-4 (van Ham et al., 2010). Silencing of MOAG-4 leads to a suppression of polyQ aggregation and toxicity, while the overexpression significantly aggravates toxicity, shifting polyQ towards compact misfolding intermediates. This effect is independent of conventional amyloid modifying pathways, as it is not influenced by alterations of the heat shock response, the UPS, or autophagy.

At this time, a more defined functional classification of MOAG/SERF is precluded by to the lack of detailed functional data. Of note, human homologues SERF1 and SERF2 share low-level homology with the RNA-binding domain of Matrin3 (Scharf et al., 1998) a protein that colocalises with small nuclear ribonucleoproteins (snRNPs), interacts with TDP-43, and has been recently identified as a novel disease marker for some rare forms of ALS (Johnson et al., 2014). This might suggest a possible role in RNA-associated (pathologic) processes.

CONCLUDING REMARKS

The purely pathological significance of amyloid protein aggregation has been questioned by the discovery of physiological processes that exploit some aspects of amyloid polymerization apparently for functional purposes. The structural and mechanistic similarity between pathologic and functional particles, however, anticipates that benefits of amyloid aggregation go hand in hand with toxicity. Despite these imminent hazards, the cell seems to tolerate aggregation as long as strictly confined within spatially and temporally delimited boundaries, as defined by inherently low transcription and translation rates, localized changes in protein levels, and a high protein quality control and turnover. By these means, aggregation-prone polypeptides are subjected to an exceptional physical and biological supervision at almost every level of cellular life. Yet, surveillance systems can fail upon chronic exposure to abnormally resistant aggregates, and in spite of some considerable functional backup, multiple simultaneous dysfunctions can tilt the equilibrium from functional into toxic. It is therefore conceivable, that amyloid polymerization is physiological, as long as the cell is capable of buffering any inherent toxicity. The occurrence of multiple system failures, as for example during cell senescence or upon extraordinary cellular challenges, might lead to the prevalence of toxic phenotypes, reflecting the multifactorial nature of the associated clinical conditions.

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ER stress and unfolded protein response in amyotrophic lateral sclerosis—a controversial role of protein disulphide isomerase

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Accumulation of proteins in aberrant conformation occurs in many neurodegenerative diseases. Furthermore, dysfunctions in protein handling in endoplasmic reticulum (ER) and the following ER stress have been implicated in a vast number of diseases, such as amyotrophic lateral sclerosis (ALS). During excessive ER stress unfolded protein response (UPR) is activated to return ER to its normal physiological balance. The exact mechanisms of protein misfolding, accumulation and the following ER stress, which could lead to neurodegeneration, and the question whether UPR is a beneficial compensatory mechanism slowing down the neurodegenerative processes, are of interest. Protein disulphide isomerase (PDI) is a disulphide bond-modulating ER chaperone, which can also facilitate the ER-associated degradation (ERAD) of misfolded proteins. In this review we discuss the recent findings of ER stress, UPR and especially the role of PDI in ALS.

Keywords: ALS, ER stress, oxidative stress, neurodegeneration, motoneuron, glia

INTRODUCTION

Intracellular protein aggregates are characteristic of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS; Ross and Poirier, 2004), the most common motoneuron disease characterized by a selective death of upper and lower motoneurons. The exact role of protein aggregates in disease pathology is still under debate (Ross and Poirier, 2005). In ALS the majority of aggregates are ubiquitinated inclusions (Wood et al., 2003) typically containing trans-activation response element (TAR) DNA binding protein 43 (TDP-43; Neumann et al., 2006) and also mutant Cu, Zn-superoxide dismutase (SOD1) in SOD1-linked familial ALS (Shibata et al., 1996) and mutant SOD1 mouse (Watanabe et al., 2001) and cell culture models (Lee et al., 2002). A likely underlying cause for protein accumulation is oxidative stress causing dissociation of SOD1 dimers to monomers and subsequently leading to aggregation (Rakhit et al., 2004). This can then together with malfunctioning proteasome degradation machinery contribute to the motoneuron dysfunction (Marino et al., 2014). Mutant SOD1s have also an ability to catalyze aberrant oxidative reactions which damages SOD1 itself (Andrus et al., 1998) possibly resulting in aggregation of mutant SOD1 (Valentine and Hart, 2003). Recent studies have further highlighted the importance of protein aggregation, especially in the case of TDP-43, in ALS pathology, as they have demonstrated that the induction of autophagy can enhance the TDP-43 turnover and neuronal survival (Barmada et al., 2014).

ER STRESS AND UNFOLDED PROTEIN RESPONSE IN ALS

As the first compartment in the secretory pathway, endoplasmic reticulum (ER) is responsible for protein synthesis, posttranslational processing, folding of newly synthesized proteins, and finally delivering the biologically active proteins to their proper target sites. The rate-limiting step in the secretory pathway is the transit to Golgi complex. If ER capacity is transcended and influx to the ER is excessive, the normal physiological state of the ER is disrupted leading to ER stress. There turn to its normal physiological balance then requires activation of the unfolded protein response (UPR) signaling pathway. If the UPR fails to restore the cell integrity, cell death signaling cascades are activated and the cell undergoes apoptosis (Schröder and Kaufman, 2005).

Changes in ER morphology have been observed in ALS patients and the G93A-SOD1 mice (Lautenschlaeger et al., 2012). G93A-SOD1 mice exhibit dilated rough ER (rER) accompanied with ribosomal detachment at preclinical and early symptomatic stages (Dal Canto and Gurney, 1995). Similar findings were demonstrated in postmortem samples of sporadic ALS (sALS) patients as researchers reported fragmentation of the rER, irregular distension of the rER cisternae and a detachment of ribosomes in degenerating anterior horn cells (Oyanagi et al., 2008). A recent study demonstrated deposits of granular or amorphous material in the ER lumen of sALS patients, indicating accumulation of misfolded proteins which could then in turn cause ER stress (Sasaki, 2010). In addition, Golgi apparatus become fragmented both in ALS patients (Fujita et al., 2000) and in G93A-SOD1

mice (Stieber et al., 2000). Although the results indicate an early interference in the ER balance, the manifold disease course and specific disease stage of an individual cell make it difficult to draw an accurate picture of the ongoing process (Lautenschlaeger et al., 2012).

CAUSES OF ER STRESS

Accumulation of SOD1 has been considered one of the reasons for ER stress in ALS, as mutant SOD1 colocalizes with ER markers, including glucose-related protein 78 (Grp78) and calnexin (Wate et al., 2005; Kikuchi et al., 2006). Further evidence for linking UPR to SOD1 accumulation was gained by a discovery that protein disulphide isomerase (PDI), an ER chaperone, is up-regulated both in ALS patients and G93A-SOD1 mice (Atkin et al., 2006) co-localizing with accumulated mutant SOD1 (Atkin et al., 2006). Mutant SOD1 may interact with Derlin-1 and cause dysregulation of ER-associated degradation (ERAD), thereby leading to activation of ER stress-induced apoptosis signal-regulating kinase 1 (ASK1) and apoptosis (Nishitoh et al., 2008).

Another possible cause for ER stress in ALS is the imbalance of ER calcium homeostasis (Grosskreutz et al., 2010) as protein processing and folding are calcium dependent (Kuznetsov et al., 1992). Importantly, decreased ER calcium content contributes to ER stress in ALS (Jaiswal and Keller, 2009). The ER mitochondria calcium cycle hypothesis proposes that the increased calcium release from ER is coupled with calcium uptake by mitochondria and that calcium is then transported back to ER (Grosskreutz et al., 2010). This hypothesis could explain why dysfunction in the mitochondrial calcium storage (Damiano et al., 2006) would lead to disruption of the ER refilling and subsequently to ER stress and UPR (Grosskreutz et al., 2010).

UNFOLDED PROTEIN RESPONSE IN ALS

Three major ER stress sensors detect accumulation of unfolded proteins: double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6; Schröder and Kaufman, 2005). Inositol required enzyme 1 and PERK, are type I transmembrane proteins with protein kinase activity (Liu et al., 2002) while ATF6 is a type II transmembrane protein, whose cytosolic domain can translocate to the nucleus and activate UPR relevant genes (Haze et al., 1999). While the ER luminal domains of all three ER stress sensors normally bind to the ER chaperone GRP78, under ER stress GRP78 dissociates from these sensors to bind to the misfolded proteins and enables the activation of UPR sensors (Kaufman, 1999). As stated before, mutant SOD1 has been demonstrated to bind to and co-localize with GRP78, which increases its expression in ALS mice prior to the motor symptoms (Tobisawa et al., 2003).

THE DOUBLE-STRANDED RNA-ACTIVATED PERK

Dimerization and trans-autophosphorylation result in activation of the PERK kinase domain (Harding et al., 1999). Activation of PERK in turn leads to phosphorylation of the eukaryotic initiation factor-2 (eIF2α) inhibiting general translation initiation and protecting ER against an overload of newly synthesized proteins (Harding et al., 1999). Paradoxically eIF2α phosphorylation increases translation of activating transcription factor 4 (Lu et al., 2004). ER kinase has also another substrate, nuclear factor erythroid 2-related factor 2 (Nrf2; Cullinan and Diehl, 2006). ER kinase pathway has been implicated in sALS (Hetz et al., 2009). Moreover, increased amounts of phospho-PERK-PERK (Atkin et al., 2006, 2008; Saxena et al., 2009) and phospho-eIF2α (Saxena et al., 2009) both in G93A-SOD1 mice and Neuro2a cells transfected with mutant SOD1 have been reported.

INOSITOL REQUIRING ENZYME 1 (IRE1)

Endoplasmic reticulum stress elicits autophosphorylation of IRE1 inducing its RNAse activity (Liu et al., 2002). IRE1 mediated endoribonuclease activity consequently leads to non-conventional splicing of XBP1 (X-box binding protein 1; Calfon et al., 2002). Spliced XBP1 in turn translocates to the nucleus and controls genes related to protein quality control, protein folding, components of the ERAD pathway and genes required for lipid synthesis (Sriburi et al., 2004). Postmortem spinal cord samples from ALS and ALS mice manifest increased amounts of IRE1. Interestingly, ALS mice had augmented IRE1 amounts before the onset of symptoms (Atkin et al., 2006, 2008). Further studies have revealed up-regulation of phosphorylated IRE1 and increased amount of spliced XBP1 in G93A-SOD1 mice (Kikuchi et al., 2006). Accordingly, studies with Neuro2a cells expressing G85R-SOD1 demonstrated increased splicing and nuclear translocation of XBP1 mRNA (Oh et al., 2008). The unphosphorylated and unspliced forms of IRE1 and XBP1 levels are not changed indicating that these forms could operate as an activable pool (Lautenschlaeger et al., 2012). Surprisingly, knocking down IRE1 and XBP1 by shRNA in NSC-34 cells transfected with SOD1 mutant caused decreased SOD1 aggregation and improved cell survival (Hetz et al., 2009). Augmented autophagy has been hypothesized to be the reason for the protective outcome. Generation of a knockout/transgenic mouse line by crossbreeding G86R-SOD1 and XBP1 Nes−/− (Hetz et al., 2008) demonstrated several autophagic signs further strengthening the autophagy hypothesis. XBP1 Nes−/−—G86R-SOD1 mice had a slightly prolonged life span in females whereas males showed no improvement (Hetz et al., 2009).

THE ACTIVATING TRANSCRIPTION FACTOR 6 (ATF6)

Endoplasmic reticulum stress translocates ATF6 to Golgi apparatus where it is cleaved by two proteases (Haze et al., 1999). Following cleavage, the cytosolic domain of ATF6 is translocated to nucleus where it activates UPR-related genes (Gotoh et al., 2002; Yoshida et al., 2003) Elevated levels of ATF6 have been reported in ALS patients and G93A-SOD1 mice (Atkin et al., 2006, 2008). In addition, cleavage and translocation of ATF6 in Neuro2a cells transfected with mutant SOD1 has been verified (Oh et al., 2008) and knocking down ATF6 in NSC-34 cells transfected with mutant SOD1 was found to increase SOD1 aggregation (Hetz et al., 2008). Importantly, mutations in vesicle-associated membrane protein-associated protein B (VAPB), which have been connected with late-onset motoneuron disease, associates with intracellular membranes (Nishimura et al., 2004) as well as with UPR (Gkogkas et al., 2008). Both native and mutant

VABP interact with ATF6 and reduce its capability to promote transcription of XBP1. The mutant VAPB is a much more potent inhibitor of ATF6 than the wild type VAPB, which may contribute to the pathological mechanisms of ALS (Gkogkas et al., 2008).

CONTROVERSIAL ROLE OF PDI IN ALS

As seen above, the majority of studies related to UPR in ALS have concentrated on motoneuronal UPR. However, damaged white matter has been reported in several neurological disorders (Matute, 2006) and, interestingly, loss of large myelinated fibers in the corticospinal tracts and ventral roots has been demonstrated in ALS patients (Underwood et al., 2011). Importantly, motoneurons that develop ER stress response are coupled with microglial activation and consequent axonal degeneration (Saxena et al., 2009). Moreover, up-regulation of UPR markers PDI and GADD34 have been demonstrated in glial cells in the spinal cord of G93A-SOD1 mice right after disease onset and shown segregation of UPR into ventral horn astrocytes and white matter microglia (Jaronen et al., 2013). This segregation is likely to reflect variable roles of UPR in astrocytes around degenerating motoneuron cell bodies and microglia around both motoneuron cell bodies and neurites. In view of the early degenerative changes in motoneuron axons and the role of microglia in front line defense, it is not surprising that microglial UPR precedes and/or dominates over astrocytic UPR during early motoneuron degeneration.

PDI INACTIVATION MAY CONTRIBUTE TO PROTEIN AGGREGATION

Protein disulphide isomerase is an enzyme of a thioredoxin superfamily primarily functioning in the ER as a chaperone protein. It facilitates the rearrangements of disulphide bonds via catalysis of thiol-disulphide exchange (Wilkinson and Gilbert, 2004; Ellgaard and Ruddock, 2005). In addition to its well-known role in the ER, PDI has been found in other cellular localizations, such as cytosol and mitochondria, where its physiological role is not yet completely clear (Rigobello et al., 2001; Turano et al., 2002; Wilkinson and Gilbert, 2004). However, the mitochondrion-associated PDI can induce apoptosis through mitochondrial outer membrane permeabilization when accumulating at high levels in response to misfolded proteins (Hoffstrom et al., 2010). Up-regulation of PDI has been demonstrated in ALS (Atkin et al., 2006, 2008). In G93A-SOD1 rats PDI expression is increased early in the disease progression declining sharply towards the end stage (Ahtoniemi et al., 2008). The up-regulation of PDI in the early symptomatic stages of ALS (Ahtoniemi et al., 2008) might be due to the attempt to resolve the misfolding and aggregating SOD1, but as the oxidative damage increases (Goldsteins et al., 2008) PDI becomes oxidized and loses its ability to function as a disulphide bond-rearranging enzyme (**Figure 1**). Recent report has demonstrated that S-nitrosylated and inactivated PDI can increase mutant SOD1 aggregation and trigger neuronal cell death (Jeon et al., 2014).

PDI UPREGULATION MAY CAUSE NOX ACTIVATION

Regardless of the fact that PDI is generally thought to act as a compensatory survival supporting enzyme, the other side of the PDI coin might not be as beneficial as once assumed. Recent studies have shown that in non-neuronal cells, such as vascular cells and peripheral macrophages, PDI is associated with NADPH oxidase (NOX) and act as a redox-sensitive regulatory protein of several NOX isoforms (Janiszewski et al., 2005; Laurindo et al., 2008; Santos et al., 2009a). NADPH oxidase is an enzyme that generates superoxide by transferring electrons from NADPH inside the cell across the membrane and coupling these to molecular oxygen to produce superoxide anion, a reactive free-radical. Indeed, *in vivo* ALS studies indicate that NOX activation and superoxide production are elevated in microglia and may contribute to motoneuron death (Wu et al., 2006). Furthermore, ER stress capable of inducing UPR has been previously shown to result in NOX activation, leading to increased superoxide production in peripheral macrophages (Li et al., 2010). As UPR has been demonstrated in microglia in the spinal cords of G93A-SOD1 mice (Jaronen et al., 2013), we hypothesize that PDI activity might be coupled to NOX-mediated reactive oxygen species (ROS) production during UPR. The view is supported by the finding that induction of UPR results in NOX activation and this activation is PDI-dependent (Jaronen et al., 2013). Moreover, siRNA-mediated down-regulation of PDI expression was found to reduce NOX activation. Similar results were obtained when human primary monocytes, rat primary microglia and murine macrophage type of cells were used. These *in vitro* data suggest that PDI is a significant regulator of UPR-induced NOX activation in cells of hematopoietic origin. Transient expression of G93A-SOD1 inflicted augmented NOX activation in microglia BV-2 cells suggesting that mutant SOD1 is capable of triggering the UPR and finally superoxide production (**Figure 1**). Although several models have been proposed where PDI interacts with catalytical or regulatory subunits of NOX (Laurindo et al., 2008), the exact mechanism of how PDI activates NOX remains unclear. Based on the current knowledge PDI reductase activity may be required as bacitracin, an inhibitor of PDI reductase activity (Dickerhof et al., 2011), is able to suppress superoxide production in several cell types.

EXCESSIVE PDI CATALYZED REFOLDING MAY CONTRIBUTE TO OXIDATIVE STRESS

The main site of PDI function is the ER, where the redox conditions are very different from cytosol, enabling the protein folding. Glutathione is one of the key players in controlling the redox status of ER as it has been shown that glutathione can provide oxidizing equivalents for disulphide formation (Hwang et al., 1992). However, oxidoreductin Ero1 is thought to act as a primary electron acceptor in the disulphide bond formation, transferring oxidizing equivalent to its substrate PDI (Sevier et al., 2007). Ero1 oxidizes the active cysteinyl thiol groups in PDI, enabling it then in turn to oxidize the client protein and create a disulphide bond. As Ero1 acts as an acceptor of electrons from PDI, it passes the electrons to molecular oxygen creating harmful hydroperoxide (**Figure 1**; Higa and Chevet, 2012). Furthermore, reduced glutathione may be necessary for isomerization of improper disulphide bonds, resulting in oxidized glutathione (Margittai and Bánhegyi, 2010). These hydroperoxide

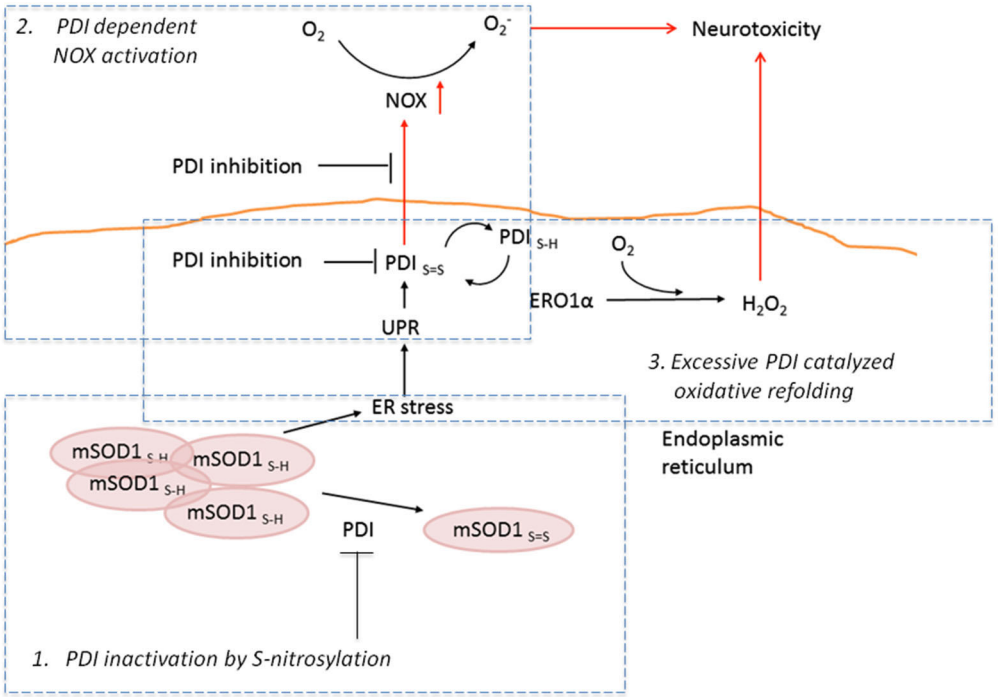

FIGURE 1 | The role of PDI in ALS. At the early stages of ALS, PDI can prevent SOD1 aggregation by re-assembling SOD1 to its active form. Protein disulphide isomerase inactivation by S-nitrosylation increases ER stress (1). Further on in the disease, the aggregation of SOD1 can lead to ER stress following UPR. Interestingly, augmented UPR can result in increased PDI activation. This can in turn result in NOX activation, leading to increased superoxide production and finally neurotoxicity (2). The second possible route for PDI-related neurotoxicity is through ERO1α; PDI activity will activate ERO1α, which will then in turn produce hydroperoxide and result in neurotoxicity (3).

and oxidized glutathione byproducts are thought to be dangerous (Tu and Weissman, 2002; Margittai and Bánhegyi, 2010) and form a link between ER stress and oxidative stress (Harding et al., 2003; Haynes et al., 2004; Malhotra et al., 2008). However, no clear consensus on whether the Ero1-mediated extensive oxidation in the ER leads to augmented oxidative stress or acts as a part of homeostatic redox control mechanisms, has been reached (Appenzeller-Herzog, 2011). Interestingly, a recent study by Shepherd et al. (2014) shed more light over the companionship of PDI and Ero1, demonstrating that PDI has ability to catalyze both the activation and inactivation of its own catalyst Ero1.

CONCLUSION

Endoplasmic reticulum stress is a characteristic of neurodegenerative diseases, including ALS. While UPR is thought to be an adaptive and protective reaction of cells to overwhelming ER stress, the cellular response triggered by protein aggregation and UPR together may lead to misbalance in protein folding pathway and result in increased ROS production. The following increased oxidative stress upon UPR can be regarded as a union of a number of both proapoptotic and proadaptive mechanisms (Santos et al., 2009b), increased PDI expression being an integral part of the latter. However, keeping in mind that high levels of PDI in response to misfolded proteins, is also capable of promoting a cell death cascade (Hoffstrom et al., 2010), the control of PDI expression offers an interesting therapeutic strategy. In microglia cells ROS production may indeed depend on PDI, which associates with NOX and regulates its function. In agreement with our findings, recent studies have shown that overexpression of PDI promotes NOX activation in vascular smooth muscle cells (Fernandes et al., 2009). Furthermore, our notion is also supported by findings that PDI closely associates with p22phox subunit of phagocyte NOX, and that NOX activation directly correlates with PDI expression levels (Santos et al., 2009a). Currently the main scope for the role of PDI in protein aggregation linked neurodegeneration has been focused at its function in maintenance of native protein structure in neurons. Nevertheless upon excessive protein misfolding the penalty of oxidative stress originating from oxidative folding may exceed ER adaptive capabilities in neuronal cells and cause aberrant NOX activation in microglia.

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Anti-apoptotic BCL-2 family proteins in acute neural injury

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Cells under stress activate cell survival and cell death signaling pathways. Cell death signaling frequently converges on mitochondria, a process that is controlled by the activities of pro- and anti-apoptotic B-cell lymphoma 2 (BCL-2) proteins. In this review, we summarize current knowledge on the control of neuronal survival, development and injury by anti-apoptotic BCL-2 family proteins. We discuss overlapping and differential effects of the individual family members BCL-2, BCL-extra long (BCL-X_L), myeloid cell leukemia 1 (MCL-1), and BCL2-like 2 (BCL-W) in the control of survival during development and pathophysiological processes such as trophic factor withdrawal, ischemic injury, excitotoxicity, oxidative stress and energy stress. Finally we discuss recent evidence that several anti-apoptotic BCL-2 proteins influence mitochondrial bioenergetics and control neuronal Ca^{2+} homeostasis independent of their classical role in cell death signaling.

Keywords: BCL-2, apoptosis, mitochondria, neuronal injury, neuronal development, neurodegeneration, ischemia, excitotoxcity

THE BCL-2 PROTEIN FAMILY

The BCL-2 gene was first identified in B-cell follicular lymphomas (Tsujimoto et al., 1985). The BCL-2 protein family act as key regulators in the intrinsic or "mitochondrial" apoptosis pathway. The different BCL-2 protein family either trigger or constrain apoptosis (Youle and Strasser, 2008). They are classified into three different classes depending on their structural and functional properties: (a) anti-apoptotic BCL-2 proteins including BCL-2 itself, BCL-X_L (BCL-extra long), MCL-1 and BCL-W which contain four BH(1-4) domains (Czabotar et al., 2014); (b) pro pro-apoptotic proteins BAX (BCL-2-associated × protein), BAK (BCL-2-antagonist/killer-1) (Wei et al., 2001) and potentially BOK (BCL-2 related ovarian killer) that contain three conserved BH domains and interacts strongly with some anti-apoptotic proteins (Hsu et al., 1997); and (c) BH3-only proteins including BIM (BCL-2 interacting mediator), PUMA (p53 upregulated modulator of apoptosis), BID (BH3 interacting domain death agonist), BIK (BCL-2 interacting killer), BAD (BCL-2 associated death promoter), BMF (BCL-2 modifying factor, Hrk (Hara-kiri) and NOXA (Latin for "damage") that have homology to the BCL-2 family proteins in only a single domain, the BH3 domain (Giam et al., 2008; Happo et al., 2012). While anti-apoptotic BCL-2 family proteins as well as BAX and BAK are often constitutively expressed in cells, BH3-Only proteins are typically transcriptionally or post-translationally activated in response to apoptotic stress signaling (Engel et al., 2011). Activation of the mitochondrial apoptosis pathway through pro-apoptotic BCL-2 proteins is able to activate different cell death pathways including apoptosis (Kilbride and Prehn, 2013). The key upstream event that leads to the activation of these different pathways is mitochondrial outer membrane permeabilization (MOMP). This process is triggered by the membrane insertion and oligomerization of the pro-apoptotic members BAX and BAK, with subsequent release of apoptosis-activating factors such as cytochrome c (cyt c) from the mitochondrial intermembrane space to the cytosol. Two models have been proposed for the activation of BAX and BAK during apoptosis: (a) the direct activation model where BAX and BAK activation occurs directly through conformational changes induced by BH3-only proteins (Letai et al., 2002) and (b) indirect activation model where cell death signals induce the binding of BH3-only pro-apoptotic initiators to anti-apoptotic BCL-2 proteins, facilitating the release and activation of BAX and BAK (Uren et al., 2007; Willis et al., 2007). Anti-apoptotic BCL-2 proteins are integral membrane proteins, possessing a C-terminal transmembrane domain that localizes these proteins to intracellular membranes, notably the mitochondrial outer membrane (MOM), but also the endoplasmic reticulum and the nuclear envelope (Cory and Adams, 2002; Chipuk et al., 2006; Brunelle and Letai, 2009; Tait and Green, 2010) and where they are also able to inhibit the process of MOMP by binding to pro-apoptotic BCL-2 proteins (Gonzalez-Garcia et al., 1994; Yang et al., 1995, 1997).

ACUTE NEURONAL INJURY AND BCL-2 PROTEINS

Neurons are highly specialized, excitable cells that communicate to other target neurons through the process of synaptic transmission. Glutamate is the principal excitatory neurotransmitter in the CNS. Glutamate release from presynaptic nerve terminals activates post-synaptic glutamate receptors including NMDA, AMPA, and Kainate receptors (Fykse and Fonnum, 1996). However, overactivation of glutamate receptors can be neurotoxic, a process termed excitotoxicity (Olney et al., 1972). Excitotoxic neuronal cell death is primarily mediated by excessive Ca^{2+} influx via NMDA receptors (Choi, 1985, 1987, 1988) and has been implicated in neurological disorders including stroke, traumatic brain injury, ischemia, Huntington's disease and amyotrophic lateral sclerosis (Dirnagl et al., 1999; Waggie et al., 1999; Mehta et al., 2013). Glutamate neurotoxicity but also

other processes such as ischemia/reperfusion injury also invoke oxidative stress (Barnham et al., 2004).

Recent studies have shown that excitotoxic and oxidative stress-induced neuronal injury involves BCL-2 family proteins. Specifically, injury conditions that produce a more delayed neuronal injury are often triggered by the transcriptional and post-translational activation of BH-3 only proteins such as BIM, PUMA, and BID (Konig et al., 2007; Steckley et al., 2007; Concannon et al., 2010). Similarly, the pro-apoptotic BAX protein has been shown to be implicated in excitotoxicity, oxidative stress and trophic factor deprivation-induced neuronal apoptosis (Deckwerth et al., 1996; D'Orsi et al., 2012). In this review we focus on recent advancements in describing the role of individual anti-apoptotic BCL-2 family proteins during neuronal development, injury and neurodegeneration.

BCL-2

BCL-2 is widely expressed in the developing brain including, neuroepithelial cells of the ventricular zones as well as the post-mitotic cells of the cortical plate, cerebellum, hippocampus and spinal cord (Merry et al., 1994). BCL-2 knockout mice show normal embryonic development but present with lymphoid apoptosis, neuronal and intestinal lesions and terminal kidney disease (Veis et al., 1993). High expression of *BCL-2* mRNA was observed in the developing nervous system and reduced significantly in the post-natal brain (Abe-Dohmae et al., 1993; Merry et al., 1994). Interestingly, high level of BCL-2 expression was maintained in sensory and sympathetic adult neurons (Merry et al., 1994). BCL-2 acts as an important regulator of cell death in developing sympathetic neurons after neuronal growth factor deprivation, whereas BCL-2 is not involved in the survival of mature sympathetic neurons (Greenlund et al., 1995). Overexpression of BCL-2 inhibited BAX-mediated cytochrome-c release, caspase activation and cell death in nerve growth factor-deprived sympathetic neurons (Putcha et al., 1999). These results taken together indicate that BCL-2 plays an important role specifically during development of the nervous system.

Functional studies in primary neuron cultures and animal models indicated that BCL-2 overexpression protected hippocampal neurons against glutamate-mediated excitotoxicity, and significantly reduced lesion size in the hippocampus resulting from NMDA induced excitotoxic damage (Wong et al., 2005). Overexpression of BCL-2 blocked translocation of apoptosis inducing factor (AIF) from mitochondria to the nucleus, resulting in improved cortical neuron survival following focal cerebral ischemia (Zhao et al., 2004). BCL-2 deficient mice show enhanced oxidative stress and alterations in antioxidants in the brain (Hochman et al., 1998) and up-regulation of BCL-2 may aid DNA repair following oxidative stress (Deng et al., 1999). Interestingly, BCL-2 expression also inhibited apoptosis of newborn neurons following MCAO in adult rat brains (Zhang et al., 2006). Transgenic mice overexpressing BCL-2 in neurons resulted in hypertrophy of the nervous system caused by reduced naturally occurring cell death but also showed a 50% reduction in brain infarct volume compared to wild type mice after permanent ischemia induced by MCAO(Martinou et al., 1994). Furthermore, transplantation of embryonic stem cells overexpressing BCL-2 into the post-infarct brain cavity of adult rats after MCAO resulted in neuronal differentiation and improvements in functional recovery and behavioral testing (Wei et al., 2005). Of note, alterations in endoplasmic reticulum Ca^{2+} homeostasis have been shown to induce apoptosis in neurons (Mattson et al., 2000). BCL-2 also modulates ER Ca^{2+} content by decreasing ER Ca^{2+} uptake (Ferrari et al., 2002; Rudner et al., 2002) which supports axon regeneration and neurite outgrowth during energy stress and mobilizes intracellular calcium signaling (Jiao et al., 2005). These results suggest that BCL-2 may represent an interesting target in stroke recovery therapy.

BCL-X_L

The BCL-X gene can be alternatively spliced to produce two protein isoforms, BCL-X_L and BCL-X_S (Gonzalez-Garcia et al., 1994). BCL-X_L acts as an anti-apoptotic protein whereas BCL-Xs exhibits pro-apoptotic properties. BCL-X_L is found in post-mitotic cells in the adult brain whereas BCL-X_S expression is predominantly expressed in developing cells with a high turnover rate such as lymphocytes (Boise et al., 1993). BCL-X_L is highly expressed in developing neurons as they migrate away from ventricular zone, and remains up-regulated in post-mitotic neurons in the adult brain (Motoyama et al., 1995; Roth et al., 2000). BCL-X_L shows close homology to BCL-2 (Gonzalez-Garcia et al., 1994). Deletion of BCL-X induces massive apoptotic cell death in developing neurons throughout the nervous system and results in lethality at embryonic day 13 (Motoyama et al., 1995; Akhtar et al., 2004). BCL-X_L also protects cultured sympathetic neurons against nerve growth factor withdrawal (Gonzalez-Garcia et al., 1995). These data suggest that BCL-X_L plays important roles during development of the nervous system; in addition BCL- X_L is also expressed at high levels in the adult nervous system.

Overexpression of BCL-X_L protected neurons in the hippocampus and cortex against hypoxic-ischemia (Parsadanian et al., 1998). Systemic delivery of BCL-X_L fusion protein inhibited caspase-3 and -9 activities and also prevented translocation of AIF into the nucleus following hypoxic-ischemic brain injury (Yin et al., 2006). Interestingly, ischemic preconditioning blocked the assembly of BAD with BCL-X_L, cleavage of BCL-X_L to a pro-apoptotic form, and release of pro-apoptotic factors from mitochondria (Miyawaki et al., 2008). Overexpression of BCL-X_L also protected primary rat septal neurons against oxygen glucose deprivation and hypoglycaemic stress (Panickar et al., 2005). Furthermore, decreased expression of BCL-X_L has been implicated in spinal cord injury induced neuronal cell death. This was attenuated by exogenous administration of a BCL-X_L fusion protein into the spinal cord (Nesic-Taylor et al., 2005). In addition, transplantation of neural stem cells overexpressing BCL-X_L enhanced graft survival by supplying tropic factors essential for survival, and improved locomotor recovery in rats following spinal cord injury (Lee et al., 2009).

BCL-X_L is also involved in non-apoptotic processes such as synapse formation. Overexpression of BCL-X_L in hippocampal neurons increased synapse numbers and localization of mitochondria to synapses, a process that was modulated through the mitochondrial fission protein, dynamin related protein 1 (Li et al., 2008). Interestingly, BCL-X_L demonstrated a dual role in synaptic

transmission under hypoxia (Hickman et al., 2008). Inhibition of BCL-X_L resulted in reduced recovery of synaptic responses under hypoxia, but exerted neuroprotective effect (Hickman et al., 2008). Recently, it has been demonstrated that BCL-X_L is important in maintaining mitochondrial fission, fusion and biomass (Berman et al., 2009), and directly interacts with ATP synthase to stabilize the mitochondrial membrane potential (Chen et al., 2011). BCL-X_L has also been demonstrated to influence Ca^{2+} signaling in astrocytes induced by the activation of inositol 1,4,5-triphosphate (IP_3)-generating metabotropic type 5 glutamate receptors (mGluR5) during the process of motoneuron degeneration. Administration of the BH4 domain of BCL-X(L) fused to the protein transduction domain of the HIV-1 TAT protein was sufficient to restore Ca^{2+} homeostasis in astrocytes overexpressing the ALS-associated SOD1 (G93A) mutation, and chronic treatment of SOD1(G93A) transgenic mice with the TAT-BH4 peptide delayed the onset of the disease and improved motor function and lifespan (Martorana et al., 2012).

BCL-w

BCL-w also known as BCL2-like 2 (Bcl2l2) is a highly conserved gene located on the mouse chromosome 14 and the human chromosome 14 at band q11. BCL-w is highly expressed particularly in the brain, colon and testes and is also associated with intracellular membranes (O'Reilly et al., 2001). Although BCL-w is widely expressed, mice deficient in BCL-w failed to show large abnormalities with the exception of increased apoptosis in sperm cells during spermatogenesis, resulting in a sterile male phenotype (Print et al., 1998). The level of BCL-w has been shown to increase during neuronal development and BCL-w has been localized to specific regions of the mature brain where it may play a crucial role in maintaining their neuronal survival (Hamner et al., 1999). In this context, BCL-w overexpression increased neuronal survival in NGF-dependent trigerminal neurons and BDNF- dependent nodose neurons over the period of development in response to neurotrophin withdrawal (Middleton et al., 2001).

Functional studies revealed that BCL-w interacts with the pro-apoptotic protein BAD and blocks neuronal death induced by growth factor deprivation in sympathetic neurons (Hamner et al., 2001). Interestingly, BCL-w expression was found to be increased in mouse neurons up to 72 h after transient middle cerebral artery occlusion (MCAO). Moreover, BCL-w co-localized with mitochondria in non-fragmented neurons and protected neurons against Ca^{2+} mediated brain injury by inhibiting cytochrome-c release and maintaining mitochondrial membrane potential following MCAO (Yan et al., 2000). Mouse deficient in BCL-w showed an increased neuronal loss and nuclear fragmentation in the hippocampus after status epilepticus with a neurophysiological phenotype leading to earlier onset of seizure, and was suggested to influence neuronal excitability (Murphy et al., 2007). This effect may be regulated through an effect of BCL-w on GABA-mediated currents, a disruption of which may lead to seizure induction (Murphy et al., 2007). Furthermore, in an experimental model of Alzheimer's disease, BCL-w blocked the mitochondrial release of Smac and inhibited neuronal apoptosis induced by β-amyloid (Yao et al., 2005). Using a rat model of transient MCAO and oxygen glucose deprivation in neurons, it was demonstrated that BCL-w plays an important role in neuroprotection following ischemic injury and is directly regulated by microRNA-29b (Shi et al., 2012). There results taken together suggest that BCL-w plays an important role in various neurological conditions to protect neurons and therefore also presents an attractive target to development of therapeutic agents.

Table 1 | Non-cell death related functions of anti-apoptotic proteins.

Anti-apoptotic proteins	Physiological state
BCL-2	Regulates ER Ca^{2+} homeostasis by decreasing the ER Ca^{2+} uptake (Ferrari et al., 2002; Rudner et al., 2002) Supports axon regeneration and neurite outgrowth (Jiao et al., 2005)
BCL-X_L	Involved in synapse formation Increases synapse number and localization of mitochondria to synapse (Hickman et al., 2008) Maintains mitochondrial fusion, fission, and biomass (Berman et al., 2009) Stabilizes mitochondrial membrane potential by directly interacting with ATP synthase (Chen et al., 2011) Stabilizes IP_3-receptor mediated Ca^{2+} signaling in astrocytes (Martorana et al., 2012)
BCL-w	Regulates neuronal excitability by modulating GABA-mediated currents (Murphy et al., 2007)
MCL-1	Localizes on mitochondrial outer membrane and inner membrane. Antagonizes anti-apoptotic proteins and maintains normal mitochondrial bioenergetics status (Perciavalle et al., 2012) Regulates mitochondrial fusion, fission, and cristae formation and facilitates ATP production (Perciavalle et al., 2012) Maintains cystosolic Ca^{2+} homeostasis and increases mitochondrial membrane potential (Anilkumar et al., 2013)

MCL-1

The human *MCL-1* gene is located on chromosome 1q21, and MCL-1 proteins were originally isolated from myeloid leukemia cells (Kozopas et al., 1993). MCL-1 is prominently expressed in neuroendocrine cells, sympathetic neurons, cardiac and skeletal muscles (Krajewski et al., 1995). Importantly, diffuse expression and rapid induction of transcription of *MCL-1* is found in neurons (Mori et al., 2004). MCL-1 germline deletion in mice resulted in peri-implantation lethality at embryonic day 3.5, showing the most severe phenotype amongst the anti-apoptotic BCL-2 family members (Rinkenberger et al., 2000). MCL-1 is associated with membranes through its C-terminal hydrophobic tail and has a predominant mitochondrial localization (Yang et al., 1995). Recently, it has been demonstrated that MCL-1 is spliced into two variants and resides in two distinct mitochondrial regions: outer membrane (MCL-1^{OM}) and matrix

(MCL-1^{MATRIX}), and has been proposed to exert differential effects depending on its location: MCL-1^{OM} antagonizes apoptosis, whereas MCL-1^{MATRIX} facilitates ATP production, membrane potential, respiration, cristae ultrastructure and mitochondrial fusion (Perciavalle et al., 2012).

MCL-1 is essential for neuronal development. Conditional deletion of MCL-1 in mice induced apoptosis of neuronal progenitors and newly committed neurons as they commence their migration away from the ventricular zone (Arbour et al., 2008). In addition, conditional deletion of MCL-1 *in vitro* in neuronal precursor cells showed a two-fold increase in apoptosis (Malone et al., 2012). These results suggested that MCL-1 is crucial for survival of neuronal precursor cells. Recently, it has been demonstrated that MCL-1 can act as a switch between autophagy and apoptosis in a developmentally regulated manner under energetic stress conditions (Germain et al., 2011). Being an anti-apoptotic BCL-2 family member, MCL-1 inhibited apoptosis by binding and sequestering the pro-apoptotic BCL-2 family member BAK (Willis et al., 2005) and blocking the activation and translocation of BAX (Chen et al., 2007). Interestingly, MCL-1 protected cortical neurons against NMDA-mediated excitotoxicity, but also increased mitochondrial bioenergetics of cortical neurons and normalized neuronal Ca^{2+} homeostasis during NMDA excitation (Anilkumar et al., 2013). Such dual effects of MCL-1 may make it an attractive target for the treatment of neurodegenerative disorders such as Alzheimer's and Parkinson's disease, in which altered bioenergetics and increased neuronal loss are so prominent. MCL-1 heterozygous mice also demonstrated increased neuronal sensitivitiy against pilocarpine-induced status epilepticus (Mori et al., 2004). Furthermore, sustained expression of MCL-1 protected and loss of MCL-1 increased DNA damage-induced neuron death (Arbour et al., 2008). A recent study has also shown that apoptosis induced by serum and KCl deprivation in cerebellar granule neuron was mediated by proteasomal degradation of MCL-1, and that stabilization of MCL-1 by blocking its ubiquitination and degradation was protective (Magiera et al., 2013). In addition, neurons depleted of Parkin, mutated gene in juvenile onset and familial forms of Parkinson's disease, became acutely sensitive to oxidative stress, and this was attributed to decreased MCL-1 levels (Ekholm-Reed et al., 2013). Although the role of MCL-1 in neurons and neurodegenrative disorders warrants further investigation, emerging evidence suggests that it may represent the most promising therapeutic target of all BCL-2 family proteins studied so far.

CONCLUSION

BCL-2 family proteins have been firmly established to play a significant role in initiating or inhibiting apoptosis during neuronal development and injury. Emerging evidence suggests that some of the anti-apoptotic protein family members are involved in maintaining both mitochondrial bioenergetics and neuronal survival, in particular BCL-X_L and MCL-1. BCL-2, BCL-X_L and MCL-1 modulation of Ca^{2+} signaling during acute neuronal injury may also play a vital role in neuroprotection. Hence manipulating the pro-survival BCL-2 family members may be beneficial in developing future therapies for neurological and neurodegenerative disorders. In addition, exploiting neural stem cells overexpressing anti-apoptotic BCL-2 family proteins may provide an attractive and powerful tool for post-neuronal injury therapies.

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J protein mutations and resulting proteostasis collapse

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Despite a century of intensive investigation the effective treatment of protein aggregation diseases remains elusive. Ordinarily, molecular chaperones ensure that proteins maintain their functional conformation. The appearance of misfolded proteins that aggregate implies the collapse of the cellular chaperone quality control network. That said, the cellular chaperone network is extensive and functional information regarding the detailed action of specific chaperones is not yet available. J proteins (DnaJ/Hsp40) are a family of chaperone cofactors that harness Hsc70 (heat shock cognate protein of 70 kDa) for diverse conformational cellular tasks and, as such, represent novel clinically relevant targets for diseases resulting from the disruption of proteostasis. Here we review incisive reports identifying mutations in individual J protein chaperones and the proteostasis collapse that ensues.

Keywords: sacsin, Tim14, Rme-8, auxilin, CSPα, HSJ1, Mrj, Hsp40

J PROTEINS: PERSONAL TRAVEL GUIDES

In neurons, there are significant demands on cellular folding events. Dynamic protein complexes are central to synaptic transmission, a process that occurs with speed, precision and plasticity. Most proteins can exist in more than one conformation and many proteins must change conformation and activity regularly. Rigorous quality control mechanisms operate at the synapse to provide defense against the detrimental effects of functionally impaired proteins and protein complexes. The balance between protecting protein functional integrity and preventing accumulation of misfolded proteins is accomplished by a network of chaperone families including: J proteins (DnaJ/Hsp40), HspA (Hsp70), HspB (small Hsp) HspC (Hsp90), HspD/E (Hsp60/Hsp10), HspH (Hsp110), CCT (TRiC) and numerous regulatory factors (**Figure 1**). Operating like a switch, J proteins control Hsc70 (70–kDa heat shock cognate protein) by directing and activating Hsc70's ATPase activity for conformational and refolding work (Kampinga and Craig, 2010; Kakkar et al., 2012). Sequence analysis has highlighted the diversity of the J protein family but has not provided much clarity into the functionality of specific J proteins. Each J protein has a 70 amino acid signature region comprised of four helices, called a J domain. Outside of the J domain, J proteins are structurally divergent, likely the basis for their distinct functional properties. Originally named Hsp40 or DnaJ after the founding members of the family (Georgopoulos et al., 1980; Ohtsuka et al., 1990), we now know that there are 49 J proteins in humans that range in molecular weight from 18–520 kDa and that while some J proteins are induced by heat most members of the J protein family are constitutively expressed (Zhao et al., 2008; Kakkar et al., 2012). The recognition that Hsc70 serves as a central hub and J proteins are the switches for diverse proteostasis events has fueled investigations into understanding the specific role of J proteins.

AGE-ASSOCIATED NEURODEGENERATIVE DISORDERS

How do folding processes go awry in age? To counteract aging and disease, neurons must routinely regulate protein machinery within tight limits. The quality control machinery constantly protects against the misfolding and aggregation of proteins that is inherent to continuous protein synthesis, generation of folding intermediates and molecular crowding of neural proteins. Neurons which don't undergo cell division are vulnerable to infectious misfolded aggregated proteins that spread (e.g., prion diseases), mutations that produce toxic proteins characteristic of late onset of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis (ALS) as well as mutations that generate ineffective chaperones. In fact, at the cellular level, pathology may start long before the onset of clinical symptoms associated with failure of the chaperone machinery to meet the demands of misfolded proteins. While it is widely accepted that J proteins maintain and restore protein balance several questions remain regarding the nature of changes to the chaperone network in age related neurodegenerative diseases (Soti and Csermely, 2002; Muchowski and Wacker, 2005; Kim et al., 2013; Labbadia and Morimoto, 2014).

DEFINING A TRAJECTORY TO UNDERSTAND THE FUNCTION OF SPECIFIC J PROTEINS

The chaperone network is elaborate and elucidating the function of individual J proteins is challenging. Chaperonopathies are a collection of diseases caused by genetically inherited mutations

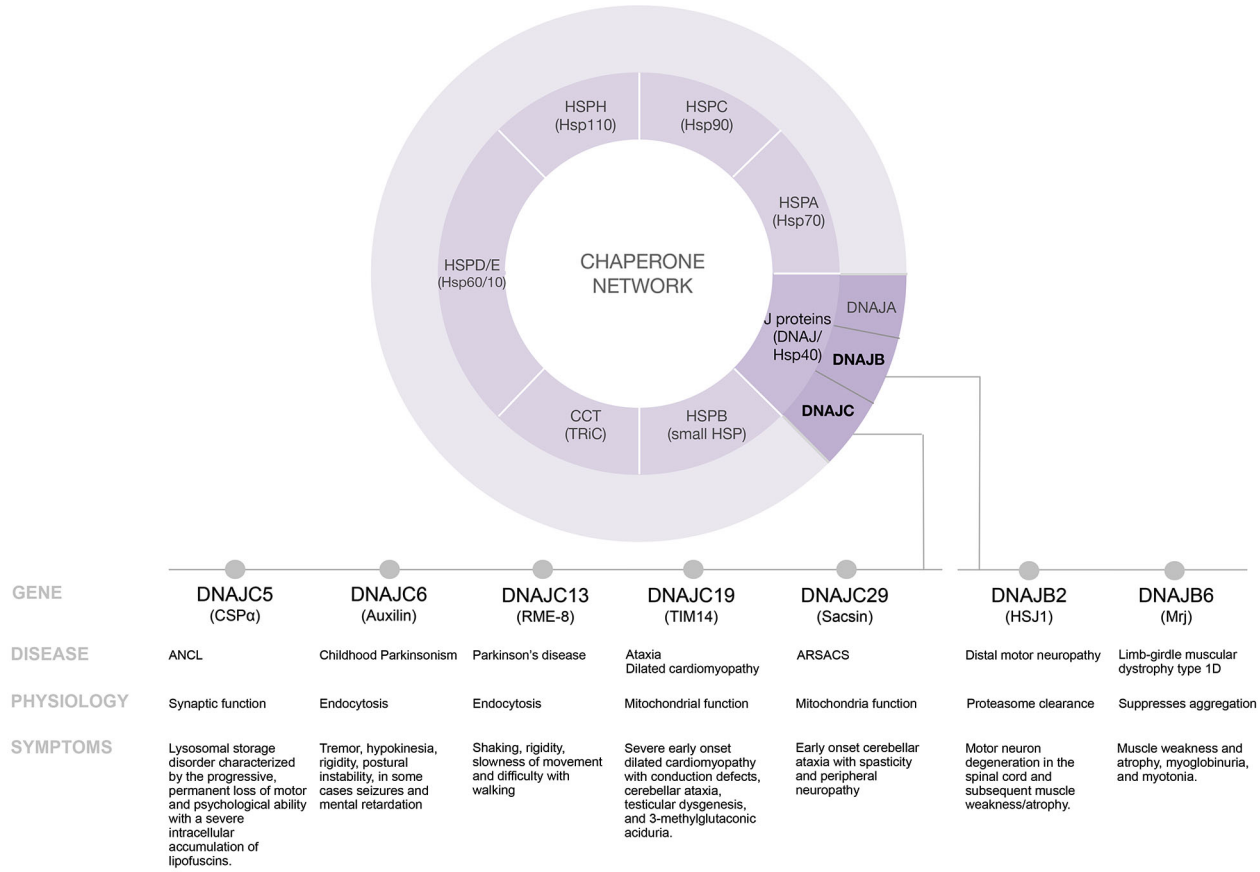

FIGURE 1 | Disease modifying pathways of select J proteins.

in chaperones. Our growing appreciation that diverse diseases in humans result from select J protein mutations provides new roads to explore the mechanisms of action of individual J proteins. To date, mutations in seven distinct J proteins; DNAJB2, DNAJB6, DNAJC5, DNAJC6, DNAJC13, DNAJC19 and DNAJC29 have been linked to distinct diseases in humans (**Table 1**). Here we review the information that these recently identified mutations provide regarding individual J protein function and the collapse of proteostasis. Understanding the mechanisms of specific J proteins is key to understanding the proteostasis machinery and will be critically important for J protein-based drug development.

DNAJB2 (HSJ1)

Mutation of DNAJB2 (HSJ1), a 32/38 kDa J protein, causes recessive distal hereditary motor neuropathy (Blumen et al., 2012). Originally identified in Morocco, individuals show a distal motor weakness, hypotonia, atrophy and paralysis of the lower limbs in young adulthood (age 20) due to progressive degeneration of motor neurons in the spinal cord.

HSJ1 was first identified via expression cloning with antiserum prepared against Alzheimer's disease brain extracts enriched in helical filaments (Cheetham et al., 1992). It is preferentially expressed in neurons where it is proposed to promote proteasome degradation of proteins tagged with polyubiquitin (Cheetham et al., 1992; Chapple and Cheetham, 2003; Westhoff et al., 2005; Howarth et al., 2007). Alternative splicing produces two isoforms. HSJ1a is a cytosolic 32 kDa form and HSJ1b is a 38 kDa form that is anchored to the cytosolic face of the endoplasmic reticulum via C terminal geranylgeranylation. In addition to its J domain, HSJ1 contains two unique ubiquitin interaction domains that bind ubiquitin to prevent aggregation and direct ubiquitinated/misfolded proteins to the proteasome. *Which misfolded proteins are targeted by HSJ1?* In cellular models HSJ1a suppresses the aggregation of polyglutamine expanded proteins and mutant superoxide dismutatse1 (SOD1; Chapple et al., 2004; Westhoff et al., 2005; Gao et al., 2011; Blumen et al., 2012). Transgene upregulation of HSJ1a reduces brain huntingtin aggregation in the mouse R6/2 model of Huntington's disease (Labbadia et al., 2012) and mutant SOD1 aggregation in the mouse SOD1^{G93A} model of ALS (Novoselov et al., 2013). Key questions arise from these findings. While transgenic expression of HSJ1 alone reduces aggregation, it does not fully reverse disease progression and correct life span. Whether reinforcing other J proteins along with HSJ1 will completely circumvent the cascade of degeneration remains an open question. Further, while the splice mutation that give rise to distal motor neuropathy in humans decreases the availability of both HSJ1a and HSJ1b which are broadly

Table 1 | J proteins mutations and resulting proteostasis consequences in human diseases.

Human disease	Chaperone	Mutation	References
Autosomal-recessive distal hereditary **motor neuropathy**	**DNAJB2 (HSJ1)** 32 kda (cytosolic) HSJ1a 38 kda (endoplasmic reticulum) HSJ1b	Splice mutation	(Blumen et al., 2012)
Autosomal-dominant limb-grid **muscular dystrophy**	**DNAJB6 (Mrj)** 26 kda	Phe93Leu Phe89Ile Pro96Arg Splice mutation	(Harms et al., 2012; Sarparanta et al., 2012; Sato et al., 2013; Suarez-Cedeno et al., 2014)
Adult onset autosomal-dominant, **neuronal ceroid lipofusinosis** (ANCL)	**DNAJC5 (CSPα)** 35 kda	Leu115Arg Leu116del	(Benitez et al., 2011; Nosková et al., 2011; Velinov et al., 2012)
Autosomal-recessive juvenile **parkinsonism**	**DNAJC6 (Auxilin) 100 kda**	80 kb deletion *(Exons 5–19)* Gln734X Splice mutation	(Edvardson et al., 2012; Vauthier et al., 2012; Köroğlu et al., 2013)
Adult onset autosomal-dominant **Parkinson's disease**	**DNAJC13 (RME-8)** 220 kda	Asn855Ser	(Vilariño-Güell et al., 2014)
Autosomal-recessive dilated cardiomyopathy (DCMA) and cerebellar **ataxia**	**DNAJC19 (TIM14)** 18 kda	Single nucleotide deletion Frameshift mutation	(Davey et al., 2006; Ojala et al., 2012)
Autosomal-recessive spastic **ataxia** of Charlevoix-Saguenay (ARSACS)	**DNAJC29 (Sacsin)** 521 kda	Asp168Tyr Leu308Phe Leu556Pro (>100 mutations)	(Bouchard et al., 1978; Engert et al., 2000; Thiffault et al., 2013)

expressed in neurons, the mutation causes selective loss of motor neuron function. Obviously, whether HSJ1 has a motor neuron specific or a general neuronal function will be the focus of future investigation.

DNAJB6 (Mrj)

Mutations in DnaJB6 (Mrj; mammalian relative of DnaJ), a 26/36 kDa J protein cause autosomal dominant limb-girdle muscular dystrophy type 1 D (Harms et al., 2012; Sarparanta et al., 2012; Sato et al., 2013; Suarez-Cedeno et al., 2014). Four mutations F96L, F96I, F89I and F93L results in adult or child onset (age 14–68 yrs) limb-girdle muscular dystrophy type 1D which is clinically characterized by elevated serum creatine kinase levels, progressive muscle weakness mainly in the legs. Limb-girdle muscular dystrophies are a heterogeneous group of inherited disorders caused by a number of dominant or recessive mutations that cause myofibrillar myopathy and the DNAJB6 mutations are specifically distinguished by characteristic protein aggregates and autophagic vacuoles (Mitsuhashi and Kang, 2012).

Mrj is a ubiquitous J protein with Hsc70-dependent and -independent activities that is most highly expressed in brain (Chuang et al., 2002; Hageman et al., 2010). Further, expression of Mrj is increased in astrocytes from Parkinson's disease patients, where it is found to be a component of Lewy bodies (Durrenberger et al., 2009). Alternative splicing produces two isoforms (Hanai and Mashima, 2003). DnaJB6a is 36 kDa and localizes to the nucleus, whereas DnaJB6b is 26 kDa and cytosolic but translocates to the nucleus in response to cellular stress (Andrews et al., 2012). *What are Mrj's targets?* Mrj suppresses aggregation and toxicity of several aggregation-prone proteins including: huntingtin, α-synuclein and parkin (Chuang et al., 2002; Durrenberger et al., 2009; Hageman et al., 2010; Kampinga and Craig, 2010; Rose et al., 2011). Possible "client proteins" in addition to disease-causing proteins include: keratin-intermediate filaments (Watson et al., 2007), histone deacetylase (HDAC; Hageman et al., 2010) and the transcription factor, NFATc3 (Dai et al., 2005). Mrj KO is embryonic lethal in mice due to a failure of chorioallantoic attachment in placental development (Hunter et al., 1999). All human mutations causing myopathy are found in a glycine-phenylaline linker region that follows the amino terminal J domain and result in a reduction in the inhibition of protein aggregation (Sarparanta et al., 2012). Furthermore, low expression of Mrj has been linked to breast cancer (Andrews et al., 2012). It remains an open question why mutations in neuronal-enriched Mrj cause myopathy and low levels are linked to cancer rather than neurodegeneration. Further investigation is required to understand these somewhat disparate pieces and develop a clear picture of the neuroprotective actions of Mrj. It is noteworthy that in the wobbler mouse, an ALS model of progressive motor neuron degeneration, DnaJB3, which shares

90% identity with Mrj(DnaJB6), shows reduced expression in the spinal cord prior to loss of motor neurons (Boillée et al., 2002). Overlap in Mrj and DnaJB3 expression as well as possible overlap in DnaJB6/DnaJB3 client proteins remains to be determined.

DNAJC5 (CSPα)

The DnaJC5 gene encodes CSPα, a 35 kDa secretory vesicle protein. Mutations in DnaJC5, cause adult onset, neuronal ceroid lipofusinosis (ANCL), a neurodegenerative disorder characterized by lysosomal accumulation of autofluorescent oxidized lipid and protein waste, called lipofuscin (Benitez et al., 2011; Nosková et al., 2011; Velinov et al., 2012). Deletion of leucine 116 (L116△), or mutation of residue 115 from leucine to arginine (L115R) results in ANCL which is clinically characterized by anxiety, depression, speech difficulties, ataxia, myoclonus, involuntary movements, progressive seizures and dementia. Neuronal ceroid lipofuscinoses (NCLs) are a heterogeneous group of inherited lysosomal storage disorders caused by mutations that lead to accumulation of lipofuscin and loss of neurons (Anderson et al., 2013). ANCL is unique among NCLs in that it is adult onset and the only NCL mutations inherited in an autosomal dominant manner.

What is the physiological function of CSPα? CSPα protects against synapse loss, however the precise molecular mechanism underlying neuroprotection is not yet known. In addition to its N terminal J domain, CSPα bears a hydrophobic region followed by the distinctive cysteine string region after which the protein is named. Most of the cysteines are palmitoylated, which anchors CSPα to the synaptic vesicle membrane. CSPα is located on synaptic vesicles (Mastrogiacomo et al., 1994), exocrine vesicles (Braun and Scheller, 1995), and endocrine vesicles (Chamberlain et al., 1996; Brown et al., 1998). At birth, CSPα KO mice appear normal and around postnatal day 20 develop progressive motor deficits and CNS degeneration, followed by early lethality (Fernández-Chacón et al., 2004). The synapse loss in CSPα null mice is activity-dependent and synapses that fire frequently are lost first (Schmitz et al., 2006; Garcia-Junco-Clemente et al., 2010). In *Drosophila*, CSPα KOs that survive to adulthood are characterized by uncoordinated movements, shaking, and temperature-sensitive paralysis (Zinsmaier et al., 1994). *What are CSPα's targets?* Presynaptic targets that demonstrate changes in protein levels early-on in the cascade of neurodegeneration include SNAP25 (Sharma et al., 2011, 2012), dynamin1 (Zhang et al., 2012) and BK channels (Kyle et al., 2013; Ahrendt et al., 2014). Other promising targets include; voltage dependent Ca^{2+} channels, heterotrimeric G proteins, syntaxin and synaptotagmin (Donnelier and Braun, 2014). Which client proteins are critical for triggering the cascade of events leading to degeneration and which changes are downstream of the primary event remains an open question. In addition to CSPα, CSPβ and CSPγ have been identified in the mammalian genomes (Fernández-Chacón et al., 2004). While brain expresses only CSPα, all CSP isoforms are expressed in testis, whereas the cochlea expresses CSPα and CSPβ (Chandra et al., 2005). In CSPα KO mice, the redundancy of CSPβ in the ribbon synapses protects against neurodegeneration in cochlea, however other J proteins do not protect against the absence of CSPα. Surprisingly, in mice, transgenic expression of α-synuclein abolishes neurodegeneration caused by deletion of CSPα but its protective mechanism has not yet been fully elucidated (Chandra et al., 2005). The L116△ and L115R mutations that result in ANCL cause CSPα to mislocalize implying both a partial loss of function (at the synaptic vesicle) and a partial gain of function (at the intracellular site of mislocalization). Although progress has been made, mislocalization is not sufficient to explain lysosome dysfunction. There are still gaps in our understanding of the molecular mechanisms underlying CSPα's neuroprotection with regards to synaptic vesicle release and recycling (Rozas et al., 2012; Sharma et al., 2012).

DNAJC6 (AUXILIN)

Mutations in DnaJC6 (auxilin), a 100 kDa nerve-specific J protein cause recessive juvenile parkinsonism characterized by early onset (age 3–18) tremor at rest, bradykinesia, rigidity and postural instability (Edvardson et al., 2012; Vauthier et al., 2012; Köroğlu et al., 2013). The severity and age of onset depends on the extent of the deletion or reduction in expression of auxilin and in some cases includes mental retardation, epilepsy and lack of responsiveness to L-Dopa.

Auxilin is one of the best studied J proteins and its role in uncoating of clathrin from clathrin-coated vesicles is well established (Ungewickell et al., 1995; Eisenberg and Greene, 2007). Neurotransmission requires a rapid continuous recycling of synaptic vesicles. Following fusion, clathrin assembles into a lattice on the presynaptic plasma membrane and deforms plasma membrane into a clathrin coated vesicle that is severed from the membrane by dynamin. After internalization auxilin binds clathrin and Hsc70 and in a J domain-dependent process uncoats clathrin to replenish the pool of synaptic vesicles (Morgan et al., 2001). Unpolymerized clathrin remains in association with Hsc70 until released by nucleotide exchange factors for additional rounds of endocytosis (Morgan et al., 2013). The clathrin binding motif and J domain are located at the C terminus of auxilin and therefore C terminal truncations are expected to render auxilin incapable of uncoating clathrin thereby impairing synaptic transmission (Morgan et al., 2001). Extending on these studies, impaired dopamine receptor recycling may explain the lack of L-Dopa responsiveness observed in some auxilin mutations. Auxilin KO mice have a high rate of postnatal mortality and surviving pups have a low body weight and show recycling and endocytosis defects (Yim et al., 2010). There is a high homology between neural-specific auxilin (DnaJC6) and ubiquitous GAK (cyclin G associated kinase; DnaJC26) with primary differences in the N terminal domain and GAK is thought to partially compensate for auxilin deletion. Interestingly, genome wide association studies have linked DnaJC26 (GAK) to Parkinson's disease susceptibility (Li et al., 2012).

DNAJC13 (RME-8 RECEPTOR MEDIATED ENDOCYTOSIS)

Mutation of DnaJC13 (RME-8 receptor mediated endocytosis 8) a 220 kDa J protein (Asn855Ser) cause adult onset autosomal-dominant Parkinson disease (Vilariño-Güell et al., 2014). Originally discovered in Saskatchewan, onset of disease is between 59 and 85 years and is characterized by slowly progressive

tremor, rigidity, bradykinesia and roughly half of the Lewy body inclusions are immunoreactive for DnaJC13. Interestingly, DanJC13 mutation (Ala2057Ser) has also been linked to Tourette syndrome/chronic tic phenotype in patients of European ancestry (Sundaram et al., 2011).

RME-8 was first identified in a screen for endocytotic defects in *C. elegans* (Zhang et al., 2001) and was subsequently shown to have a role in endocytosis in *Drosophila* (Chang et al., 2004) and endosomal function in mammals (Girard et al., 2005). Surprisingly, Rme-8 which bears a central J domain, is widely expressed but not especially abundant in brain (Girard et al., 2005). Rme-8 interacts with retromer (Popoff et al., 2009) and WASH complexes (Freeman et al., 2014) and loss of RME-8 disrupts cation independent-mannose-6-phosphate receptor and epidermal growth factor receptor trafficking (Girard and McPherson, 2008; Popoff et al., 2009). There are numerous gaps in our understanding of how disruption of normal Rme-8 function in endosomal recyclying causes Parkinson disease.

DNAJC19 (TIM14 MITOCHONDRIAL IMPORT INNER MEMBRANE TRANSLOCASE SUBUNIT 14)

Mutation of DnaJC19 (Tim14 or Pam 18), an 18 Kda J protein, cause early-childhood-onset (before 3 years) recessive dilated cardiomyopathy and cerebellar ataxia (Davey et al., 2006; Sparkes et al., 2007; Ojala et al., 2012). Originally identified in Alberta Dariusleut Hutterites, DnaJC19 mutations are clinically characterized by raised levels of 3-methylglutaconic acid, a readout of mitochondrial distress, dilated cardiomyopathy, prolongation of the QT interval in the electrocardiogram and cerebellar ataxia (Davey et al., 2006).

DnaJC19 is a transmembrane component of the TIM23 mitochondria import machinery that delivers nuclear encoded proteins to the mitochondrial matrix in an ATP dependent manner (Sinha et al., 2014). DnaJC19 activates mortalin, the mtHsp70 ATPase, and activation is counteracted by MAGMAS, a protein that contains a C-terminal J-like domain that lacks the HPD motif required to recruit and activate the Hsc70 ATPase activity. There are multiple mitochondrial J proteins and our understanding of the differences in function and specificity remains limited. Clearly, DNAJC19 and another mitochondrial J protein, DNAJC15, are not redundant as DnaJC15 does not rescue DnaJC19 mutations that lead to dilated cardiomyopathy and cerebellar ataxia. This underscores the importance of identifying the diverse disease modifying pathways of mitochondrial J proteins.

DNAJC29 (SACSIN)

Mutations in DnaJC29 (sacsin), a 521 kDa J protein, cause early-childhood-onset (age 1–2 years), recessive spastic ataxia of Charlevoix-Saguenay (ARSACS; Bouchard et al., 1978). Originally discovered in Quebec in 1978 (Bouchard et al., 1978), we now know that >100 different mutations in DnaJC29 mutation exist worldwide (Engert et al., 2000; Thiffault et al., 2013). ARSACS is clinically characterized by unsteady gait, spasticity, ataxia, muscle atrophy, due to progressive cerebellar atrophy and peripheral neuropathy. Quebec patients never walk properly and are wheelchair-bound on average by 41 years with life expectancy approximately 51 years. Outside of Quebec, mild ARSACS with onset delayed until later childhood and early 20's or severe ARSACS with mental retardation is also found (Baets et al., 2010; Thiffault et al., 2013). Deposits of lysosome derived oxidized lipid and protein waste, called lipofuscin, are found in cerebellar cortical neurons or skin (Stevens et al., 2013), somewhat reminiscent of DnaJC5 mutations that cause ANCL.

Sascin is the largest J protein, bearing C termini J domain, an N-terminal ubiquitin-like domain and a higher eukaryotic and prokaryotic nucleotide binding domain (HEPN; Parfitt et al., 2009; Kozlov et al., 2011). Sacsin is predominantly cytosolic with a mitochondria component, it interacts with dynamin-related protein 1, a GTPase required for mitochondrial fission (Kozlov et al., 2011; Girard et al., 2012; Thiffault et al., 2013). Knockdown of sacsin in SH-SY5Y cells results in an overly interconnected and functionally impaired mitochondrial network with mitochondria accumulating in the soma and proximal dendrites (Girard et al., 2012). Sacsin KO mice display age-dependent neurodegeneration of cerebellar Purkinje cells most likely due to defects in mitochondrial proteostasis (Girard et al., 2012). Much work will be needed to untangle the complexities of sacsin mutations and ensuing neurodegeneration.

FUTURE PROSPECTS

Independent threads have recently begun to provide insight into how select J proteins in the protein quality machinery monitor and adjust proteotoxic imbalances in order to maintain neural function. Many knowledge gaps in our understanding of the functionality of specific J proteins exist. It remains to be seen whether identification of further human mutations will yield insights into the biological roles of J proteins. Ultimately, systematic analysis of the mechanisms by which J proteins facilitate proteostasis will enable us to develop novel therapeutic agents and re-purpose drugs currently used for other indications.

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Prognostic role of "prion-like propagation" in SOD1-linked familial ALS: an alternative view

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"Prion-like propagation" has recently been proposed for disease spread in Cu/Zn superoxide dismutase 1 (SOD1)-linked familial amyotrophic lateral sclerosis (ALS). Pathological SOD1 conformers are presumed to propagate via cell-to-cell transmission. In this model, the risk-based kinetics of neuronal cell loss over time appears to be represented by a sigmoidal function that reflects the kinetics of intercellular transmission. Here, we describe an alternative view of prion-like propagation in SOD1-linked ALS – its relation to disease prognosis under the protective-aggregation hypothesis. Nucleation-dependent polymerization has been widely accepted as the molecular mechanism of prion propagation. If toxic species of misfolded SOD1, as soluble oligomers, are formed as on-pathway intermediates of nucleation-dependent polymerization, further fibril extension via sequential addition of monomeric mutant SOD1 would be protective against neurodegeneration. This is because the concentration of unfolded mutant SOD1 monomers, which serve as precursor of nucleation and toxic species of mutant SOD1, would decline in proportion to the extent of aggregation. The nucleation process requires that native conformers exist in an unfolded state that may result from escaping the cellular protein quality control machinery. However, prion-like propagation-SOD1 aggregated form self-propagates by imposing its altered conformation on normal SOD1-appears to antagonize the protective role of aggregate growth. The cross-seeding reaction with normal SOD1 would lead to a failure to reduce the concentration of unfolded mutant SOD1 monomers, resulting in continuous nucleation and subsequent generation of toxic species, and influence disease prognosis. In this alternative view, the kinetics of neuronal loss appears to be represented by an exponential function, with decreasing risk reflecting the protective role of aggregate and the potential for cross-seeding reactions between mutant SOD1 and normal SOD1.

Keywords: aggregate, amyotrophic lateral sclerosis, mutation, nucleation, prion, SOD1, kinetic model

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal neurodegenerative disease affecting motor neurons for which there is no effective treatment. The average disease duration is about 3 years, but it can vary significantly. Death usually results from compromised function of respiratory muscles. Since the landmark discovery in 1993 that mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene cause the familial form of ALS (FALS; Rosen et al., 1993), the underlying molecular features, and associated clinical characteristics of this disease have been extensively annotated. More than 150 different mutations of SOD1 have been found in patients with familial ALS. Studies on the clinical course of FALS suggest that the duration of illness is relatively consistent for each SOD1 mutation, but variable among the different mutations; for example, patients with the A4V mutation survive an average of 1 year after diagnosis, whereas patients with the H46R mutation survive an average of 18 years (Czaplinski et al., 2006). Aggregation of misfolded SOD1 proteins is a common pathological finding among subjects with different SOD1 mutations and is therefore believed to be central to disease pathogenesis (Johnston et al., 2000; Kato et al., 2001; Wang et al., 2003). The severity of mutant-induced destabilizing effects on the SOD1 molecule appears to correlate weakly with disease progression (Lindberg et al., 2005; Sato et al., 2005). However, the molecular mechanisms underlying variations in age of onset and disease duration of these conditions remain largely unknown.

Using ALS models derived from human embryonic stem cells or transgenic mice, recent studies have demonstrated that both cell-autonomous and non-cell-autonomous processes contribute to neurodegeneration in SOD1-linked FALS (Nagai et al., 2007; Di Giorgio et al., 2007; Yamanaka et al., 2008; Wada et al., 2012). Mutant SOD1 overexpression in differentiated motor neurons is sufficient to induce selective cell death and formation of aggregates as inclusions that mimic the *in vivo* human ALS disease. Furthermore, neuronal losses are enhanced when motor neurons are co-cultured with astrocytes expressing mutant SOD1 (Nagai et al., 2007; Di Giorgio et al., 2007). Clinically, the relationship between mean age at onset and mean survival time among patients with different SOD1 mutations are poorly correlated (Sato et al., 2005; Wang et al., 2008). These findings suggest differences in the mechanisms responsible for the initiation and progression of the neurodegenerative process in SOD1-linked FALS.

Prion disorders, such as Creutzfeldt-Jakob disease, are infectious diseases caused by the amyloid form of the prion protein, PrP^{Sc}, which endlessly self-propagates by imposing its altered conformation on the cellular prion protein, PrP^{C} (Prusiner, 1998). We use the term "prion-like propagation" to describe this molecular event—self-templating replication to cross-seed aggregation of normal cellular counterparts. Recent studies have suggested prion-like propagation as a mechanistic model of lesion spread in SOD1-linked FALS (Grad et al., 2011; Munch et al., 2011; Polymenidou and Cleveland, 2011; Grad and Cashman, 2014). Although there has been no evidence of transmission of SOD1 aggregates between individuals, using cultured neuronal cells, mutant SOD1 aggregates showed prion-like behavior, a process involving a cross-seed aggregation of normal SOD1 and cell-to-cell transmission of misfolded SOD1 aggregates (Grad et al., 2011, 2014; Munch et al., 2011). Neurodegeneration in ALS typically begins focally and then spreads spatiotemporally until motor neurons of the respiratory system are lost (Ravits et al., 2007a,b). An attractive model for this progression would be the spread of toxic aggregates from a focal site through cell-to-cell transmission.

Aggregation of misfolded proteins is a pathological hallmark of many neurodegenerative diseases and is generally considered to be controlled by nucleation-dependent polymerization—a two stage processes consisting of the energetically unfavorable formation of a nucleus (i.e., nucleation), followed by efficient elongation of that nucleus via sequential addition of monomers. There is a major controversy concerning the role of aggregates growth in disease pathogenesis. One hypothesis is that these aggregates play a vital role in both disease initiation and progression, with the misfolded versions of endogenous proteins likely to acquire toxic properties, potentially through increased hydrophobicity and/or sequestration of essential cellular components within the aggregates and other pathways. An alternative hypothesis is that the large aggregates represent not the toxic species but rather the final product of a defensive response aimed at protecting cells from more toxic oligomeric species.

Under the assumption that toxic species of misfolded SOD1, as soluble oligomers, are formed as on-pathway intermediates of nucleation-dependent polymerization, we describe here an alternative view of prion-like propagation in SOD1-related ALS mechanism. These different notions reflect uncertainties surrounding the roles of misfolded protein aggregates (toxic versus protective). In this alternative view, the ability of the aggregated form of SOD1 to cross-seed aggregate normal SOD1 appears to antagonize the protective role of aggregate growth. Thus, prion-like propagation would be expected to exert a significant influence on disease prognosis. On the basis of available data for SOD1-linked FALS, together with a risk-based modeling approach, we show that prion-like propagation may account for up to 84% of the variability in survival times among subjects with different SOD1 mutations, highlighting the prognostic value of prion-like propagation and providing potential therapeutic targets for SOD1-linked FALS.

PRION-LIKE PROPAGATION IN SOD1-LINKED FALS

Two essential steps are required for prion infectivity: self-templating replication and an efficient replication cycle (Cushman et al., 2010). For efficient infectivity, pathogenic prion proteins overwhelm the first key step of self-templating replication by converting the α-helix-rich host-encoded PrP^{C} into PrP^{Sc}, characterized by a higher β-sheet content and a polymeric state (**Figure 1A**; Prusiner, 1998; Jackson et al., 1999). This process is thought to be achieved by a nucleation-dependent polymerization mechanism. Typically, this capture and conversion process requires that native conformers exist in a transiently unfolded state or possess an intrinsically unfolded domain. The second step is associated with frangibility of the fibril structure. Amplification of conformational replication is achieved by the fragmentation of amyloid forms to liberate new fiber ends where prion replication occurs. Increased frangibility leads to more fiber ends per unit mass and, consequently, more rapid conversion of available monomers (**Figure 1B**; Tanaka et al., 2006; Colby et al., 2009).

Cu/Zn superoxide dismutase 1 aggregated form could also seed misfolding of a much larger amount of wild-type (WT) SOD1 *in vitro* (Chia et al., 2010). Moreover, in cultured cells,

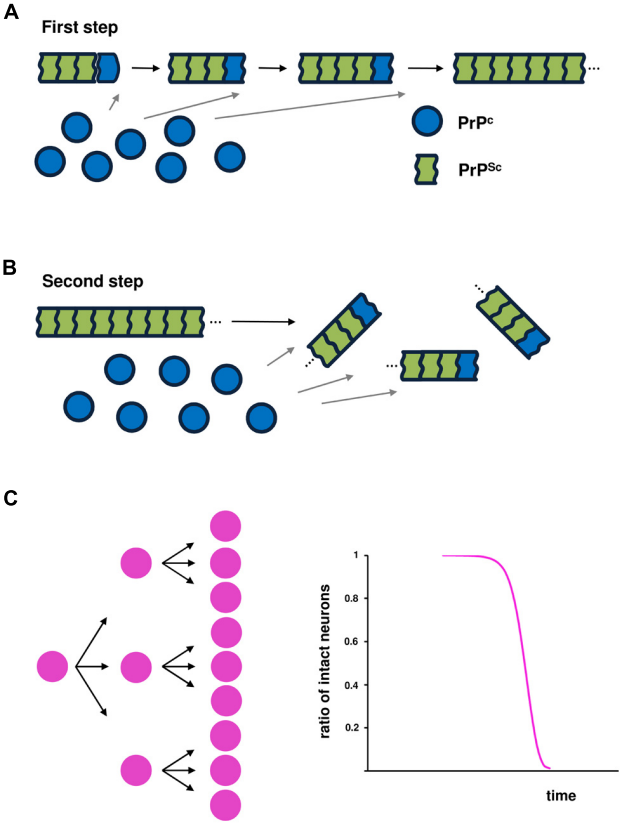

FIGURE 1 | Prion propagation based on nucleation-dependent polymerization. Two events are essential for infectivity: **(A)** conversion of the α-helix-rich, host-encoded prion protein (PrP^{C}) into a pathogenic conformer (PrP^{Sc}), characterized by a higher β-sheet content and a polymeric state, through a nucleation-dependent polymerization mechanism; and **(B)** fragmentation of the PrP^{Sc} amyloid conformer to liberate new fibril ends that are the sites of the conversion of PrP^{C} into PrP^{Sc}, leading to amplification of conformational replication. **(C)** Schematic illustration of the kinetics of infected cells over time.

SOD1 aggregated forms, either exogenously applied (Munch et al., 2011; Grad et al., 2014) or formed within cells (Grad et al., 2011), induced the misfolding and subsequent aggregation of the respective native proteins. Importantly, induced aggregation of endogenous SOD1 was shown to persist after removal of the misfolded seeds, suggesting that the newly formed aggregates could act as templates for the subsequent misfolding of additional native SOD1 (Grad et al., 2011, 2014; Munch et al., 2011). Although fragmentation of the SOD1 aggregated forms to liberate new fiber ends has not yet been verified, these behaviors are consistent with a self-perpetuating, cyclic reaction, analogous to that underlying the replication of infectious prion aggregates. These findings raise the possibility that SOD1-linked ALS progresses through the spread of toxic SOD1 aggregates from a focal site in a manner similar to that of prion spread (cell-to-cell transmission of pathological misfolded prions). However, studies of SOD1 have found no evidence that directly links the transmission of SOD1 aggregates to neuronal toxicity.

Work over the past 4 years indicates that multiple proteins associated with neurodegenerative diseases, especially tau and α-synuclein can propagate aggregates between cells in a prion-like manner. Frost et al. (2009) demonstrated, for the first time, that aggregates of tau protein were taken up into cultured cells where they could induce fibrillization of intracellular tau (Guo and Lee, 2011). Further, tau aggregates newly formed in a cell were observed to transfer to co-cultured cells (Frost et al., 2009). This work was subsequently replicated for α-synuclein (Desplats et al., 2009; Luk et al., 2009; Lee et al., 2010; Hansen et al., 2011; Volpicelli-Daley et al., 2011; Freundt et al., 2012) and SOD1(Munch et al., 2011). It is now well established that protein aggregates are mobile, and can transmit aggregates from cell to cell *in vitro*.

KINETICS OF NEURONAL CELL LOSS

In the model of prion-like propagation in SOD1-linked FALS (cell-to-cell transmission of pathological SOD1 conformers), it is predicted that the kinetics of neuronal cell death over time can be expressed by a sigmoidal function that reflects the kinetics of intercellular transmission over time (**Figure 1C**). If one amyloid conformer of a disease-specific protein liberates three particles that can penetrate a cell, then after fibril growth, the next fragmentation would yield nine particles. Thus, the risk of intercellular transmission would be accelerated in early and middle stages of the disease through fragmentation of the amyloid conformer, but the efficiency of transmission would decline in the advanced stage.

AN ALTERNATIVE VIEW OF PRION-LIKE PROPAGATION IN SOD1-LINKED FALS

Under the assumption that toxic species of disease-specific proteins, for example as soluble oligomers, are formed as on-pathway intermediates of fibril growth through nucleation-dependent polymerization, further fibril extension would be protective against neurodegeneration. According to the free energy profile as a function of aggregate size, free energy peaks at the nucleus stage, and decreases in proportion to the extent of growth of aggregates (Oosawa and Kasai, 1962). Thus, toxic species of misfolded proteins are expected to be structurally stabilized. Importantly, an additional protective effect of aggregate growth is to reduce the concentration of the unfolded/misfolded monomeric proteins. These species of disease-specific proteins are considered to serve as precursors of nucleation—a critical stage in the assembly of a polymeric structure. The fibril nucleation rate is determined as an explicit function of the concentration of the protein solution (Ferrone, 1999). The rate constants for nucleation, estimated by non-linear least-squares algorithms, are ∼10,000,000 times smaller than those for fibril growth (Lee et al., 2007). A reduction in the corresponding protein concentration inhibits the nucleation process and subsequent generation of the toxic species of disease-specific proteins in the affected cells.

In contrast to prion-like propagation in disease spread, we present an alternative view of prion-like propagation in the context of the protective-aggregation hypothesis in which SOD1 toxic species are formed as an on-pathway intermediate in nucleation-dependent polymerization. Although the cross-seeding reaction of prion-like propagation seems to have little influence on the stabilizing effect of fibril growth on the toxic species of misfolded SOD1, it can be predicted to antagonize the protective role of aggregate growth.

Instead of cell-to-cell transmission of the pathological SOD1 conformer, the endogenous, stochastic occurrence of mutant SOD1 nucleation in neuronal cells plays a pivotal role in disease initiation and spread. The subsequent generation of toxic species of SOD1 then causes damage to the cells. In the affected cells, however, further aggregate growth decreases the probability of the next generation of nucleation by reducing the concentration of unfolded mutant SOD1 monomers into the fibril under the protective-aggregation hypothesis (**Figure 2A**). This species of SOD1 is considered to serve as a precursor of nucleation and subsequent toxic species (Furukawa et al., 2004; Hough et al., 2004; Schmidlin et al., 2009). However, if the SOD1 aggregated form preferentially self-propagates by cross-seeding aggregates of normal SOD1 monomers; the result would be a failure to reduce the concentration of unfolded mutant SOD1 monomers, leading to continuous nucleation and the generation of toxic species in the affected cells (**Figure 2B**). Thus, it is assumed that the ability of the reaction underlying prion-like propagation to cross-seed normal SOD1 exerts a significant influence on disease prognosis. The concentration of unfolded mutant SOD1 monomers varies according to the degree of the cross-seeding reaction between the SOD1 aggregated form and normal SOD1, altering nucleation probability and the generation of toxic species.

KINETICS OF NEURONAL CELL LOSS

A simple analytical theory has been proposed to account for the lag time distribution for a stochastic nucleated polymerization reaction in which the lag time of nucleation probability versus the number of unaffected cells fits an exponential distribution (Szabo, 1988). Consistent with this exponential decay in the number of unaffected cells, Colby et al. (2006) showed that, in cultured striatal neurons expressing the mutant Huntington's disease (HD) protein, the probability of a cell remaining aggregate-free dropped exponentially with time.

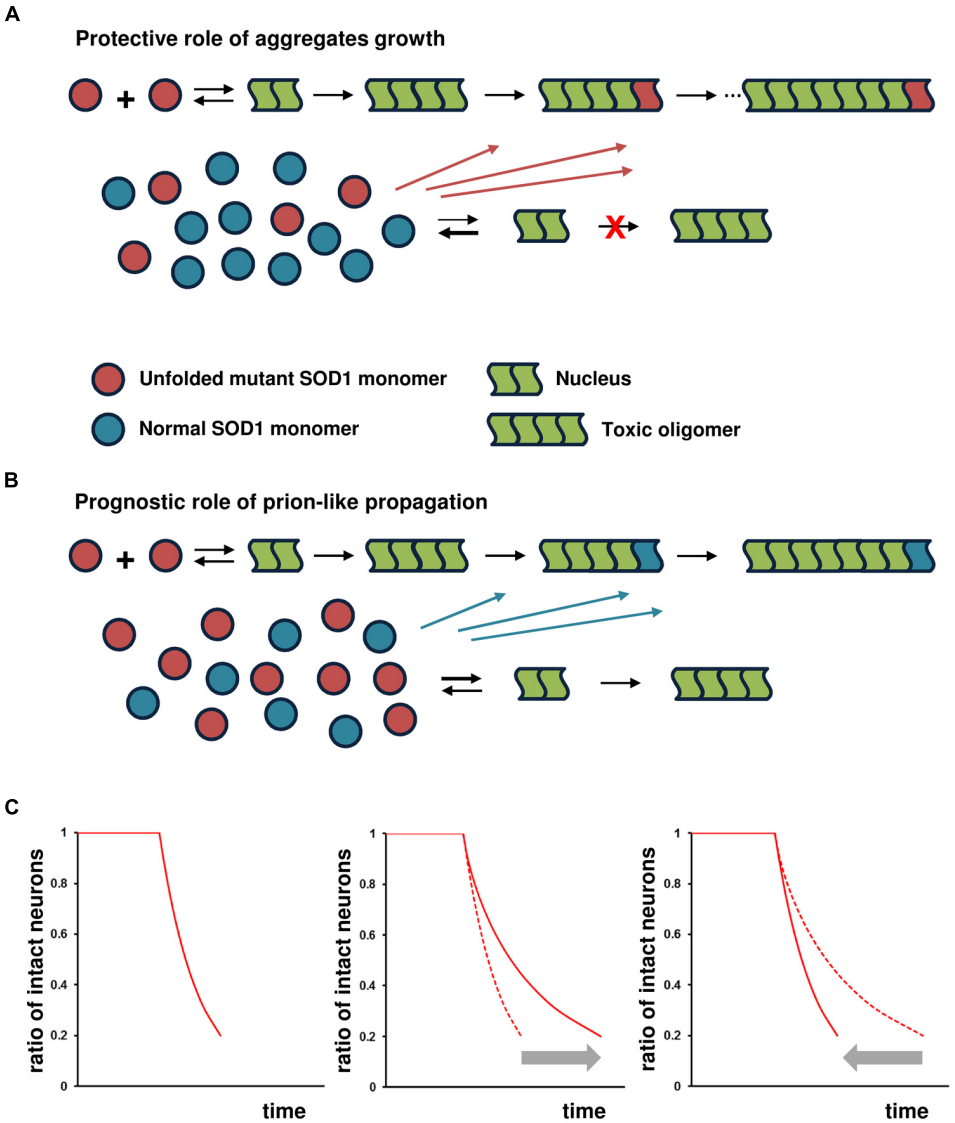

FIGURE 2 | Prognostic role of prion-like propagation in SOD1-linked FALS. Under the assumption that toxic species of misfolded SOD1 are formed via on-pathway nucleation-dependent polymerization, prion-like propagation antagonizes the protective role of aggregate growth. Small oligomeric forms may be toxic, but for the sake of simplicity, the toxic species are shown as tetramers in this schema. The number of monomeric units involved in the formation of the nucleus for SOD1 aggregation is still unknown, but shown as a dimer in this schema. **(A)** SOD1 aggregate growth decreases the probability of the next generation of nucleation and formation of toxic species by reducing the concentration of unfolded mutant SOD1 monomers in the affected cells (protective role of aggregate growth). **(B)** The ability of the SOD1 aggregated form to cross-seed with normal SOD1 results in a failure to reduce the concentration of unfolded mutant SOD1 monomers, leading to continuous nucleation and the subsequent generation of toxic species (prognostic role of prion-like propagation). **(C)** Under the assumption of constant risk for neuronal cell damage, the kinetics of neuronal loss (proportion of intact neurons as a function of time) shows a first-order exponential function (left panel). The kinetics of cell death shows an exponential function with decreasing risk, with further elongation of aggregates reducing the risk of cell death in proportion to the extent of the increase in aggregate size (middle panel). The significant influence of prion-like propagation on disease prognosis through cross-seed aggregation of normal SOD1 would closely fit the exponential function indicated by the gray arrow (right panel).

If soluble oligomers implicated in the toxicity of several neurodegenerative disorders are formed via on-pathway nucleation-dependent polymerization, the probability of nucleation would be considered to pose a constant risk for cell death regardless of its toxicity (Perutz and Windle, 2001; Sugaya and Matsubara, 2009). Nucleation is a rare event that occurs randomly in time and space, and is a rate-limiting process with a thermodynamically unfavorable state (Perutz and Windle, 2001). In a constant-risk model of neurodegeneration, the death of a neuron is initiated randomly in time by a series of single, rare catastrophic events, and the death of any given cell is independent of that of any other cell (Clarke et al., 2000). Under the constant-risk assumption, the kinetics of neuronal loss over time exhibits an exponential function (**Figure 2C**, left panel; Clarke et al., 2000). Accordingly, the

kinetics of cell death would be expected to show an exponential function, with risk decreasing as further elongation of aggregates reduced the generation of toxic, soluble oligomers; thus, risk would decrease in proportion to the extent to which aggregation increased (**Figure 2C**, middle panel). Under the scenario of the failure to reduce the concentration of unfolded mutant SOD1 monomers by cross-seeding aggregates of normal SOD1 monomers, we would expect the kinetics of cell death to closely fit an exponential function, but in the opposite direction (**Figure 2C**, right panel).

ASSOCIATION WITH CLINICAL FINDINGS

A motor pool refers to all of the individual motor neurons that innervate a single muscle. Because of motor pools in the spinal cord are clustered in distinct columns of motor neurons extending over multiple spinal cord segments, a longitudinal study for estimating the number of motor units from individual muscles in ALS patients may provide a mechanistic insight into local disease spread. A number of techniques have been developed to estimate the number of motor units in humans by defining a motor unit as the spinal motor neuron and its axon together with the muscle fibers it innervates. Motor unit number estimation (MUNE) is a technique that uses EMG to estimate the number of motor units in a muscle. Using the most reliable electrophysiological method for MUNE, the exponential nature of lower motor neuron loss over time in both ALS patients and SOD1-linked FALS patients has recently been demonstrated in all of the muscles examined (Baumann et al., 2010, 2012). Furthermore, MUNE values obtained with the Bayesian method show a solid correlation with the actual number of lower motor neurons in the spinal cord, determined by histology (Ngo et al., 2012). The exponential kinetics of neuronal cell loss is consistent with the "one-hit" model of neurodegeneration described by Clarke et al. (2000). Thus, the endogenous, stochastic occurrence of one-hit events in a homogenous population of lower motor neurons may play a pivotal role in local disease spread. The alternative view of prion-like propagation in the context of the protective-aggregation hypothesis may well explain the exponential nature of lower motor neuron loss in SOD1-linked FALS.

ASSOCIATION WITH TRANSGENIC MOUSE MODEL

Further evidence to support the prognostic role of prion-like propagation has come from co-expression studies using transgenic mice. Since most familial ALS patients with SOD1 mutations are heterozygous, recent studies have utilized transgenic mice expressing both human WT (hWT) and ALS-related mutant SOD1 to more accurately recapitulate SOD1 behavior *in vivo*. Co-expression of $SOD1^{hWT}$ exacerbates the disease phenotypes of $SOD1^{G93A}$ (Jaarsma et al., 2000; Fukada et al., 2001), $SOD1^{G85R}$ (Wang et al., 2009), and $SOD1^{A4V}$ mice (Deng et al., 2006), hastening the appearance of cellular pathologies and shortening survival times. The effect of the WT protein on $SOD1^{A4V}$ mice is particularly dramatic; even though ALS patients with this mutation exhibit particularly rapid disease progression, mice expressing $SOD1^{A4V}$ alone do not develop motor neuron disease within their lifetimes (Gurney et al., 1994; Jaarsma et al., 2000). The alternative view of prion-like propagation suggests that normal SOD1 prevents the reduction in the concentration of mutant SOD1 monomers, with a predicted shorter survival (**Figure 2**). Indeed, the concentration of monomeric G93A-SOD1 protein is markedly elevated in the tissues of transgenic mice carrying both G93A-SOD1 and hWT-SOD1 genes (G93A/hWT-mice) compared to that in G93A-mice (Fukada et al., 2001). Furthermore, in the affected tissue, SOD1 aggregates containing both WT and mutant protein have been observed (Deng et al., 2006; Wang et al., 2009), suggesting that hWT-SOD1 is "recruited" into non-native oligomers by the mutant SOD1, possibly by the cross-seeding reaction.

GENOTYPE–PHENOTYPE CORRELATIONS IN SOD1-LINKED FALS UNDER THE PROTECTIVE-AGGREGATION HYPOTHESIS

The nucleation aggregation theory predicts that the probability of nucleation is an exponential function of the free energy of nuclear formation (Perutz and Windle, 2001). In the case of HD caused by the expansion of CAG trinucleotide repeats, which creates proteins containing long, toxic polyglutamine repeats, the change in free energy per additional glutamine repeat is expected to be constant (Perutz et al., 2002). Therefore, the probability of nucleation is expected to rise exponentially with the number of repeats. This appears to be reflected by the exponential relationship between the extent of expansion and age of onset in HD (Perutz and Windle, 2001; Sugaya et al., 2007; Sugaya and Matsubara, 2009). In the case of SOD1, a thermodynamic analysis suggests that the WT protein is a so-called three-state dimer in which the individual monomers also adopt their correct folded structures in the absence of an intermolecular interface or stabilizing Zn and Cu ions (Banci et al., 1998; Stroppolo et al., 2000; Strange et al., 2003). Several recent studies have implicated such immature monomeric SOD1 species as precursors in the ALS mechanism (Furukawa et al., 2004; Hough et al., 2004; Schmidlin et al., 2009). Reduced stability shifts the folding equilibrium towards denatured monomers.

There are differences in the ability of amyloid conformers with different SOD1 mutations to cross-seed with normal SOD1 (Chia et al., 2010; Hwang et al., 2010). Using the altered free energy of the denatured monomer of SOD1 species with different mutations as an index, we examined whether the prognostic role of prion-like propagation can explain the variance in survival times among patients with different SOD1 mutations.

CHANGES IN THE STABILITY OF SOD1 MUTANT PROTEINS

Native SOD1 is an extremely stable, obligate homodimer. A key event in SOD1-linked ALS seems to be the pathological formation of toxic species of misfolded SOD1 as a result of initially unfolded SOD1 mutants. Dimeric $apoSOD1^{pWT}$ (metal depleted, pseudo-WT SOD1 with Cys-to-Ala substitutions at positions 6 and 111) has been shown to exhibit three-state folding behavior in which the monomer folds independently following a classical two-state process (Lindberg et al., 2004). After folding, the structured monomers assemble as homo-dimers according to the relationship,

$$2D \rightleftarrows 2M \rightleftarrows M_2,$$

where D is the unfolded monomer, M is the folded monomer, and M_2 is the dimer. Equilibrium studies tell us about the difference in

free energy between the folded and the denatured state. Using the stopped-flow technique, Lindberg et al. (2004, 2005) reported the effect of protein destabilization (ΔG) upon ALS-associated point mutations in SOD1 (Byström et al., 2010). Using these date, we performed a regression analysis on the relationship between mean age at disease onset (or respiratory failure death) in patients with different point mutations and the effects of each point mutation on protein destabilization, applying a mathematical model that assumes that toxic species of the misfolded SOD1 are formed via on-pathway nucleation-dependent polymerization:

$$Dimer \rightleftarrows Unfolded\ monomer \rightleftarrows Nucleus \rightarrow Fibril$$

The clinical data used in this study and protein stability changes (ΔG) for each SOD1 mutation are summarized in Supplemental Table 1. Protein stability changes (ΔG) were normalized for comparison purposes.

MATHEMATICAL MODEL OF THE PROTECTIVE-AGGREGATION HYPOTHESIS BASED ON NUCLEATION-DEPENDENT POLYMERIZATION

An exponential relationship would be expected to hold between the effect of protein destabilization (ΔG) and the nucleation lag time of SOD1 aggregation, similar to the relationship between polyQ length and nucleation lag time of polyQ aggregation (Sugaya and Matsubara, 2009, 2012). If the nucleation rate over time acts as a constant risk for neuronal cell damage in a homogenous cell population, the probability of aggregate-free neurons (or the proportion of intact neurons) as a function of time t would be expected to decline exponentially, consistent with the stochastic appearance of nucleation. The rate constants for nucleation, estimated by non-linear least-squares algorithms, are ∼10,000,000 times smaller than those for fibril growth (Lee et al., 2007). Therefore, under the assumption that toxic species of misfolded SOD1 are formed via on-pathway nucleation-dependent polymerization, the proportion of intact neurons at time t is largely determined by two integral elements: one reflects the probability distribution function for nucleation lag time (t_N), and the other corresponds to an extension time (t_E), reflecting the protective effects of aggregate growth attributable to the decreased risk of neuronal cell loss. Their relationships can be described by $t^2 = {t_N}^2 + {t_E}^2$ in a linear regression model. We initially made the assumption that the extent of motor neuron loss at the site of onset would be nearly identical at disease onset regardless of the nature of the SOD1 mutation. Then, mean age at onset (t_A) can be expressed as a function of protein stability change (x):

$$(\text{mean } {t_A}^2 - {t_{EA}}^2)^{1/2} = t_{NA} = f(x) \quad (1)$$

where t_{EA} is t_E at disease onset, t_{NA} is t_N at disease onset, and $f(x)$ is the integration of the probability distribution function of nucleation lag time at disease onset as an exponential function of the change in protein stability. Similarly, mean age at respiratory failure death (t_R) can be expressed as

$$(\text{mean } {t_R}^2 - {t_{ER}}^2)^{1/2} = t_{NR} = g(x) \quad (2)$$

where t_{ER} is t_E at respiratory failure death, t_{NR} is t_N at respiratory failure death, and $g(x)$ is the integration of the probability distribution function of nucleation lag time at respiratory failure death as an exponential function of the change in protein stability (x). Therefore, mean survival time among subjects with different SOD1 mutations can be expressed as a function of protein stability change as

$$\text{mean } t_R - \text{mean } t_A = ({t_{ER}}^2 + [g(x)]^2)^{1/2} - ({t_{EA}}^2 + [f(x)]^2)^{1/2} \quad (3)$$

We employed linear regression with logarithmic transformation of Eqs 1 and 2, thus invoking an intrinsically linear model. Linear regression analysis was then applied to determine t_E values by identifying the points at which R^2 values were identical for a quadratic curve and a linear model that best fit a linear relationship. Models were evaluated using R^2 values, the F-test, and analyses of residual error to test whether the assumptions of the regression were reasonably satisfied.

PRION-LIKE PROPAGATION IS REFLECTED IN VARIABILITY OF SURVIVAL TIME

The relationship between mean age at onset in patients with different SOD1 mutations and changes in the stability of their SOD1 mutant proteins showed a good fit with a linear regression model of logarithmically transformed Eq. 1 ($R^2 = 0.85$, $F = 126.0$) when ${t_{EA}}^2 = 140$ (**Figure 3A**). The variability of age at onset is well fit by the model when the values of ${t_{EA}}^2$ are identical as expected by an exponential decline of neuronal cell loss. Accordingly, the alternative role of prion-like propagation, together with Eq. 3, suggests that the variability in mean survival time among patients with different SOD1 mutations is largely dependent on the differences in extension time at respiratory failure death (t_{ER}). The concentration of unfolded mutant SOD1 monomers varies according to the efficacy of the cross-seed reaction between the SOD1 aggregated form and normal SOD1. Because the nucleation process is concentration dependent, this alters the probability of nucleation and subsequent generation of toxic species of misfolded SOD1, leading to the variable values of ${t_E}^2$ (extension time due to the protective effects of aggregate growth versus toxic species of misfolded SOD1) from short survival to long survival. We first considered that the values of t_{ER} are nearly identical in subjects with SOD1 mutations that show a mean survival time less than 2.5 years, and determined t_{ER} value using the regression model of logarithmically transformed Eq. 2 to provide the best fit to a linear model. In a similar way, we next determined each value of t_{ER} in patients with different SOD1 mutations. The relationship between mean age at respiratory failure death in patients with different SOD1 mutations and changes in the stability of their mutant SOD1 proteins showed a good fit with a linear regression model of logarithmically transformed Eq. 2 ($R^2 = 0.93$, $F = 172.1$) for the variable ${t_{ER}}^2$ values shown in Supplementary Table 1 (**Figure 3B**). Remarkably, the values of ${t_{ER}}^2$ accounted for 84% of the variability of mean survival time in patients with different SOD1 mutations (**Figure 3C**). However, the values of ${t_{ER}}^2$ showed no significant correlation with the aggregation propensity of individual SOD1 mutants (Vassall et al., 2011). Although data are available for only a limited number of SOD1 mutants (Chia et al., 2010; Hwang et al., 2010), the results appear to

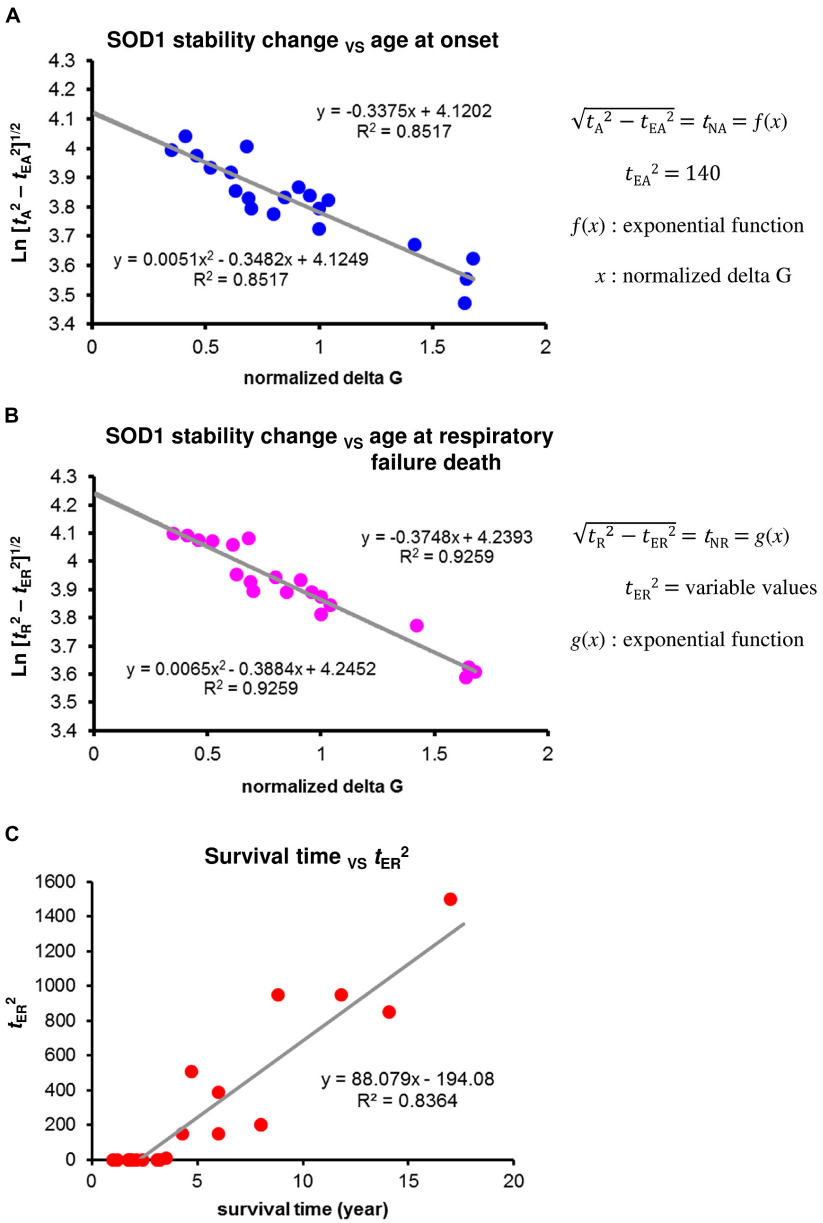

FIGURE 3 | Changes in the stability of SOD1 proteins and phenotypic variability of SOD1-linked FALS. (A) Correlation between mean age at onset among patients with different SOD1 mutations and changes in the stability of mutant SOD1 protein. A linear regression analysis using a logarithmic transformation of Eq. 1 provided the best fit to a linear model when $t_{EA}{}^2$ = 140. The $t_{EA}{}^2$ value was determined by identifying the points at which R^2 values were identical for a quadratic curve and a linear model. **(B)** Correlation between mean age at respiratory failure death among patients with different SOD1 mutations and changes in the stability of mutant SOD1 protein. A linear regression analysis using a logarithmic transformation of Eq. 2 provided the best fit to a linear model when the values of $t_{ER}{}^2$ were as shown in Supplementary Table 1 (variable values of $t_{ER}{}^2$). Each value of $t_{ER}{}^2$ was determined by identifying the point at which the R^2 value was identical for a quadratic curve and a linear model. **(C)** Correlation between the values of $t_{ER}{}^2$ from the result of **(B)** and mean survival time among patients with different SOD1 mutations.

be consistent with differences in the ability of SOD1 amyloid conformers with different mutations to cross-seed with normal SOD1.

DISCUSSION

In inherited neurodegenerative disorders, delayed clinical onset, in which symptoms may not appear for years or decades, is often assumed to reflect the occurrence of age-dependent cumulative damage. One prediction of the cumulative-damage hypothesis is that the probability of cell death will increase over time, and the kinetics of neuronal death over time can be expressed by a sigmoidal function. However, Clarke et al. (2000) reported that the kinetics of neuronal loss in many forms of neurodegeneration appeared to be exponential, and in fact could be better

explained by a mathematical model in which the risk of cell death remains constant (one-hit model). The exponential decimation of remaining lower motor neurons over time in ALS patients is also consistent with the one-hit model of neurodegeneration (Baumann et al., 2010, 2012). Furthermore, Clarke and Lumsden (2005) showed that the one-hit model of neurodegeneration can be improved using stretched exponential decay models, which most easily fit data in which the rate of death decreases over time, consistent with multiple populations of neurons coexisting, each with a different constant risk of death. However, there are some scenarios in which it may also be possible to explain such kinetics as an exponentially decreasing risk of neurodegeneration. For example, if dying neurons released a cyto-protective substance such as a neurotrophic factor into their environment, then the concentration of that factor would increase as more neurons were affected, causing a concomitant decline in the risk of cell death. One scenario has also emerged in the context of the protective-aggregation hypothesis in which toxic species of misfolded proteins, as soluble oligomers, are formed as an on-pathway intermediate in nucleation-dependent polymerization. The expectation under this scenario is that the kinetics of cell death would reveal an exponentially decreasing risk, with further fibril elongation reducing the risk posed by soluble oligomers in proportion to the increased extent of aggregates. These scenarios suggest that, although initiation of the neurodegenerative process occurs randomly in time as a series of independent events for each neuron, under the condition of exponentially decreasing risk, the progression of the neurodegenerative process may be influenced by other cells.

One major unresolved question regarding the molecular mechanism underlying neurodegenerative process in patients with SOD1-linked FALS is why the prognoses are so varied despite a pathogenic mechanism in common. The duration of illness is relatively consistent for each SOD1 mutation, but is variable among different mutations. Wang et al. (2008) reported that two synergistic properties, increased protein aggregation propensity and decreased protein stability, account for 69% of the variability in mutant Cu/Zn-superoxide-dismutase-linked familial ALS patient survival times (Wang et al., 2008). However, in their regression analysis, about half of the patients had A4V mutation. Its corrected value account for about 20% of the variability in survival times among subjects with different mutation. Recently, Vassall et al. (2011) reported that in their depth analyses, there is minimal to no correlation between observed aggregation, predicted aggregation propensity, and disease duration. Thus, the major factor, which contributes to the disease progression, is still unknown. The correlation between polyglutamine repeat length and the effects of repeat length on the rate of disease progression, as well as age of onset, were well explained by the regression models of Eqs 1 and 2 when polyglutamine repeat length was used as an index (x; Sugaya and Matsubara, 2009, 2012). However, one distinct feature in the results of SOD1-linked FALS stands out: the extension time (t_E) is nearly identical among subjects with different polyglutamine repeat-length, even those with late-stage HD (Sugaya and Matsubara, 2012). Compared with the mechanisms of prion-like propagation in SOD1 aggregates, one clear difference is that mutant huntingtin aggregates do not cross-seed *in vitro* with huntingtin containing normal lengths of polyglutamine tracts (Busch et al., 2003). These features support the idea that differences in the ability of SOD1 amyloid conformers with different mutations to cross-seed with normal SOD1 is reflected in the variability of survival time. These findings, together with the results of **Figure 3** suggest the prognostic value of prion-like propagation under the protective-aggregation hypothesis, showing that it may account for up to 84% of the variability in survival times across subjects with different SOD1 mutations.

CONCLUSION

At first glance, several major aspects of the pathogenesis of SOD1-linked ALS, including cell-autonomous and non-cell-autonomous processes, prion-like propagation and exacerbation of disease phenotypes in transgenic mice expressing both WT and mutant SOD1, appear to be independent events, like pieces of a puzzle. Viewed in the context of the protective-aggregation hypothesis, the molecular events in prion propagation raise the possibility of "prion-like propagation," providing an alternative disease model to account for the mechanism of SOD1-linked ALS. Considerable work remains to verify this disease model; however, a central issue for future studies is connecting the factors involved in the pathogenesis of SOD1-linked FALS with each other to delineate the prognosis and spread of the disease.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00359/abstract

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The two-hit hypothesis for neuroinflammation: role of exogenous ATP in modulating inflammation in the brain

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Brain inflammation is a common occurrence following responses to varied insults such as bacterial infections, stroke, traumatic brain injury and neurodegenerative disorders. A common mediator for these varied inflammatory responses is prostaglandin E_2 (PGE_2), produced by the enzymatic activity of cyclooxygenases (COX) 1 and 2. Previous attempts to reduce neuronal inflammation through COX inhibition, by use of nonsteroidal anti-inflammatory drugs (NSAIDs), have met with limited success. We are proposing the two-hit model for neuronal injury—an initial localized inflammation mediated by PGE_2 (first hit) and the simultaneous release of adenosine triphosphate (ATP) by injured cells (second hit), which significantly enhances the inflammatory response through increased synthesis of PGE_2. Several evidences on the role of exogenous ATP in inflammation have been reported, including contrary instances where extracellular ATP reduces inflammatory events. In this review, we will examine the current literature on the role of P2 receptors, to which ATP binds, in modulating inflammatory reactions during neurodegeneration. Targeting the P2 receptors, therefore, provides a therapeutic alternative to reduce inflammation in the brain. P2 receptor-based anti-inflammatory drugs (PBAIDs) will retain the activities of essential COX enzymes, yet will significantly reduce neuroinflammation by decreasing the enhanced production of PGE_2 by extracellular ATP.

Keywords: ATP, microglia, neuroinflammation, NSAIDs, P2 receptors, PBAIDs, prostaglandin E2

INFLAMMATION WITHIN THE BRAIN

Various environmental factors can lead to inflammation within the brain. These range from bacterial infections that cause acute inflammation to neurodegenerative disorders that mediate chronic inflammation. The inflammation may be restricted to a local region in focal ischemia or occur in a wider zone during traumatic brain injury. Inflammation could also result from an autoimmune response such as multiple sclerosis or in response to toxins and nerve agents (for general reviews, see Lucas et al., 2006; Aguzzi et al., 2013). Recent reports implicate inflammation contributing to the pathology of psychiatric disorders such as stress, depression and schizophrenia (Najjar et al., 2013), in metabolic syndromes such as obesity and type 2 diabetes (Purkayastha and Cai, 2013), and even as a response to increased neuronal activity (Xanthos and Sandkuhler, 2014). Irrespective of the type of inflammation, the molecular mediators are oftentimes the same –prostaglandin E_2 (PGE_2) or cytokines such as interleukin-1β (IL-1β), produced by the activity of resident microglial cells. Despite brain inflammation playing such a major role in various CNS disorders, successful therapeutic strategies to overcome it are still lacking.

PGE_2 is produced by the action of cyclooxygenases (COX) which mediate the first committing step in its synthesis from arachidonic acid (Akundi et al., 2005). The constitutively active COX-1 isoform is believed to be responsible for the majority of PGE_2 formed in the body. However, it is the growth factor-, cytokine- or mitogen-inducible COX-2 that emerged as the isoform responsible for the massive release of PGE_2 during inflammation of all types—systemic, central, acute or chronic. The COX enzymes have been a therapeutic target in a multitude of disorders, ranging from fever and pain to cancer, rheumatoid arthritis, and Alzheimer's disease (AD; Yedgar et al., 2007). Their importance can be judged from the widespread use of aspirin as an analgesic and antipyretic; and the promise of nonsteroidal anti-inflammatory drugs (NSAIDs) against spreading neurodegeneration in AD (Szekely and Zandi, 2010). However clinical trials failed to not only halt the progression of dementia in AD patients but also showed increased risks of myocardial infarction and stroke (Jüni et al., 2004). An essential lesson learnt from the debacle of NSAIDs was that the two isoforms of COX do not functionally substitute one another but each remains indispensable in certain

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ATP, adenosine triphosphate; COX, cyclooxygenase; IL-1β, interleukin-1β; IRF8, interferon regulatory factor 8; LPS, lipopolysaccharide; MCP1, monocyte chemotactic protein 1; MMP-9, matrix metalloproteinase 9; NSAIDs, non-steroidal anti-inflammatory drugs; PARP, poly (ADP-ribose) polymerase; PBAIDs, P2 receptor-based anti-inflammatory drugs; PGE_2, prostaglandin E_2; PD, Parkinson's disease; TNF-α, tumor necrosis factor α; UTP, uridine triphosphate.

functions. COX-2 stands on a delicate balance—the neuronal isoform plays an important role in synaptic plasticity, memory consolidation and cortical development while the microglial isoform mediates neuroinflammation. Targeting COX enzymes, therefore, requires a careful consideration of the benefit-to-risk ratio.

NEUROINFLAMMATION: A TWO-HIT MODEL

An interesting observation in the past decade and half showed that the inflammatory response of microglia—the release of mature IL-1β from lipopolysaccharide (LPS)-primed cells—could be significantly modulated with the addition of exogenous adenosine triphosphate (ATP; Ferrari et al., 1997). More diversified studies showed that ATP is able to mediate the release of PGE_2 in IL-1β-treated astrocytes (Xu et al., 2003) or in LPS-activated macrophages (Barberà-Cremades et al., 2012). We found a similar synergistic effect of ATP on LPS-mediated PGE_2 release in primary rat microglial cells (unpublished observation). These studies conclusively showed that exogenous ATP significantly modulates inflammation. In this review, we are proposing the two-hit model for neuroinflammation. The *first hit* is the injury itself—nerve injury, bacterial infections, hypoxia-ischemia, autoimmune reactions or proteopathies associated with neurodegeneration—leading to the activation of glial cells (**Figure 1**). The *second hit* is the release of large pools of cytosolic ATP from damaged neurons into the extracellular milieu, in response to direct injury or following glial cell activation. This excess ATP, despite mechanisms regulating their concentration outside the cell, activates a wide variety of purinergic receptors present on cells in the vicinity, thus modulating glial activity and neuronal response to inflammation. Such a model was earlier proposed for the release of mature IL-1β following bacterial infections (Ferrari et al., 2006). Identifying the pro-inflammatory receptors of ATP, and targeting them pharmacologically, will significantly diminish the dramatic release of prostanoids and cytokines to clinically manageable levels; thus, balancing their functional roles in active defence and tissue repair.

ATP: THE COMMON DENOMINATOR FOR VARIED INFLAMMATORY INSULTS

Both healthy neurons and glial cells carry ATP, in millimolar concentrations, within presynaptic vesicles and granules, respectively (Abbracchio et al., 2009). Neuronal ATP serves as a neurotransmitter while astrocytic ATP allows distant astrocytes to communicate with each other and modulate neuronal response. However, the release of ATP by neurons or astrocytes is usually very low, in the nanomolar range. Furthermore, the extracellular concentration of ATP is dependent on the regional distribution and local activity of synaptic ectonucleotidases CD28/CD39 which convert ATP to ADP and AMP, CD73 which converts AMP to adenosine, and nucleoside diphosphate kinase whose transphosphorylating activity maintains the exogenous levels of various nucleotides in steady state (Lazarowski et al., 2003). This steady state balance is, however, disrupted during pathological conditions when damaged neurons and chronically activated glial cells release dramatic levels of ATP, uridine triphosphate (UTP) and other intracellular nucleotides.

Not only neuropathological conditions even systemic inflammation leads to an increase in exogenous ATP within the CNS (Gourine et al., 2007). In fact the release of ATP in response to tissue injury is a universal phenomenon also seen in plants at sites of physical wounding (Choi et al., 2014). Efflux of ATP into the extracellular space is a common universal "stress signal", leading to the evolution of receptors for ATP to recognize this "danger" and initiate a stress response. Mammals evolved purinergic receptors with varying specificities for ligands such as ATP, ADP, UTP, UDP, UDP-sugars or adenosine, and diverse range of intracellular signaling mechanisms downstream to receptor activation. Nucleotides act on P2 receptors, with seven ionotropic P2X receptors gating Na^+, K^+, and especially Ca^{2+}

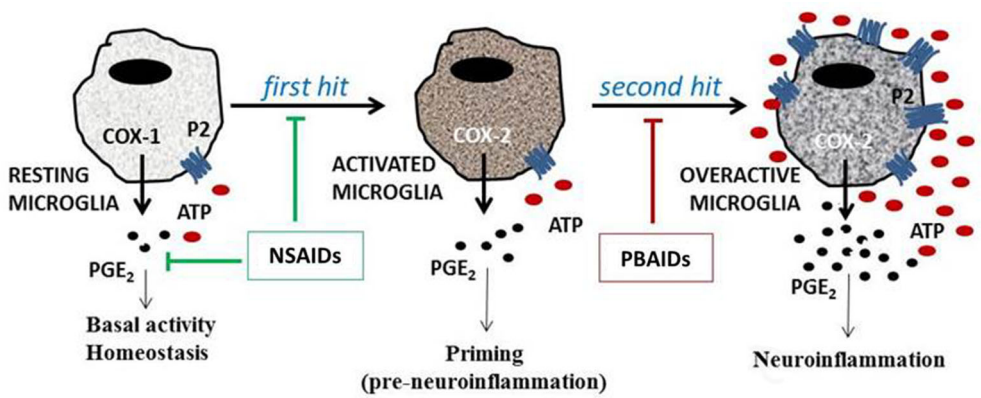

FIGURE 1 | The two-hit model of neuroinflammation. The ATP-mediated enhancement of neuroinflammation can be explained through the two-hit model. A variety of insults, such as bacterial LPS, various cytokines, or amyloid peptides, can act as the *first hit*, resulting in microglial activation, COX-2 induction and PGE_2 release. The *second hit*, following neuronal injury, death or persistent glial cell activation, results in the release of ATP, which acts on both neuronal and glial P2 receptors, leading to enhanced microglial PGE_2 release. NSAIDs target COX enzymes affecting the housekeeping roles of PGE_2. ATP potentiates the effects of first hit multi-fold, and thus, would be the most relevant target for therapeutic intervention. By acting on P2 receptors, PBAIDs are believed to reduce PGE_2 to pre-inflammatory levels without affecting the activity of COX enzymes.

ions, and eight G protein-coupled metabotropic P2Y receptors (Abbracchio et al., 2009). Adenosine, on the other hand, acts on adenosine receptors, of which A_1 and A_3 adenosine receptors inhibit, while A_{2A} and A_{2B} adenosine receptors stimulate adenylyl cyclase (Fredholm, 2010). Recently ATP receptors have also been identified in plants which are activated in response to tissue wound (Choi et al., 2014). Called DORN1 (Does not Respond to Nucleotides 1), these receptors, much like their mammalian counterparts, show high affinity to ATP and alter Ca^{2+} flow.

EXOGENOUS ATP HAS BOTH POSITIVE AND NEGATIVE ROLES IN INFLAMMATION

The presence of functionally active purinergic receptors on microglia indicates the likelihood of astrocyte-microglia crosstalk (Verderio and Matteoli, 2001). Such a communication enhances microglial surveillance system and their response to inflammation within the CNS. Indeed, neuronal and astrocytic release of ATP during traumatic brain injury causes rapid microglial chemotactic response (Davalos et al., 2005). At the site of injury, exogenous ATP mediates the release of pro-inflammatory cytokines and PGE_2 (Xu et al., 2003; Ferrari et al., 2006; Xia and Zhu, 2011). The end effect of this synergism is the production of pathological levels of inflammatory cytokines and prostanoids.

While the above reports suggest extracellular ATP as proinflammatory, others have reported to the contrary. In LPS-primed cells, ATP inhibited the release of cytokines from spinal cord microglia (Ogata et al., 2003), nitric oxide (NO) release in BV-2 microglia (Brautigam et al., 2005), and pro-inflammatory markers such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and NO in primary microglia (Boucsein et al., 2003). Since TNF-α and IL-6 also have neuroprotective roles (Suzuki et al., 2004; Noguchi et al., 2013), the above inhibitory action of ATP may actually be detrimental to the organism. Increases in exogenous ATP need not always be hazardous—extracellular levels of adenosine increases 6- to 31-fold within the hippocampus of patients with epilepsy, but acts as a natural anticonvulsant terminating seizure (During and Spencer, 1992). Interestingly, cancer cells evade surveillance by up-regulating a subpopulation of regulatory T cells expressing ectonucleotidases CD39 and CD73 to exploit the immunosuppressive nature of adenosine (Whiteside and Jackson, 2013). Early development of nervous system is dependent on purinergic signals, which in concert with growth factors, regulate the number of proliferating and differentiating neural stem cells (Ulrich et al., 2012).

P2 RECEPTORS DETERMINE THE EVENTUAL EFFECT OF EXTRACELLULAR ATP

$P2X_7$ RECEPTORS

The specific roles of P2 receptors in neuroinflammation are still being uncovered. The subject is still in its teens, starting with the discovery of ATP enhancing IL-1β release in activated immune cells (Ferrari et al., 1997). LPS-mediated activation of toll-like receptor 4 leads to the formation of the inflammasome complex wherein IL-1β processing occurs (Martinon et al., 2002). However, release of IL-1β requires loading of the inflammasome complex into the secretory lysosome, or the formation of membrane blebs—either mechanism triggered through $P2X_7$ receptor-mediated K^+ efflux (Ferrari et al., 2006; di Virgilio, 2007). As a result, the effect of ATP is dependent on cells primed with LPS, and conversely, LPS does not release IL-1β in the absence of $P2X_7$ receptors (Solle et al., 2001). Pannexin 1, a gap junction-related protein, has been shown to be responsible for the release of ATP from dying cells, leading to the activation of the inflammasome and recruitment of phagocytes (Dahl and Keane, 2012). Intraperitoneal injection of LPS results in two-fourfold higher detection of ATP in the mouse peritoneum (Barberà-Cremades et al., 2012). Systemic administration of LPS markedly increases the expression of $P2X_7$ receptors in the brain (Choi et al., 2007). LPS- or IL-1β-mediated febrile response is greatly reduced in mice with genetic or pharmacological loss of $P2X_7$ receptors (Barberà-Cremades et al., 2012). ATP and the preferential $P2X_7$ agonist, 2′(3′)-*O*-(4-benzoylbenzoyl) ATP (BzATP) induce the secretion of cytokines IL-6 and TNF-α in wildtype microglia but not in cells derived from $P2X_7^{-/-}$ mice (Shieh et al., 2014). These reports underline the importance of $P2X_7$ receptors in mediating inflammation, especially in the release of IL-1β (Ferrari et al., 2006). The low affinity of $P2X_7$ receptors for extracellular ATP ensures their activation occurs only under pathological conditions where excess ATP is found, further supporting the notion of exogenous ATP as an "alarm" signal.

Immunohistochemical analysis of AD brains reveal significant levels of $P2X_7$ receptors colocalized with activated microglia, an observation that was also found in the hippocampus of rats injected with $A\beta_{1\text{-}42}$ (McLarnon et al., 2006). Aβ triggers ATP release, membrane permeabilization and IL-1β secretion in wildtype but not in $P2X_7^{-/-}$ mouse (Sanz et al., 2009). In fact, overexpression of $P2X_7$ receptor itself, in the absence of any pro-inflammatory stimuli, can drive the activation and proliferation of microglial cells (Monif et al., 2009). Similarly, exposure to high levels of extracellular ATP can also tilt the signaling mechanism from a P2X7-phosphatidylinositol 3-kinase/Akt-mediated growth pathway to a novel P2X7-AMPK-mammalian target of rapamycin (mTOR)-mediated autophagic pathway, as observed in tumor cells (Bian et al., 2013). Rapamycin reduces neuroinflammation and brain lesions in a mouse model of Leigh syndrome (Johnson et al., 2013). In astrocytes, inhibition of mTOR significantly reduces the stability of inducible nitric oxide synthase (iNOS) mRNA (Lisi et al., 2011). However, in microglia, blocking of mTOR pathway in activated cells leads to enhanced PGE_2 synthesis (de Oliveira et al., 2012). Activated microglia downregulate microRNA, miRNA-200b, which leads to increased c-Jun N-terminal kinase (JNK) activity leading to increased iNOS expression (Jadhav et al., 2014). These observations suggest that the role of mTOR in neuroinflammation is cell-type specific and depends on both epigenetic factors and the presence of inflammatory stimuli.

Gene expression studies in Aβ-treated microglia derived from human post-mortem brains, in fact, suggest that the expression of pro-inflammatory genes are largely up-regulated at the expense of genes involved in Aβ phagocytosis and removal (Walker et al., 2006). Activated $P2X_7$ receptors impair lysosomal function and instead stimulate the release of autolysosomal contents into the

extracellular space, possibly leading to the increased secretion of IL-1β or amyloidogenic proteins (Takenouchi et al., 2009). In corollary, silencing of $P2X_7$ receptors in Aβ-stimulated cells leads to a decreased release of pro-inflammatory cytokines and a marked increase in the phagocytosis of $A\beta_{1–42}$ peptide (Ni et al., 2013). $P2X_7$ receptors, therefore, turn phagocytic (neuroprotective) microglia into inflammatory (neurodegenerative) phenotype.

Expression of $P2X_7$ receptors is also up-regulated in Huntington's disease and amyotrophic lateral sclerosis (ALS; Díaz-Hernández et al., 2009). In microglia isolated from superoxide dismutase SOD1-G93A mutant mouse model of ALS, activation of $P2X_7$ receptors enhances oxidative stress (Apolloni et al., 2013). Oxidative stress drives the nitration of 90 kDa heat-shock protein (Hsp90), which mediates cell death through $P2X_7$ receptors (Franco et al., 2013). Nitrated Hsp90 is found in the motor neurons of patients with ALS; and as expected, deletion of $P2X_7$ receptors prevents the neurotoxic effects of nitrated Hsp90.

Imbalances in energy homeostasis are associated with neurodegenerative disorders (Akundi et al., 2013). Over-activation of poly (ADP-ribose) polymerase 1 (PARP1) contribute towards dopaminergic degeneration in Parkinson's disease (PD), which is completely absent in $PARP1^{-/-}$ mice (Kim et al., 2013). PARP1 activation leads to depletion of cytosolic NAD^+. Replenishment of NAD^+ prevents PARP1-mediated neuronal death (Alano et al., 2010). Exogenous NAD^+, surprisingly, enters neurons through the dilated $P2X_7$ receptor-gated channels, marking a neuroprotective role for the otherwise proinflammatory $P2X_7$ receptors.

Among other neuroprotective roles, various *in vitro* models show that activation of $P2X_7$ receptors stimulates α-secretase activity leading to the shedding of non-amyloidogenic soluble amyloid precursor protein (APP; Darmellah et al., 2012). On the contrary, inhibition of $P2X_7$ receptors in a transgenic mouse for mutant human APP show a significant decrease in the number of amyloid plaques through increased activity of α-secretase (Diaz-Hernandez et al., 2012). Such opposing roles could be best explained with the discovery of a shorter, natural, splice variant of $P2X_7$ receptor that exhibits neurotrophic properties (Adinolfi et al., 2010). Though it remains to be investigated, it is probable that the truncated $P2X_7$ receptors induce α-secretase activity while the longer isoforms are inhibitory. The factors that mediate the retention or deletion of the C-terminal part of $P2X_7$ receptors are not yet known. Such contrasting roles of $P2X_7$ receptors have also been identified in other cellular systems such as cancer (Feng et al., 2006). The distribution of short and long isoforms of $P2X_7$ within the receptor heterotrimer most likely determines its overall trophic or toxic nature.

$P2X_4$ RECEPTORS

An interesting use of neuronal P2 receptors as "flags" for microglial recognition has been reported. In the mutant superoxide dismutase SOD1 mouse model of ALS, degenerating motor neurons typically express $P2X_4$ receptors for the recruitment and eventual engulfment by activated microglia (Casanovas et al., 2008). Unlike a typical cell undergoing apoptosis, $P2X_4$-positive neurons neither show chromatin condensation nor caspase 3 activity; rather exhibit loss of neuronal NeuN marker and recruitment of microglial cells. It is not just restricted to motor neurons but to other degenerating neurons affected with ALS—serotonergic neurons of raphe nucleus, noradrenergic neurons of locus coeruleus, and Purkinje cells in the cerebellum. In $A\beta_{1\text{-}42}$-treated neurons that do undergo caspase 3-mediated apoptosis, increased surface expression of $P2X_4$ receptors occurs due to the unique presence of a putative caspase 3 cleavage site within the C terminus region (Varma et al., 2009). Hence, overexpression of $P2X_4$ receptors enhances Aβ-induced neuronal death, while receptor inhibition subdues cell death. These reports form the basis for our hypothesis that surface expression of $P2X_4$ receptors may serve as markers for degenerating neurons, attracting microglial cells for eventual engulfment.

On the other hand microglial $P2X_4$ receptor expression is associated with increased neurophagic activity (Cavaliere et al., 2003). Knocking out $P2X_4$ receptors results in poorer microglial activation and loss of PGE_2-mediated inflammatory pathway (Ulmann et al., 2010). $P2X_4$ receptor forms a large conductance pore on the cell surface affecting ionic balance, thus mediating the release of proinflammatory substances. Constitutively, $P2X_4$ receptors are trafficked into late endosomes and remain resistant to lysosomal degradation (Robinson and Murrell-Lagnado, 2013). Such a mechanism prevents the "flagging" of healthy neurons or the "activation" of microglia under normal physiology.

OTHER P2X RECEPTORS

Slow neurodegeneration, following axotomy, shows an upregulation of $P2X_1$ and $P2X_2$ receptors, synchronous with upregulation of neuronal nitric oxide synthase (nNOS; Viscomi et al., 2004). P2X receptors further mediate translocation of nNOS to the plasma membrane (Ohnishi et al., 2009). In an animal model of PD, dopamine denervation upregulates $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_6$ receptors on nigral GABAergic neurons to compensate the loss of dopamine (Amadio et al., 2007). Coincidentally, within the substantia nigra, two out of five groups of GABAergic neurons, but none of the five groups of dopaminergic neurons, express nNOS (González-Hernández and Rodríguez, 2000). Whether P2X and nNOS are upregulated within the same cell during neurodegeneration is not known; however, coordinated activation of purinergic and nitrergic mediators seems a likely event during neuroinflammation.

P2Y RECEPTORS

The metabotropic P2Y receptors play a major role in neuron-glia communication. Neuronal injuries activate astrocytic $P2Y_1$ receptors leading to the release of PGE_2, causing reactive gliosis (Xia and Zhu, 2011), or glutamate, mediating synaptic modulation (Domercq et al., 2006). Blocking of $P2Y_1$ receptors therefore reduces glial activity (Davalos et al., 2005) and improves cognitive outcome following traumatic brain injury (Choo et al., 2013). In the AD brain, $P2Y_1$ receptors are localized in the neurofibrillary tangles and neuritic plaques (Moore et al., 2000). In contrast, there is a selective loss of $P2Y_2$ receptors correlating with worsening neuropathological scores (Lai et al., 2008).

This is not surprising since $P2Y_2$ receptors stimulate α-secretase activity (Camden et al., 2005). In addition, $P2Y_2$ receptors mediate microglial phagocytosis of fibrillar forms of Aβ in a mouse model of AD (Ajit et al., 2014). The soluble Aβ peptides are instead cleared through ATP-dependent $P2Y_4$ receptor-mediated pinocytosis (Li et al., 2013). In fact soluble $A\beta_{1\text{-}42}$ itself induces ATP release, auto-stimulating $P2Y_4$ receptors in microglia, thus mediating its own clearance. Degradation of extracellular amyloid peptides is also performed by metallopeptidases such as matrix metalloproteinase 9 (MMP-9), whose secretion is upregulated following inhibition of the tonically active $P2Y_{14}$ receptors (Kinoshita et al., 2013). These reports suggest that the loss of $P2Y_2$ and $P2Y_4$ receptors or an overactivation of $P2Y_1$ and $P2Y_{14}$ receptors alter the steady state levels of amyloid peptides leading to AD.

Both $P2Y_2$ and $P2Y_4$ receptors are preferentially expressed in perivascular astrocytes, and in response to exogenous ATP, mediate increased levels of cytosolic calcium within their endfeet processes (da Silva et al., 2009). As a result, P2Y receptors influence the permeability of blood-brain barrier through induction of endothelial nitric oxide synthase (eNOS). Activated glial cells also induce the expression of chemokines such as monocyte chemotactic protein 1 (MCP1) leading to the CNS recruitment of monocytes (Kim et al., 2011). Interestingly, MCP1 deficiency decreases microglial phagocytosis of Aβ oligomers, thus contributing to progressive amyloidosis (Kiyota et al., 2013).

UDP is the ligand of choice for $P2Y_6$ receptors. Activated $P2Y_6$ receptors trigger a change in microglial phenotype—from active motile/surveillance cells to active neuron devouring/phagocytic cells (Koizumi et al., 2007). The neurophagic activity of microglial cells is potentiated by TNF-α, LPS or Aβ peptides, and delayed through $P2Y_6$ receptor antagonists (Neher et al., 2014). Furthermore, activated $P2Y_6$ receptors block dilation of $P2X_4$ receptor-mediated channels, shifting microglial phenotype from inflammatory to phagocytic cells (Bernier et al., 2013). Blocking of $P2Y_6$ receptors increases neuronal survival suggesting that phagocytosis is not limited to degenerating neurons alone but non-specifically targets "stressed-but-otherwise-viable" neurons as well (Emmrich et al., 2013). Irrespective of the type of insult, the release of UDP signals microglia to initiate indiscriminate phagocytosis found in neurodegenerative disorders.

The migration of microglial cells to the site of injury is mediated by $P2Y_{12}$ receptors (Haynes et al., 2006). In mice lacking $P2Y_{12}$ receptors, microglia fail to polarize and migrate towards the lesion site while overactivation of $P2Y_{12}$ receptors enhances neuroinflammation. As a result $P2Y_{12}{}^{+/-}$ mice show lesser severity of neuronal injury following cerebral ischemia compared to $P2Y_{12}{}^{+/+}$ littermates (Webster et al., 2013). Interestingly, loss of the transcriptional factor interferon regulatory factor 8 (IRF8) suppresses microglial chemotaxis (Masuda et al., 2014). $Irf8^{-/-}$ microglia show reduced expression of $P2Y_{12}$, $P2X_4$ and adenosine A_3 receptors—all involved in microglial activation and migration to the site of injury. As a result, $Irf8^{-/-}$ mice are resistant to experimental autoimmune encephalitis (EAE)—a mouse model of multiple sclerosis (Yoshida et al., 2014).

ADP, formed by the activity of ectonucleotidases on extracellular ATP, is the preferred ligand for $P2Y_{13}$ receptors. Ubiquitination at its C-terminal end leads to proteasomal degradation and poor surface expression (Pons et al., 2014). However its surface expression increases in response to oxidative stress and genotoxins such as cisplatin or UV irradiation (Morente et al., 2014). In the red blood cells, ADP-activated $P2Y_{13}$ receptors show a negative feedback loop by inhibiting ATP release (Wang et al., 2005). Such a mechanism ensures additional regulation of extracellular ATP during neuronal injuries by restricting the lesion area such that undamaged and far away neurons which are exposed to ADP are not activated.

ADENOSINE RECEPTORS

Enhanced neuroinflammation and microglial activity is a feature of A_1 adenosine receptor knockout mice ($A_1AR^{-/-}$), suggesting that activation of A_1ARs is neuroprotective under pathological conditions (Luongo et al., 2014). On the other hand, A_{2A} receptors facilitate glutamate release, and their association with ectonucleotidase, CD73, implies A_{2A} receptors are activated under pathological conditions when excess extracellular ATP is found (Augusto et al., 2013). Hence A_{2A} receptor antagonists such as caffeine limit the pathology of neurodegenerative disorders such as AD and PD. Interestingly, in mouse models of senescence, A_1ARs significantly decrease with age while A_{2A} receptors increase with age (Castillo et al., 2009). A higher density of A_{2A} receptors in the putamen of PD patients also correlates with increasing motor symptoms (Varani et al., 2010). A_{2A} receptors induce microglial COX-2 expression (Fiebich et al., 1996) and inhibit astrocyte glutamate uptake by interacting with the α2 subunits of Na^+/K^+-ATPase (Matos et al., 2013). Though activation of A_1ARs and inhibition of A_{2A} receptors provide neuroprotection in the adult brain, the opposite is true for the embryonic brain. Chronic hypoxia mediates accelerated maturation of oligodendrocyte progenitor cells leading to hypomyelination and ventriculomegaly in mice (Akundi and Rivkees, 2009). Deletion of A_1ARs or early intervention with caffeine rescues embryos against hypoxia-mediated white matter injury (Back et al., 2006). Similarly, hypoxic ischemia-mediated brain damage was more profound in newborn $A_{2A}{}^{-/-}$ mice compared with their wildtype littermates (Adén et al., 2003). Similarly, it has been observed that A_{2A} receptor agonists show synergy with agonists of certain toll-like receptors, such as TLR2, 4, 7 and 9, in selectively upregulating the expression of vascular endothelial growth factor and downregulating the release of TNF-α (Pinhal-Enfield et al., 2003). Such a synergistic mechanism provides an angiogenic role for macrophages making it relevant in the aftermath of cerebral ischemia.

Adenosine A_3 receptors mediate microglial process extension (Ohsawa et al., 2012). Agonists of A_3 receptors thereby provide neuroprotection against ischemia (Choi et al., 2011). However, during chronic neuroinflammation microglia undergo process retraction through upregulation of adenosine A_{2A} receptors (Orr et al., 2009). A_{2A} receptor-dependent process retraction is also seen in the substantia nigra of mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) for 5 days (Gyoneva et al., 2014). A_{2A} receptor-mediated loss of process extension (due to A_3 receptors) and chemotaxis (due to $P2Y_{12}$ receptors) thereby dampens microglial response to injury. Microglial cells

also express α7 nicotinic acetylcholine receptor. Activation of these receptors attenuates neuroinflammation in a mouse model of MPTP (Liu et al., 2012). Coincidentally, nicotinic acetylcholine receptor stimulation mediates dopamine release in the rat striatum which is negatively regulated by agonists of adenosine A_{2A} receptors (Garção et al., 2013). These observations suggest that adenosine A_{2A} receptors regulate various neurotransmitter systems, and use of specific antagonists of A_{2A} receptors, therefore, show potential as therapeutic alternatives for PD (Threlfell et al., 2012).

TARGETING P2 RECEPTORS MAY BE AN ATTRACTIVE THERAPEUTIC APPROACH OVER COX2 INHIBITION

Neuroinflammation in AD was promisingly approached through the use of NSAIDs (Szekely and Zandi, 2010). Following failures in clinical trials, the AD Anti-inflammatory Prevention Trial (ADAPT) group found that neither celecoxib nor naproxen prevented AD in adults with a family history of dementia (Breitner et al., 2013). The drawback of using COX inhibitors lies in the ubiquitous presence and role of its product, PGE_2. PGE_2 plays an important role in gastro-intestinal (GI secretion, bowel motility), cardiovascular (regulates blood pressure), renal (hemodynamics in the glomeruli), and reproductive (embryo implantation, uterine contraction) systems; and within the CNS, regulates body temperature, sleep-wake cycle, memory consolidation and synaptic plasticity. The relative contribution of either COX isoforms in mediating the above functions is unclear. Selective COX-2 antagonists were designed to target the excess PGE_2 formed during neuroinflammatory episodes with the consideration that the more ubiquitous COX-1 would suffice for the production of physiological levels of PGE_2. However, despite better gastrointestinal safety ratio, selective COX-2 inhibitors showed increased risks of myocardial infarction, stroke, systemic and pulmonary hypertension, and sudden cardiac death (Jüni et al., 2004). The multiple deaths led to the eventual withdrawal of COX-2 inhibitors such as rofecoxib leading to sweeping lawsuits and wider criticism of the drug licensing procedures.

In this scenario targeting P2 receptors, which modulate PGE_2 synthesis, comes as a promising therapeutic possibility (**Figure 1**). The rat COX-2 promoter carries consensus sequences for transcription factors such as nuclear factor κB (NF-κB), NF-IL6, AP-1 and cAMP-responsive element (Tanabe and Tohnai, 2002). In addition, COX-2 can also be epigenetically regulated with hypermethylation responsible for its silencing in various types of cancer (Lodygin et al., 2005; Castells et al., 2006). Epigenetic contribution in the development of multiple sclerosis and neurodegenerative disorders is slowly being recognized, although evidences for such changes on P2 receptor genes is not yet known (Noh et al., 2012; Koch et al., 2013; Qureshi and Mehler, 2013). Furthermore, COX-2 is posttranscriptionally regulated as well. The human COX-2 mRNA contains at least 23 AU-rich elements (AREs) in the 3′-untranslated region (UTR) conferring to its instability (Shaw and Kamen, 1986). The interactions of ARE-binding protein with the 5′-methylguanosine cap-binding protein and polyadenosine tail-binding protein can either further stabilize the mRNA or lead to its degradation through recruitment of deadenylases (Dean et al., 2004). The p38 mitogen-activated protein kinase plays a critical role in the post-transcriptional regulation of several proinflammatory genes through controlling the phosphorylation status of these binding proteins (Clark et al., 2003). Other proteins that bind to COX-2 ARE and lead to its mRNA stabilization include the heat shock protein hsp70 (Kishor et al., 2013), and the RNA-binding protein HuR which inhibits the destabilization of COX-2 mRNA mediated by microRNA miR-16 (Young et al., 2012). Another regulator is the heterogeneous nuclear riboprotein A1 (hnRNP-A1) whose declining levels have been correlated with the severity of symptoms in various neurodegenerative diseases including AD (Bekenstein and Soreq, 2013). It was recently reported to regulate IL-6 transcription, with overexpression of hnRNP-A1 increasing IL-6 expression and knockdown leading to reduced IL-6 synthesis (Zheng et al., 2013). Post-transcriptional and post-translation regulation of various P2 receptors is not yet known, although the various alternate splicing mechanisms as shown in P2X7 (Adinolfi et al., 2010) and adenosine A1 receptor (Ren and Stiles, 1994) suggest their involvement. Transcription factors downstream of P2 receptor activation bind to most of the COX-2 promoter consensus sequences (Brautigam et al., 2005; Ferrari et al., 2006; Lenertz et al., 2011). $P2X_7$ receptor antagonists present themselves as appropriate therapeutic alternatives to specific COX-2 inhibitors based on several evidences implicating them in neuroinflammation. Currently a few P2 receptor antagonists have advanced to clinical trials (Arulkumaran et al., 2011; North and Jarvis, 2013). It sets the stage for the potential role of P2 receptor-based anti-inflammatory drugs (PBAIDs) in targeting neuroinflammation.

A considerable roadblock in the design of PBAIDs is inherent in the diversity of P2 receptors. The conflicting reports on the neuroprotective and pro-inflammatory roles of various P2 receptors stems from the limited understanding of the actual types of receptors expressed, which may differ between cell types, species, age, and physiological status (Crain et al., 2009; Serrano et al., 2012). Nerve injury indiscriminately releases nucleotides of all kinds, including functionally opposing ones. The predominant ligand concentration depends on the distribution and activity of ectonucleotidases. Most studies utilize pharmacological agents which exhibit receptor promiscuity and have been characterized on purified homomers. However, not only surface P2 receptor density changes under pathological conditions, there is also the possibility of formation of heteromultimers (such as $P2X_{2/6}$, or $P2X_{4/6}$, and even $P2X_{2/4/6}$) with altered ligand affinity and functions (Robinson and Murrell-Lagnado, 2013). The long and short splice variants of $P2X_7$ receptor have opposing roles on cell growth and death (Adinolfi et al., 2010). Finally, an ideal PBAID has to overcome potential possibilities of receptor compensation. This was particularly evident in the failure of neuroprotection in $P2X_7^{-/-}$ mice where higher numbers of functionally compensating $P2X_4$ receptors were found instead (Hracskó et al., 2011). In another example, $P2Y_{12}^{-/-}$ mice show delayed microglial response to injury; however, other signaling mechanisms did ensure that microglia reached the site of injury despite the delay (Haynes et al., 2006). The $P2X_2$-$P2X_5$ heterotrimer is functionally analogous to $P2X_7$ receptors, including pore dilation, membrane

blebbing and phosphatidylserine exposure (Compan et al., 2012). Such rich receptor diversity allows P2X receptors to functionally compensate the loss of other family members. Therefore, more studies are required to correctly identify the aggravating P2 receptor contributing to neuroinflammation. Because of the functional diversity of P2 receptors, PBAIDs should be carefully chosen to target the disease at the appropriate stage where benefit outweighs risk.

SUMMARY

Conventionally COX-2 has been a target in various inflammatory disorders. However, the failure of NSAIDs and selective enzyme inhibitors reveal the importance of COX-2 not only in various physiological activities but also in tissue repair following neuronal injury. The COX enzymes maintain a delicate balance of tissue scavenging and tissue repair during neuroinflammation. An imbalance could lead to excessive PGE_2 activity leading to increased tissue damage or chronic inflammation. All cells within the vertebrate system upon damage (*hit one*) release large amounts of ATP (*hit two*) into the extracellular space. The effect of released ATP depends on the nature of two downstream factors—(1) the type of receptors present on cells within the vicinity of the injury; and (2) the distribution and activity of hydrolyzing ectonucleotidases. In large quantities, ATP potentiates the inflammatory reaction while other nucleotides have various modulatory roles in shaping the outcome of inflammation (summarized in **Figure 2**). PBAIDs aim to reduce the effect of *second hit* by targeting P2 receptors responsible for

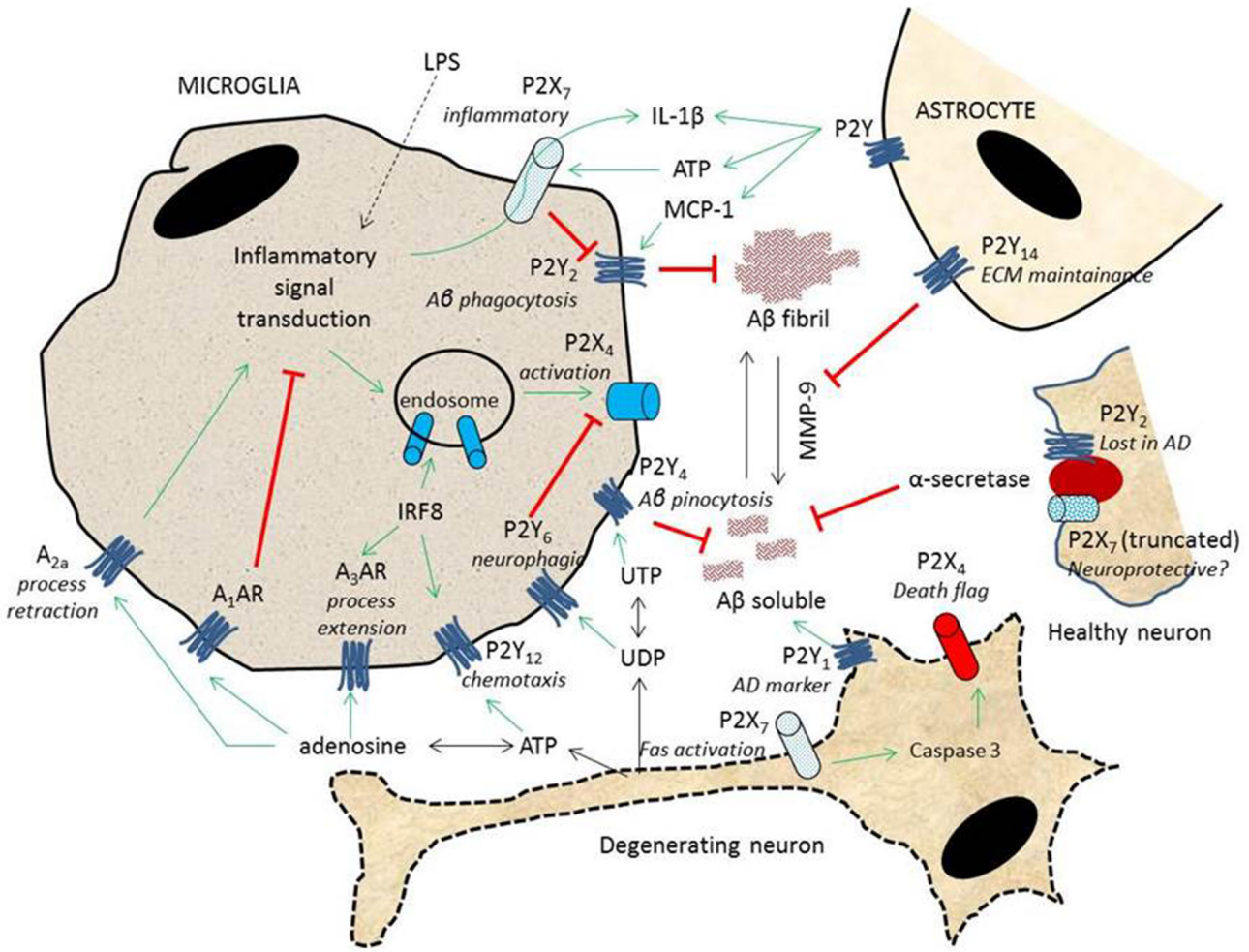

FIGURE 2 | P2 receptors modulate neuroinflammation. A simplified model based on the literature mentioned in this review summarizes the interactions between neurons and glial cells. Pro-inflammatory signals modulate $P2X_7$-mediated release of IL-1β and surface expression of $P2X_4$ receptors in the presence of ATP released by degenerating neurons and reactive astrocytes. On the surface of neurons, $P2X_7$ receptors mediate apoptosis with caspase 3-dependent expression of $P2X_4$ receptors as "flags" for microglial engulfment. Microglial migration to sites of insult is mediated by $P2Y_{12}$ and adenosine A_3 receptors and its neurophagic activity through $P2Y_6$ receptors. While A_1 adenosine receptors inhibit general inflammatory pathways, A_{2a} receptors activate COX-2 as well as retract microglial processes. In healthy neurons, truncated $P2X_7$ and $P2Y_2$ receptors enhance α-secretase activity, preventing the formation of amyloid deposits. Amyloid formation is also immediately cleared through microglial phagocytosis, mediated by $P2Y_2$ receptors; pinocytosis, through $P2Y_4$ receptors; and activity of MMP-9, inhibited by the tonic activity of $P2Y_{14}$ receptors.

inflammation-enhancement rather than the COX enzymes mediating PGE_2 synthesis. By not interfering with the COX system PBAIDs, unlike NSAIDs, retain the housekeeping functions of PGE_2, but vastly reduce the pathology through P2 receptor inhibition. Identifying the target P2 receptor, and designing a selective PBAID, remains a challenge for future therapeutic successes in neuroinflammation. Surface expression of P2 receptors under certain pathological conditions may depend on epigenetic stimuli. Silenced P2 receptors which were once active during neural development could be reprogrammed in the event of tissue injury. A global study of P2 receptor density and mutations that affect their binding to specific nucleotides, may identify newer insights into the susceptibility of neurodegenerative disorders to specific populations. Furthermore, it is essential to understand the activity of various ectonucleotidases since the steady-state levels of various nucleotides have contrasting outcome in neuroinflammation. Therapeutically increasing the activity of specific ectonucleotidases following excessive ATP release is another approach to counter neuroinflammation. Finally, the two-hit hypothesis can also be extended to various other inflammatory disorders such as arthritis, toxin exposures including nerve gas poisoning, in the inflammatory model of cancer, and in psychological stress and depression. More studies in these areas will provide new roles for PBAIDs as effective anti-inflammatory drugs.

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Mechanisms of NOS1AP action on NMDA receptor-nNOS signaling

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NMDA receptors (NMDAR) are glutamate-gated calcium channels that play pivotal roles in fundamental aspects of neuronal function. Dysregulated receptor function contributes to many disorders. Recruitment by NMDARs of calcium-dependent enzyme nNOS via PSD95 is seen as a key contributor to neuronal dysfunction. nNOS adaptor protein (NOS1AP), originally described as a competitor of PSD95:nNOS interaction, is regarded an inhibitor of NMDAR-driven nNOS function. In conditions of NMDAR hyperactivity such as excitotoxicity, one expects NOS1AP to be neuroprotective. Conditions of NMDAR hypoactivity, as thought to occur in schizophrenia, might be exacerbated by NOS1AP. Indeed GWAS have implicated NOS1AP and nNOS in schizophrenia. Several studies now indicate NOS1AP can mediate rather than inhibit NMDAR/nNOS-dependent responses, including excitotoxic signaling. Yet the concept of NOS1AP as an inhibitor of nNOS predominates in studies of human disease genetics. Here we review the experimental evidence to evaluate this apparent controversy, consider whether the known functions of NOS1AP might defend neurons against NMDAR dysregulation and highlight specific areas for future investigation to shed light on the functions of this adaptor protein.

Keywords: NOS1AP, nNOS, NMDA receptor, PSD95, PDZ, nitric oxide, excitotoxicity, schizophrenia

NOS1AP AND DISEASE

The *nos1ap* gene is linked to diseases including schizophrenia (Brzustowicz et al., 2004; Xu et al., 2005), post-traumatic stress disorder and depression (Lawford et al., 2013), autism (Delorme et al., 2010), sudden cardiac death and long QT syndromes (Newton-Cheh et al., 2009; Kapoor et al., 2014) and diabetes (Becker et al., 2008). The gene encodes NOS1 Adaptor Protein (NOS1AP), initially called carboxy-terminal PSD95-Dlg-ZO1 (PDZ) ligand of nNOS (CAPON; Jaffrey et al., 1998) because it binds the N-terminal PDZ-containing region of nNOS. The protein contains a C-terminal class II PDZ-motif (ψxψ-COOH) and an N-terminal phosphotyrosine binding (PTB) domain but no other recognizable domains. NOS1AP was originally described as an inhibitor of NMDA receptor (NMDAR)-driven nNOS actions because in cell-free assays it reduced interaction between nNOS and PSD95, the protein recruiting nNOS to NMDAR (Jaffrey et al., 1998). In contrast, later studies suggested NOS1AP *mediates* NMDAR-driven actions of nNOS (Fang et al., 2000; Cheah et al., 2006; Li et al., 2013). Nevertheless, in human disease studies, NOS1AP continues to be described as an inhibitor of nNOS (Eastwood, 2005; Xu et al., 2005; Qin et al., 2010; Weber et al., 2014). Despite this disparity of views, a consensus is emerging that nNOS:NOS1AP interaction is a potential drug target for neurological and cardiovascular disorders (Li et al., 2013; Kapoor et al., 2014; Weber et al., 2014). The rapidly accumulating reports linking NOS1AP to psychiatric and cardiovascular diseases increase focus on the druggability of NOS1AP functions. We therefore believe it is timely to discuss models for NOS1AP regulation of NMDAR-driven nNOS signaling.

NMDAR-DRIVEN nNOS SIGNALING AND THE INVOLVEMENT OF PSD95

To address the possible significance of NOS1AP regulation of NMDAR-driven nNOS functions, we briefly overview the relationship of nNOS to NMDAR signaling. NMDARs regulate neuronal development, survival and physiology but also contribute to neuronal dysfunction and disease, from stroke and neurodegenerative disorders to psychiatric disorders and chronic pain (Kemp and McKernan, 2002; Salter and Pitcher, 2012; Citrome, 2014). NMDAR signaling through nNOS contributes to excitotoxicity and thus lesions in both stroke and neurodegenerative diseases (Aarts et al., 2002; Lai et al., 2014), while atrophy caused by excitotoxicity may contribute to depression (Rajashekaran et al., 2013; Vu and Aizenstein, 2013; Stein et al., 2014). NMDAR signaling attracts interest as a potential therapeutic target because inhibitors of steps in the pathway from NMDAR to nNOS are effective in models of many disorders (Kemp and McKernan, 2002; Hashimoto, 2009; Doucet et al., 2012; Mellone and Gardoni, 2013; Lai et al., 2014; Mukherjee et al., 2014). However, side-effects of NMDAR antagonists have limited their clinical potential. After decades of disappointing results in clinical trials targeting the NMDAR and calcium influx, 2012 saw the first successful stroke

trail targeting the signaling pathway downstream from NMDAR activation and calcium influx (Hill et al., 2012).

The interaction between NMDARs and nNOS is well understood. NMDARs gate flux of calcium as well as sodium across the plasma membrane, and sustained activation of the receptor leads to substantially increased intracellular concentrations of both ions in neurons (Courtney and Nicholls, 1992). It is calcium/calmodulin that activates nNOS. nNOS has long been recognized as a major player in disorders from excitotoxic lesions to chronic pain (Florio et al., 2009; Mukherjee et al., 2014), but catalytic inhibitors have yet to benefit patients. Perhaps they would not be tolerated given the physiological importance of nNOS and other isoforms in the heart, vasculature and other sites. Importantly, calcium influx alone does not strongly activate nNOS; PSD95 is necessary to efficiently couple NMDAR-gated calcium influx to nNOS activation (Christopherson et al., 1999; Aarts et al., 2002; Ishii et al., 2006). The ternary complex assembling NMDAR, PSD95 (or related MAGUKs) and nNOS was characterized over 15 years ago (Christopherson et al., 1999; recently reviewed in Doucet et al., 2012) and has become particular interesting for development of therapeutic agents. Protein interactions have recently emerged as viable druggable targets, even in the most challenging conditions (Blazer and Neubig, 2009; Hill et al., 2012), and may provide alternative more selective approaches than inhibiting nNOS or NMDAR directly. Clearly, understanding interactions between proteins is essential for optimal development of novel drug leads that target protein-protein interactions, which could contribute to new treatments for clinically relevant conditions such as stroke, schizophrenia, chronic pain and cardiovascular diseases.

Key events downstream of NMDAR-evoked nNOS activation of relevance to neuronal disorders have remained rather nebulous. Substantial evidence supports a role for nNOS in excitotoxicity, but discrepancies exist. In hippocampal slices for example, μM of exogenous NO had no deleterious effects while excitotoxic stress was found to only generate 1000-fold lower concentrations of NO as measured by *in situ* assay (Keynes et al., 2004). NO generated by nNOS may reach high concentrations in the immediate vicinity of the active NMDAR/nNOS complex, but even a micrometer from the complex, diffusion and metabolism would considerably lower [NO] (Keynes et al., 2004; Philippides et al., 2005). The average cellular [NO] may therefore appear below the threshold for regulation of most NO targets. In such a scenario, interactions of nNOS with target proteins become critical determinants of downstream actions of the limited amounts of NO generated (Li et al., 2013).

SIGNALING PROTEINS DOWNSTREAM OF nNOS

Candidate downstream mediators of nNOS include p38MAPK and JNKs. Both are involved in disorders including neurodegeneration, cerebral ischemia and chronic pain (reviewed in Ji et al., 2009; Lai et al., 2014). Activation of p38MAPK in neurons is induced by NO, generated either from donors (Ghatan et al., 2000) or NMDAR-stimulation (Cao et al., 2005; Soriano et al., 2008; Li et al., 2013). JNK activation, not an obligate component of excitotoxicity (Cao et al., 2004; note isoforms may be regulated in opposite ways, Brecht et al., 2005), may involve distinct pathways (Soriano et al., 2008). NO can even inhibit JNK via S-nitrosylation (Park et al., 2000). NMDAR-driven nNOS activation may be specifically coupled to p38MAPK responses (Cao et al., 2005; Soriano et al., 2008) via NOS1AP (Li et al., 2013). NMDAR stimulation recruits NOS1AP to nNOS in neuronal cells and siRNAs targeting NOS1AP inhibits excitotoxic death and p38MAPK activation (Li et al., 2013). Moreover, a peptide designed to selectively disrupt the binding of nNOS to NOS1AP inhibits both p38MAPK and cell death/lesions in *ex vivo* and *in vivo* excitotoxicity models. Cell death was also prevented by overexpression of the nNOS-PDZ binding pocket without reducing calcium or nitric oxide responses. This suggests that competition for NOS1AP:nNOS interaction can be achieved by targeting either one or other side of the interaction i.e., providing either nNOS ligand peptide or the free nNOS ligand-binding domain, thereby inhibiting events downstream of PSD95:nNOS interaction. The observation that NOS1AP contributes to nNOS-dependent cell death is significant as it potentially expands the range of druggable targets in multiple disorders. However, this conclusion appears controversial as NOS1AP was originally described as a competitive inhibitor of nNOS function, and continues to be referred to as such, even though several other studies suggest that NOS1AP in fact mediates actions of NMDAR-driven nNOS (see Section NOS1AP and disease). To address this controversy, here we review how NOS1AP might contribute to NMDAR signaling pathways, taking into account the structural motifs critical for interactions between these three proteins. We discuss previously proposed models and develop for consideration an additional model of nNOS/PSD95/NOS1AP function that is more consistent with experimental findings.

REGULATION OF nNOS INTERACTIONS

THE EXCLUSION MODEL

The N-terminal region of nNOS that binds both PSD95 and NOS1AP contains a PDZ domain followed by additional sequences. PDZ domains are conserved 90 amino acid regions typically recognizing short C-terminal peptide motifs (Doyle et al., 1996). Some PDZ domains, like in nNOS, have flanking sequences or extensions which may confer additional properties, but these are not part of the core PDZ domain (Wang et al., 2010). The canonical PDZ interaction involves the docking of the last three residues at the extreme C-terminus of the peptide ligand into a "binding pocket" formed between β-sheet 2 and α-helix 2 of the PDZ domain (Doyle et al., 1996; Harris and Lim, 2001). The interaction between nNOS and PSD95 is unlike this canonical PDZ interaction. The initial finding that the N-terminal region of nNOS (containing a PDZ domain) interacts with a PDZ domain of PSD95 was taken to indicate that core PDZ domains could form a dimer, instead of merely binding C-terminal peptides. The only known interaction site at the time was the ligand-binding pocket, so PDZ dimerization was thought to exclude binding of C-terminal ligands (Jaffrey et al., 1998). We refer to this original concept as the *Exclusion Model* (**Figure 1A**). Consistent with this, recombinant NOS1AP C-terminus inhibits the interactions between nNOS and PSD95 interaction, both by GST-PSD95 pull-down of nNOS from 293T cell lysates and by

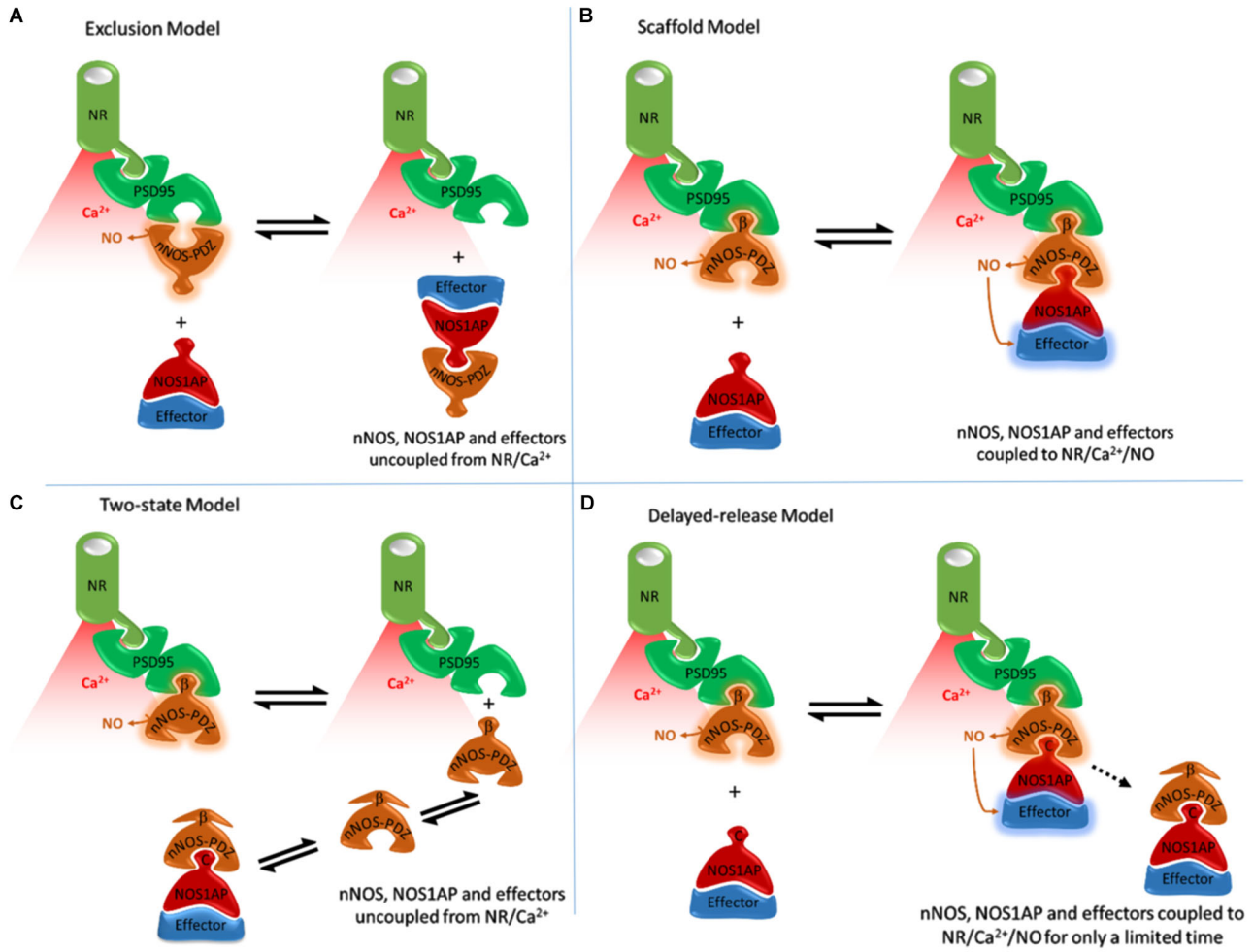

FIGURE 1 | Alternative models of NOS1AP interaction with nNOS and their anticipated consequences to exposure of NOS1AP effector to nitric oxide. **(A)** The Exclusion Model, based on Jaffrey et al. (1998) and Eastwood (2005). Binding of PSD95 to nNOS excludes binding of NOS1AP by PDZ-PDZ interaction and direct competition and vice versa. A PDZ-PDZ interaction was originally envisioned (Jaffrey et al., 1998), but this is consistent neither with structural nor functional data. Coupling of nNOS to NMDAR/Ca2+-influx is important for activation (Aarts et al., 2002; Ishii et al., 2006). Therefore in all schemes nNOS, when coupled to NMDAR (via PSD95) is shown producing NO (active), whereas nNOS displaced from NMDAR/Ca2+-influx (red shading) is depicted without NO production. In this model the nNOS/NOS1AP complexes with effectors such as DexRas would not be directly localized to the receptor and calcium influx-associated NO produced. **(B)** The Scaffold Model, based on Christopherson et al. (1999) and Li et al. (2013). Binding of nNOS β-finger to PSD95 facilitates an extended complex incorporating NOS1AP (or other ligands with C-terminal motifs). This model places nNOS close to the source of calcium influx, and NOS1AP effectors close to NO produced. This is consistent with NOS1AP mediating actions of NMDAR activated nNOS (Fang et al., 2000; Cheah et al., 2006; Li et al., 2013). But it is not consistent with cell-free experiments in which NOS1AP competes with PSD95 for binding nNOS (Jaffrey et al., 1998). **(C)** The Two-state model. The extended PDZ domain of nNOS is proposed to exist in two conformational states. One can bind PSD95 not NOS1AP, the other NOS1AP not PSD95. This could explain competition between PSD95 and NOS1AP. This model, however, places the nNOS-NOS1AP complex at a distance from the NMDA receptor, limiting activation of nNOS in the nNOS-NOS1AP complex. This is not consistent with NOS1AP mediating NMDAR/nNOS-dependent pathways (Fang et al., 2000; Cheah et al., 2006; Li et al., 2013). **(D)** The Delayed-release model. Here NOS1AP can interact with the unoccupied PDZ pocket seen in **Figure 2**, allowing the coupling of NMDAR/nNOS signaling to NOS1AP dependent pathways. But undefined mechanisms gradually lead to the loss of PSD95 binding by the beta-finger, presumably via conformational changes, resulting in delayed dissociation of the nNOS-NOS1AP complex from the receptor. In this model, the nNOS/NOS1AP effector complex is localized with the receptor and associated calcium influx for a limited time only. This model potentially explains the apparently conflicting data on NOS1AP function.

co-immunoprecipitation of PSD95 with nNOS from 293T cells overexpressing nNOS, PSD95 and NOS1AP (Jaffrey et al., 1998). Thus nNOS:PSD95 interaction was seen as a dimerization of core PDZ domains, and nNOS-PDZ ligands like NOS1AP would compete with PSD95 for interaction i.e., the dimerization occludes both ligand-binding pockets (**Figure 1A**).

THE SCAFFOLD MODEL

Subsequent structural studies clearly showed the core PDZ domain of nNOS is *not* itself the binding partner of PSD95-type PDZ domains (Hillier et al., 1999; Tochio et al., 1999, 2000; **Figure 2**), directly contradicting the exclusion model. The core PDZ domains that contain ligand-binding pockets do not

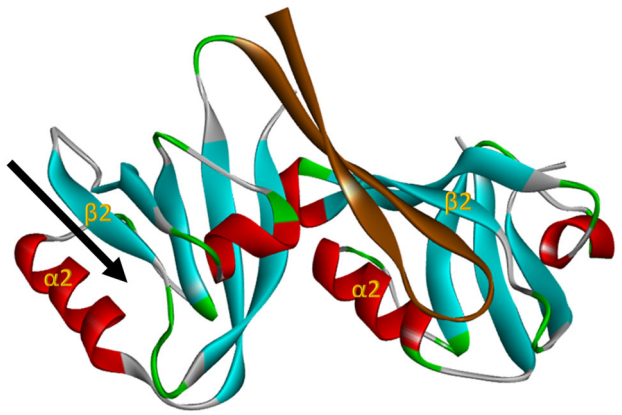

FIGURE 2 | The nNOS:PSD95 interaction. Projection of crystal structure 1QAV.pdb (Hillier et al., 1999) of the nNOS extended PDZ domain (left) docking with a classI PDZ domain (right, from syntrophin in this case). The parallel β-sheet 2/α-helix 2 regions that form the ligand binding pockets of the PDZ domains are labelled in each domain. The β finger of the nNOS extended PDZ that docks in the classI PDZ domain is shown in brown, revealing that the PDZ ligand binding pocket of nNOS (arrow, left) remains unoccupied. This projection was generated with Accelrys Discovery Studio.

interact (Christopherson et al., 1999). Instead, the flanking motif outside the core nNOS PDZ domain, known as the β-finger, mediates the interaction with PSD95-type PDZ domains. Thus nNOS:PSD95 is a heterodimer in which each protein contains one or more PDZ domains (**Figure 2**). Although not the PDZ dimer originally envisaged (**Figure 1A**), this dimeric interaction is still not a canonical PDZ domain:C-terminal peptide interaction and is referred to as a non-canonical PDZ interaction (Lenfant et al., 2010). Core PDZ domains in some cases form dimers by domain-swapping (Chen et al., 2008; reviewed in Lee and Zheng, 2010), but dimer formation does not occlude ligand binding pockets. The revised model (**Figure 1B**) allows the intriguing possibility that the ligand-binding pocket of this class III-type PDZ domain remains available for further recruitment of its own specific targets (Christopherson et al., 1999; **Figures 2**, **1B**, *Scaffold Model*).

NOS1AP may be one such target. In contrast to nNOS:PSD95 interaction, the core PDZ domain of nNOS (residues 1–100) *is* sufficient to interact with NOS1AP. Furthermore, C-terminal residues of NOS1AP are required for interaction (Jaffrey et al., 1998). On this basis, nNOS:NOS1AP binding is a canonical PDZ interaction. The scaffold model is consistent with this and with the original data describing the interaction between PSD95 and the N-terminal region of nNOS (Brenman et al., 1996). However it conflicts with the observed inhibition of nNOS:PSD95 interaction by NOS1AP C-terminus (Jaffrey et al., 1998) and potentially other ligands as envisaged in the exclusion model.

THE TWO-STATE MODEL

Although the behavior of NOS1AP in cell-free systems is inconsistent with the scaffold model, peptide ligands for nNOS-PDZ terminating with the motif G(D/E) × V did not inhibit nNOS:PSD95 interaction and YAGQWGESV peptide co-precipitated in a ternary complex with nNOS and PSD95-PDZ2 (Christopherson et al., 1999; Li et al., 2013). Notably, inhibition of PSD95:nNOS interaction by NOS1AP has been assumed to be competitive, but this was not demonstrated (Jaffrey et al., 1998). Inhibition could instead be non-competitive or allosteric. If the C-terminal peptide of NOS1AP would dock in the nNOS-PDZ binding pocket in a manner distinct from non-inhibitory peptides mentioned above, stabilizing an allosteric change of conformation that precludes PSD95 from binding the beta-finger of nNOS, this could explain the inhibition of PSD95:nNOS interaction by NOS1AP. We call this the *Two-State Model* (**Figure 1C**).

However, the Zhang lab specifically considered this in their NMR spectroscopy study but found no evidence to support it. They noted that NOS1AP peptide does *not* compete with PSD95 and concluded NOS1AP was "unlikely to compete with PSD-95 for nNOS" (Tochio et al., 1999). The two-state model predicts that events increasing nNOS-NOS1AP interaction should be accompanied by reduced nNOS-PSD95 interaction. Acquisition of a distinct conformation of nNOS driven by NMDAR activity might cause release from PSD95, and explain the increased nNOS:NOS1AP interaction and consequential downstream signaling reported (Li et al., 2013). However, PSD95-nNOS co-immunoprecipitation *increases* upon excitotoxic stimulation in neuronal cultures, hippocampal slices and ischemic brain (Zhou et al., 2010). Furthermore, the laboratory that discovered the inhibition of nNOS:PSD95 interaction by NOS1AP later proposed NOS1AP *mediates* NMDAR-driven nitrosylation and activation of NOS1AP ligand DexRas (Fang et al., 2000), which participates in NMDA-evoked activation of iron uptake (Cheah et al., 2006). The two-state model (**Figure 1C**) is not consistent with this, as nNOS/NOS1AP complexes do not interact with the NMDAR/PSD95 complex gating calcium influx (**Figure 1C**) and yet proximity of nNOS with NMDAR is considered important for nNOS activation and downstream functions (Cao et al., 2005; Ishii et al., 2006; Soriano et al., 2008; Li et al., 2013). Indeed, so important that inhibiting this interaction is a valid and successful strategy for neuroprotection from NMDAR/nNOS-dependent toxicity (Aarts et al., 2002; Hill et al., 2012).

THE DELAYED-RELEASE MODEL

Here we formulate an alternative model which proposes that competition with PSD95 observed in cell-free conditions (that utilize prolonged incubations) *does* occur in intact systems but only after a delay. The PDZ ligand-binding pocket of nNOS is clearly separate from the beta-finger ligand of nNOS that binds PSD95 (**Figure 2**), and docking of ligand in the pocket itself was reported *not* to affect the beta finger structure (Tochio et al., 2000). Thus any competition between NOS1AP and PSD95 necessitates secondary allosteric alteration of the nNOS:PSD95 interface. This may not necessarily take place instantaneously. Signaling downstream of nNOS, such as p38MAPK activation, shows transient activation in stimulated cells (Cao et al., 2005) and therefore possesses an obligate inhibitory or normalization phase. Perhaps NOS1AP, in the cellular context, acts as an inhibitor of nNOS:PSD95 interaction only after a delay, to shut down signaling once it has been activated. This delayed-release model, shown in **Figure 1D**, might explain the coupling of NOS1AP effectors to NMDAR-driven nNOS activation and the opportunities to inhibit

signaling by competition at the nNOS-PDZ domain. This may be the most attractive model as it can reconcile most if not all apparently conflicting experimental observations. Currently however, no experimental evidence directly supports such a sequence of events nor are there any known mechanisms to explain how this might occur.

NOS1AP—INHIBITOR OR MEDIATOR?

NOS1AP was regarded as an *inhibitor* of nNOS function (Jaffrey et al., 1998), particularly when discussing the possible relevance of NOS1AP to human disease (Xu et al., 2005; Qin et al., 2010; Weber et al., 2014). However, functional studies suggest NOS1AP is a *mediator* of nNOS signaling and contributor to NMDAR/nNOS-dependent regulation of neuronal functions (Fang et al., 2000; Cheah et al., 2006). Decreased expression of NOS1AP by RNAi or peptide competitors of nNOS:NOS1AP interaction inhibited NMDAR/nNOS-evoked events in neurons (Li et al., 2013). Notably, NOS1AP competition of nNOS:PSD95 interaction has been demonstrated entirely in cell-free experiments and no actual evidence of *functional* inhibition of nNOS has actually been reported (Jaffrey et al., 1998). Importantly, NOS1AP does *not* directly inhibit the enzyme activity of nNOS (Jaffrey et al., 1998). Inhibition of NMDAR-evoked nNOS activity has merely been inferred from its ability to inhibit PSD95:nNOS interaction in cell-free systems. In contrast, evidence for NOS1AP as a facilitator of nNOS-mediated NMDAR signaling to activation of DexRas, iron transport, p38MAPK and neurodegeneration derives from intact cells and animal models (Fang et al., 2000; Cheah et al., 2006; Li et al., 2013). Similarly, the effects of NOS1AP on neurite architecture are reduced by nNOS inhibitor L-NAME (Carrel et al., 2009). This supports the role of NOS1AP as a positive mediator of nNOS signaling. Apparent discrepancies may arise from differences between cell-free and more physiological systems used. Additional components or dynamic properties (like the proposed delayed release, **Figure 1D**) of intact neuronal environments absent from those cell-free binding experiments showing inhibition by NOS1AP of nNOS:PSD95 interaction are among the possible contributing factors. The evidence for NOS1AP mediating NMDAR/nNOS signaling in intact systems is therefore more convincing than evidence to the contrary.

FUTURE PERSPECTIVES FOR NOS1AP RESEARCH

The potential of nNOS:NOS1AP interaction as a candidate drug target for neurological and cardiovascular disorders (Li et al., 2013; Kapoor et al., 2014; Weber et al., 2014) highlights the need to address a number of issues. Is NOS1AP interaction with nNOS more complex than assumed (e.g., **Figure 1D**)? How is interaction between NOS1AP and nNOS regulated? Does NOS1AP have functions independent of nNOS, for which targeting nNOS:NOS1AP interaction may be irrelevant or even potentiating? Most challenging perhaps, do functions differ under conditions of health, in response to trauma or stress, during disease? Is it more desirable to promote or disrupt NOS1AP function? Ultimately does NOS1AP act as the inhibitor originally envisioned, playing a role in self-defence of the neuron against excessive input to nNOS signaling?

Pre-conditioning should also be considered. While p38MAPK mediates excitotoxicity, paradoxically it also facilitates the resistance to toxic insults preceded by prior sub-toxic stimuli, in brain and tissues such as heart and liver (Hausenloy and Yellon, 2006; Alchera et al., 2010; Zhao et al., 2013). As NOS1AP mediates NMDAR-evoked p38MAPK activation (Li et al., 2013), does it also contribute to pre-conditioning pathways? Should the same question be addressed for NMDAR:PSD95 and PSD95:nNOS interactions, which are considered as therapeutic targets, and for NR2B and extra-synaptic NMDARs which are much discussed as contributors to excitotoxic pathways (Hardingham and Bading, 2010) and may be preferentially linked to p38MAPK activation (Dau et al., 2014)?

Clearly more work is required to understand the impact of NOS1AP on NMDAR-driven nNOS signaling pathways, both at the molecular level as well as in a range of translational models for those diseases and conditions urgently needing new therapeutic approaches, including neurodegenerative diseases, stroke, chronic pain, as well as depression and other psychiatric conditions. Only then can we determine whether we should seek to boost endogenous functions of NOS1AP or to inhibit its function to achieve desirable therapeutic outcomes.

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Elevated mRNA-levels of distinct mitochondrial and plasma membrane Ca^{2+} transporters in individual hypoglossal motor neurons of endstage SOD1 transgenic mice

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Disturbances in Ca^{2+} homeostasis and mitochondrial dysfunction have emerged as major pathogenic features in familial and sporadic forms of Amyotrophic Lateral Sclerosis (ALS), a fatal degenerative motor neuron disease. However, the distinct molecular ALS-pathology remains unclear. Recently, an activity-dependent Ca^{2+} homeostasis deficit, selectively in highly vulnerable cholinergic motor neurons in the hypoglossal nucleus (hMNs) from a common ALS mouse model, the endstage superoxide dismutase $SOD1^{G93A}$ transgenic mouse, was described. This functional deficit was defined by a reduced hMN mitochondrial Ca^{2+} uptake capacity and elevated Ca^{2+} extrusion across the plasma membrane. To address the underlying molecular mechanisms, here we quantified mRNA-levels of respective potential mitochondrial and plasma membrane Ca^{2+} transporters in individual, choline-acetyltransferase (ChAT) positive hMNs from wildtype (WT) and endstage $SOD1^{G93A}$ mice, by combining UV laser microdissection with RT-qPCR techniques, and specific data normalization. As ChAT cDNA levels as well as cDNA and genomic DNA levels of the mitochondrially encoded NADH dehydrogenase ND1 were not different between hMNs from WT and endstage $SOD1^{G93A}$ mice, these genes were used to normalize hMN-specific mRNA-levels of plasma membrane and mitochondrial Ca^{2+} transporters, respectively. We detected about 2-fold higher levels of the mitochondrial Ca^{2+} transporters MCU/MICU1, Letm1, and UCP2 in remaining hMNs from endstage $SOD1^{G93A}$ mice. These higher expression-levels of mitochondrial Ca^{2+} transporters in individual hMNs were not associated with a respective increase in number of mitochondrial genomes, as evident from hMN specific ND1 DNA quantification. Normalized mRNA-levels for the plasma membrane Na^{+}/Ca^{2+} exchanger NCX1 were also about 2-fold higher in hMNs from $SOD1^{G93A}$ mice. Thus, pharmacological stimulation of Ca^{2+} transporters in highly vulnerable hMNs might offer a neuroprotective strategy for ALS.

Keywords: mCU, choline acetyltransferase ChAT, UCP2, Letm1, NCX1, mitochondrial DNA, ND1, GFAP

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is the most common motor neuron (MN) degenerative disease, with an adult onset and an annual incidence of 1–3 cases per 100,000 people worldwide (Ludolph et al., 2012; Valori et al., 2014). Typical histopathological hallmark of ALS is the loss of upper and lower motor neurons, which is accompanied by reactive gliosis (Valori et al., 2014). Although first described in 1869 (Charcot and Joffroy, 1869), the causes of ALS remain largely unknown and, as effective long-term treatment strategies are not available, most patients die 3–5 years after symptom-onset (Hardiman et al., 2011; Vucic et al., 2014). There is a family history in around 10% of ALS patients, that can be attributed to several gene defects (Renton et al., 2013). Most common is an abnormal hexanucleotide expansion of the chromosome 9 open reading frame 72 gene (C9ORF72), present in many familial as well as some sporadic ALS cases (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Haeusler et al., 2014). However, causes for neurodegeneration in most sporadic ALS patients still remain mostly unresolved (Vucic et al., 2014). Disease mechanisms of both, sporadic and familial ALS, share common pathogenetic features, in particular glutamate excitotoxicity, calcium (Ca^{2+}) overload, mitochondrial dysfunction, oxidative stress and protein dysfunction and/or aggregation, e.g., particularly of the 43-kDa trans-activating response region binding protein (TDP-43) or cytoplasmic Cu^{2+}/Zn^{2+}-superoxide dismutase 1 (SOD1) (Neumann et al., 2006; Ilieva et al., 2009; Ferraiuolo et al., 2011; Lee et al., 2012; Matus et al., 2013; Rotunno and Bosco, 2013; Muyderman and Chen, 2014; Tadic et al., 2014). There is also strong evidence for a crucial role of astroglia cells for MN degeneration in ALS (Valori et al., 2014). In both, ALS patients and its transgenic animal models, there is evidence of ubiquitinated protein inclusions in MNs as well as

in glial cells (Bruijn et al., 1997; Pasinelli et al., 2000; Mendonça et al., 2006).

Independent of the cause of ALS, one neuro-pathological hallmark of the disease is the differential vulnerability of MN populations to neurodegenerative triggers, e.g., MNs in spinal cord or hypoglossal nucleus are particularly vulnerable to ALS-trigger factors, while other MN populations, e.g., in particular in the oculomotor nucleus, remain relatively spared (Cleveland and Rothstein, 2001; Kanning et al., 2010; Kaplan et al., 2014). Vulnerable MNs display low endogenous Ca^{2+} buffering capacity due to a lack of cytosolic Ca^{2+} binding proteins (like $calbindin_{d28k}$ or parvalbumin) (von Lewinski and Keller, 2005), accompanied by expression of AMPA glutamate receptor subtypes that are highly permeable to Ca^{2+} (Van Den Bosch et al., 2000; Grosskreutz et al., 2010). Thus, these vulnerable MNs depend particularly on mitochondrial Ca^{2+} uptake to recover from transient Ca^{2+} increase during electrical activity (Grosskreutz et al., 2007; Jaiswal and Keller, 2009). However, mitochondria in highly vulnerable MNs show substantial functional and morphological changes in ALS animal models and human patients (Kawamata and Manfredi, 2010; Barrett et al., 2011; Martin, 2011; Cozzolino and Carrì, 2012; Vehviläinen et al., 2014). In particular, increased cytosolic Ca^{2+} transients and significantly reduced mitochondrial Ca^{2+} uptake have been described in ALS mouse models (Jaiswal and Keller, 2009; Coussee et al., 2011). The mitochondrial membrane potential, the driving force for mitochondrial Ca^{2+} uptake, has been described to be depolarized, and/or Ca^{2+} induced depolarization was increased (Carrì et al., 1997; Damiano et al., 2006; Jaiswal and Keller, 2009; Nguyen et al., 2009). By functional comparison of MNs from the hypoglossal (hMN) and from the oculomotor nucleus (oMN) in the most commonly utilized mouse model of ALS that express a human disease causing G93A SOD1 mutation ($SOD1^{G93A}$ mice), we recently identified a Ca^{2+} homeostasis deficit, selectively in highly vulnerable hMNs at disease endstage (Fuchs et al., 2013). More precisely, in response to elevated electrical activity, a reduced mitochondrial Ca^{2+} uptake, and an enhanced Ca^{2+} extrusion across the plasma membrane was observed in hMNs but not oMNs (Fuchs et al., 2013).

The main assumed uptake route of Ca^{2+} into mitochondria is the mitochondrial Ca^{2+} uniporter (mCU), driven by the mitochondrial membrane potential (Drago et al., 2011). It consists of a pore-forming subunit, named mitochondrial Ca^{2+} uniporter (MCU) and at least two regulatory subunits, mitochondrial Ca^{2+} uptake 1 (MICU1) and mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1) (Perocchi et al., 2010; Baughman et al., 2011; de Stefani et al., 2011; Mallilankaraman et al., 2012a; Marchi and Pinton, 2014). mCU has a relatively low Ca^{2+} sensitivity, and probably achieves mitochondrial Ca^{2+} import mainly at endoplasmic reticulum (ER) mitochondria microdomains, where Ca^{2+} concentrations are high enough (Drago et al., 2011). The mitochondrial uncoupling proteins UCP2 and UCP3 can also contribute to the mitochondrial Ca^{2+} uptake machinery. First reported to be a component of the uniporter itself (Trenker et al., 2007), they were later supposed to operate independently of other Ca^{2+} uptake pathways, particularly when Ca^{2+} is released from the ER (Waldeck-Weiermair et al., 2011). In addition, the high Ca^{2+} affine leucine zipper EF-hand containing transmembrane protein 1 (Letm1) is supposed to function as mitochondrial Ca^{2+}/H^{+} exchanger at the relatively low cytosolic concentration increases across the plasma membrane that follow Ca^{2+} depletion of the ER (store operated calcium entry, SOCE) (Jiang et al., 2009; Waldeck-Weiermair et al., 2011; Nowikovsky et al., 2012). The mitochondrial Na^{+}/Ca^{2+}exchanger (MNCX) achieves Ca^{2+} efflux from mitochondria under physiological conditions (Palty et al., 2010; de Marchi et al., 2014) but might revert its operation mode when mitochondria undergo pathological de- or hyperpolarization (Kim and Matsuoka, 2008; Chinopoulos and Adam-Vizi, 2010). Apart from mitochondrial Ca^{2+} uptake, the sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCA) are transporting Ca^{2+} into the ER, and thus provide additional Ca^{2+} clearance capacity (Hovnanian, 2007; Chaudhari et al., 2014; Hajnóczky et al., 2014). Furthermore, Ca^{2+} clearance via the plasma membrane is present and mediated by the plasma membrane Ca^{2+} ATPases (PMCA1-4) (Strehler, 2013) and the Na^{+}/Ca^{2+} exchangers (NCX1-3) (Sharma and O'Halloran, 2014). While NCX1-3 mediate extrusion of steep Ca^{2+} increases after cell stimulation via the plasma membrane, PMCA isoforms are regarded as "fine tuners" of cytosolic Ca^{2+} extrusion (Brini and Carafoli, 2011).

To molecularly define the described complex altered functional Ca^{2+} clearance phenotype in hMNs of endstage $SOD1^{G93A}$ mice (Fuchs et al., 2013), here we examined mRNA-levels of all described potential mitochondrial (MCU/MICU1/MCUR1, Letm1, UCP2/3, MNCX) and plasma-membrane (PMCA1-4, NCX1-3) Ca^{2+} transport proteins in choline-acetyltransferase (ChAT) positive hMNs of endstage $SOD1^{G93A}$ and wildtype (WT) mice, by combining UV laser microdissection (UV-LMD) with quantitative RT-PCR analysis. To assess if a possible cell-specific transcriptional Ca^{2+} transporter dysregulation is associated with an altered number of mitochondria/mitochondrial genomes in $SOD1^{G93A}$ mice (Keeney and Bennett, 2010), we quantified mitochondrial genomic DNA in individual hMNs via quantification of the mitochondrially encoded NADH dehydrogenase subunit 1 (ND1) gene (He et al., 2002; Bender et al., 2006; Krishnan et al., 2007). As cDNA levels for ChAT as well as cDNA and genomic DNA levels for ND1 were not altered in hMNs from $SOD1^{G93A}$ mice compared to WT, we utilized these genes for normalization of respective qPCR expression-data for plasma membrane and mitochondrial Ca^{2+} transporters. With this stratified analysis, we detected a selective transcriptional up-regulation of the mitochondrial MCU/MICU1 complex, similar as previously described (Fuchs et al., 2013), as well as of Letm1, UCP2, and the plasma membrane transporter NCX1. These findings point to an activity-dependent increased need of Ca^{2+} clearance capacity in hMNs of endstage $SOD1^{G93A}$ mice that is only partly met by an increased expression of mitochondrial Ca^{2+} transporters. Accordingly, Ca^{2+} extrusion via the plasma-membrane is elevated in hMNs of endstage $SOD1^{G93A}$ mice—not only functionally (Fuchs et al., 2013), but also at the molecular level, via elevated NCX1 expression. Thus, pharmacological stimulation of Ca^{2+} transporters might offer a novel neuroprotective strategy for highly vulnerable MNs in ALS.

MATERIALS AND METHODS

ETHICAL APPROVAL

All animal procedures were approved by Regierungspräsidium Tübingen, Germany (AZ 35/9185.81-3 TV No. 1090, and O-147), and conducted according to the guidelines of the German Tierschutzgesetz.

MICE

For all experiments, male transgenic mice of the strain B6SJL-TgN(SOD1-G93A) (Jackson Laboratory, Bar Harbor, US) and wildtype (WT) littermates of the same genetic background were used (Gurney et al., 1994). Mice were bred in Ulm in respective in-house breeding facility and genotyped according to the protocol recommended by Jackson Laboratory. For analyses, $SOD1^{G93A}$ mice between P115 and P145 were used after they were no longer able to pass a paw grip endurance test (clinical score 4, referred to as endstage) (Solomon et al., 2011). Data were derived from six individual $SOD1^{G93A}$ and six respective age-matched WT mice.

TISSUE PREPARATION, UV LASER MICRODISSECTION (UV-LMD) AND REVERSE TRANSCRIPTION (RT)

Carried out essentially as described (Fuchs et al., 2013; Schlaudraff et al., 2014). Briefly, $SOD1^{G93A}$ and WT mice were deeply anesthetized with isoflurane (Abbott, Wiesbaden, Germany) and decapitated. Coronal tissue blocks containing hypoglossal nuclei were separated. The blocks were mounted on a specimen disk and immediately frozen by insertion into the snap-freeze holder (−35°C) of a cryostat (Leica CM 1850). Twelve µm serial coronal brainstem sections (for exact location see **Figure 1A**) were cut using a microtome blade (type R35, Feather, Osaka, Japan), and mounted on 2 mm PEN-membrane slides (Microdissect, Herborn, Germany), fixed with an ascending ethanol series, stained with cresyl violet, dried and stored at −80°C. UV-LMD of individual hMNs was performed using a Leica LMD7000 setup. 10 pools of 15 hMNs each were laser microdissected from each endstage $SOD1^{G93A}$ and age-matched WT mouse. After cell-lysis and reverse transcription (RT) with random hexamer primers, cDNA was ethanol precipitated as described (Liss, 2002), resolved in 17 µl molecular biology grade water and stored at −20°C until PCR amplification. Note that hMNs of $SOD1^{G93A}$ mice were about 5% larger than hMNs of WT control mice, according to area-quantifications after UV-LMD.

QUALITATIVE AND QUANTITATIVE PCR

Qualitative and quantitative PCR was carried out, essentially as described (Gründemann et al., 2011; Dragicevic et al., 2014; Schlaudraff et al., 2014). Quantitative PCR (qPCR) was carried out using TaqMan assays and a GeneAmp 7900HTqPCR cycler (Applied Biosystems, Darmstadt, Germany). All qPCR assay details and assay-specific standard curve parameters are given in **Table 1**. Standard curves were generated using serial dilutions of cDNA (stock concentration: 750 ng/µl, RNA integrity number (RIN): 9.8; derived from brainstem mRNA of an age-matched C57Bl/6 mouse). Five µl of standard cDNA or of the purified hMN cDNA was used as a template for each qPCR in a final volume of 20 µl, using QuantiTect Probe PCR Master Mix (Qiagen, Hilden, Germany), 1 µl TaqMan assay, and the following cycling conditions: 60°C 2 min, 95°C 15 min, (94°C 15 s, 60°C 1 min) 50 cycles.

To test for absence of contaminations in harvested hMN pools, 5 µl of purified hMN cDNA was subjected to either multiplex-nested PCR for qualitative (essentially as described, Dragicevic et al., 2014) or to qPCR for quantitative analysis of marker gene expression: We chose choline-acetyltransferase (ChAT) as marker for motor neurons, L-glutamate decarboxylase ($GAD_{65/67}$) as marker for GABAergic cells, and glial fibrillary acidic protein (GFAP) as marker for astroglia cells. In addition, we used NADH dehydrogenase subunit 1 (ND1) as marker for mitochondrial genomic DNA copies. Only ChAT positive and $GAD_{65/67}$ negative pools were further processed for Ca^{2+} transporter mRNA quantification. Please note that we detected in pools (∼50–100%) as well as in individual hMN (100%) from $SOD1^{G93A}$ but not from WT mice (0 and 11% respectively) consistently robust GFAP signals (compare **Figure 2A** and **Table 2A**). As GFAP positive and GFAP negative hMN pools from $SOD1^{G93A}$ showed however no significant differences in MCU/MICU1 Ca^{2+} transporters expression levels, GFAP positive hMN pools were included into the analysis. **Figure 1** illustrates the UV-LMD and RT-PCR workflow.

DETERMINATION OF MITOCHONDRIAL GENOME COPY NUMBER

For quantification of mitochondrial genome copy number, single hMNs were laser microdissected and DNA isolation was performed with the QiaAmp DNA Micro Kit (Qiagen) according to the manufacturer protocol with the following adaptations: 0.1 µg/µl polyA carrier-RNA (included in kit) was added to each reaction, and all mixing steps were performed by pipetting with cell saver tips (Kisker, Steinfurt, Germany) to minimize sheering stress. DNA was eluted in 30 µl of molecular biology grade water and stored at 4°C until PCR amplification. For determination of mitochondrial DNA copy numbers, the mitochondrially coded ND1 gene was quantified, that is almost never affected by genomic deletion (He et al., 2002; Bender et al., 2006; Krishnan et al., 2007). Five µl of the eluted genomic DNA was used in a 20 µl reaction with QuantiTect Probe PCR Master Mix (Applied Biosystems), 1 µl ND1 TaqMan Primer Probe Mix, and 4 µl H_2O for qPCR amplification in duplicate reactions in a GeneAmp 7900HT using the following cycling conditions: 60°C 2 min, 95°C 15 min, (94°C 15 s, 60°C 1 min) 50 cycles. ND1 qPCR assay details, and assay-specific standard curve parameters are given in **Table 1**.

DATA ANALYSIS

Data analysis, graphical representations, correlation and linear regression analysis were performed with SDS2.3 software (Applied Biosystems) and GraphPad Prism 6 (GraphPad Software Inc., San Diego, US). The cDNA amount per neuron in relation to the utilized standard was calculated as described (Gründemann et al., 2011; Schlaudraff et al., 2014) according to:

$$cDNA\ amount\ per\ cell = \frac{S^{[(Ct - Y_{intercept})slope]}}{No_{cells} \bullet cDNA\ fraction}$$

With S = serial dilution factor of the standard curve (i.e., 10), No_{cells} = number of harvested neurons per UV-LMD sample (i.e., 15), *cDNA fraction* = fraction of the UV-LMD cDNA-reaction

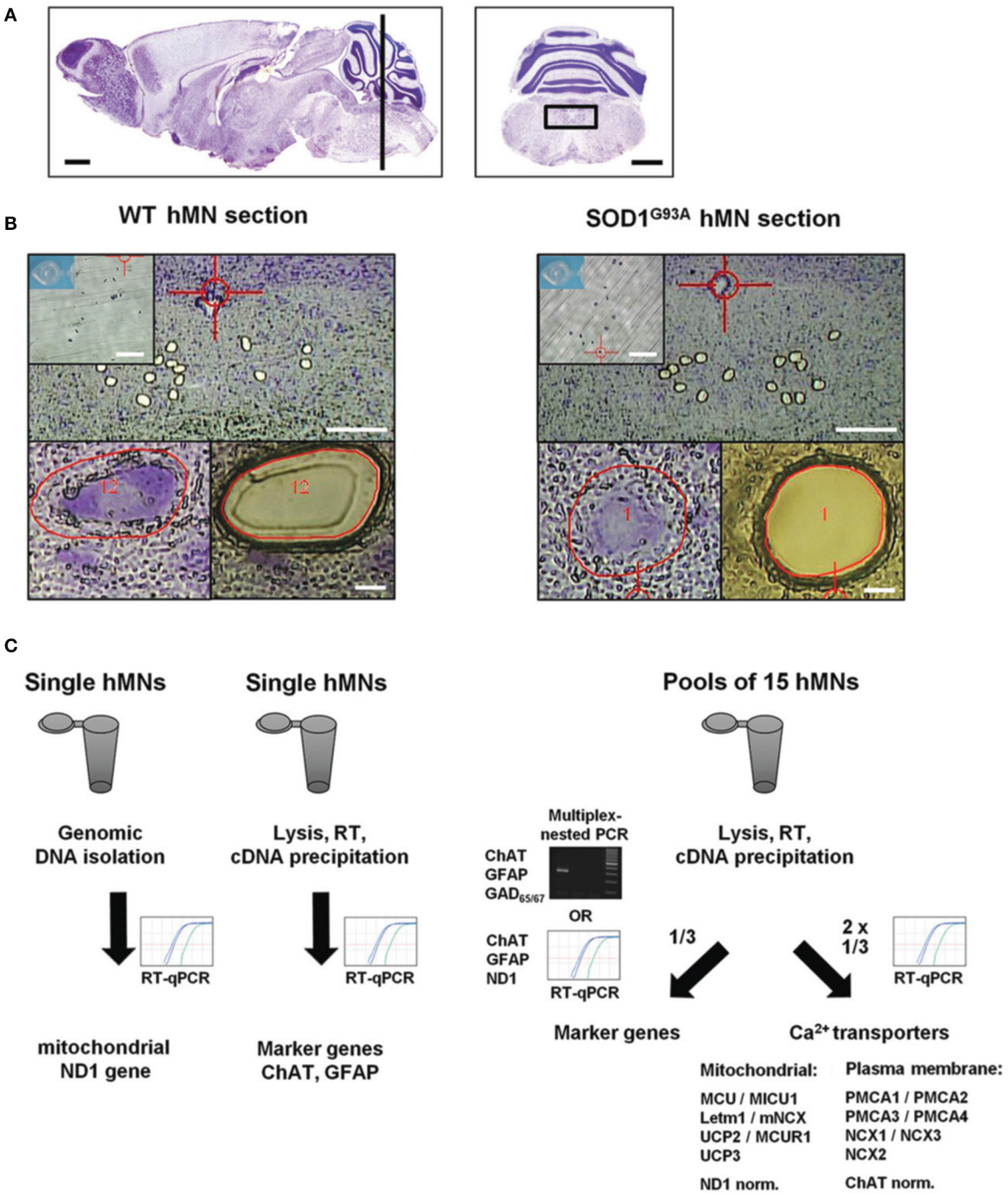

FIGURE 1 | Scheme of UV-LMD and RT-qPCR protocol for quantitative expression analysis of Ca^{2+} transporters in individual hypoglossal motor neurons from endstage $SOD1^{G93A}$ and WT mice.
(A) Nissl-stained sagittal (left) adult mouse brain section. The black bar indicates the zone (6–7 mm posterior to the Bregma) within the brainstem, where coronal sections (right) containing hypoglossal motor neurons (hMNs) (black box) were cut for UV-LMD. Scale bars: 1 mm. Pictures taken from Paxinos and Franklin (2001). **(B)** Upper: Overview of a WT (left) and endstage $SOD1^{G93A}$ mouse (right) coronal brainstem section after UV-LMD of 15 individual hMNs each. Scale bars: 250 μm. Inserts: photograph of the reaction tube cap for inspection of proper collection of all 15 neurons after UV-LMD, prior to cell lysis and reverse transcription (RT). Scale bars: 500 μm. Lower: individual hMNs before and after UV-LMD. Scale bars: 10 μm. **(C)** Workflow after UV-LMD. Left: genomic ND1 DNA-copy number of single hMNs was determined via qPCR with using genomic DNA as template, after genomic DNA isolation. ChAT and GFAP cDNA levels were determined for single hMNs after cell-lysis via individual RT-qPCR reactions with 50% of single cell cDNA each as templates. Right: For quantification of Ca^{2+} transporter expression-levels, cDNA derived from pools of 15 hMNs each were splitted, and 1/3 was used for marker gene expression profiling (either multiplex-nested PCR for ChAT, GFAP, and $GAD_{65/67}$, or alternatively individual qPCRs for ChAT, GFAP, and ND1), and 2/3 was used for RT-qPCRs for quantification of Ca^{2+} transporter mRNA levels. For details, please see methods.

Table 1 | Mouse TaqMan quantitative PCR assay information.

Assay ID	Target gene reporter-context sequence-quencher (primer sequences)	Genbank accession no. (NCBI)	Ampl. Length [bp]	Standard curve data					
				Exon spanning	Threshold	Y-Intercept	Slope	R^2	*n*
Mm01168774_m1	MCU (Ccdc109a) FAM-CACCAAAGAGAGACCTCCTAAGCCA-NFQ	NM_001033259.3	92	4–5	0.6	45.77 ± 0.13	−3.43 ± 0.02	0.99 ± 0.00	8
Mm01173692_m1	MICU1 (Cbara1) FAM-AGACAGAAAAGTGATGGAGTATGAG-NFQ	NM_144822.2	67	3–4	0.6	44.73 ± 0.16	−3.39 ± 0.05	0.99 ± 0.00	8
Mm01351581_m1	MCUR1 (Ccdc90a) FAM-AAAGCAACAAGTGATGGATGAAGTG-NFQ	NM_001081059.3	98	5–6	0.6	46.13 ± 0.18	−3.14 ± 0.00	0.99 ± 0.01	2
Mm00522265_m1	Letm1 FAM-GCCAGCTGAAACAGTGGCTGGACTT-NFQ	NM_019694.1	74	7–8	0.6	44.30 ± 0.44	−3.31 ± 0.11	1.00 ± 0.00	3
Mm01197102_m1	mNCX (Slc24a6)* FAM-TAGTCAAGTTGCCTGTGGAGTTCTT-NFQ	NM_133221.2, NM_001177594.1, NM_001177595.1	61	9–10/10–11	0.6	49.70 ± 0.71	−3.68 ± 0.17	0.98 ± 0.01	3
Mm01274107_g1	UCP2 FAM-GGTCCGGCTGCAGATCCAAGGGGAG-NFQ	NM_011671.4	82	3–4	0.6	44.72 ± 0.18	−3.33 ± 0.06	0.99 ± 0.00	4
Mm00494077_m1	UCP3 FAM-GTCTCACCTGTTTACTGACAACTTC-NFQ	NM_009464.3	69	5–6	0.6	48.62 ± 0.48	−3.33 ± 0.11	0.98 ± 0.01	5
Mm01245805_m1	PMCA1 (Atp2b1) FAM-GGGGACCTTACTCTGGGGCCAGCTT-NFQ	NM_026482.2	77	19–20	0.6	44.85 ± 0.32	−3.33 ± 0.09	0.99 ± 0.01	3
Mm00437640_m1	PMCA2 (Atp2b2)* FAM-ATAGGCAAGGCGGGCCTGGTGATGT-NFQ	NM_001036684.2, NM_009723.3	79	7–8	0.6	42.32 ± 0.19	−3.29 ± 0.04	0.99 ± 0.00	4
Mm00623641_m1	PMCA3 (Atp2b3) FAM-AGACAAGAAAGGCAAGCAGCAGGAT-NFQ	NM_177236.3	71	6–7	0.6	43.94 ± 0.31	−3.30 ± 0.08	1.00 ± 0.00	3
Mm01285597_m1	PMCA4 (Atp2b4)* FAM-TGAAAACCTCCCCTATAGAAGGTCT-NFQ	NM_213616.3, NM_001167949.1	86	1–2	0.6	46.86 ± 0.25	−3.23 ± 0.05	0.99 ± 0.01	3
Mm01232255_m1	NCX1 (Slc8a1)* FAM-ACTGTCAGCGCTGGGGAAGATGACG-NFQ	NM_001112798.1, NM_011406.2	65	7–8/9–10	0.6	45.60 ± 0.38	−3.38 ± 0.06	0.99 ± 0.00	3
Mm00455836_m1	NCX2 (Slc8a2) FAM-AGGTGTAGTCCAGGTGTGGGAGGCA-NFQ	NM_148946.2	100	2–3	0.6	46.75 ± 0.24	−3.26 ± 0.08	0.98 ± 0.02	3
Mm00475520_m1	NCX3 (Slc8a3)* FAM-CATCACTGTTAGTGCAGGAGGGGAT-NFQ	NM_080440.3, NM_001167920.1	71	5-6/6–7	0.6	46.14 ± 0.53	−3.28 ± 0.13	0.99 ± 0.00	3
Mm01221882_m1	ChAT FAM-TAGCTGTGAGGAGGTGCTGGACTTA-NFQ	NM_009891.2	67	3–4	0.8	46.13 ± 0.64	−3.04 ± 0.10	0.99 ± 0.00	3
Mm01253033_m1	GFAP* FAM-AGAAAACCGCATCACCATTCCTGTA-NFQ	NM_001131020.1, NM_010277.3	75	6–7	0.8	42.41 ± 0.10	−3.29 ± 0.04	0.99 ± 0.00	3
Mm04225274_s1	ND1 FAM-ACAACCATTTGCAGACGCCATAAAA-NFQ	NC_005089_ND1.0	81	–	1.0	35.60 ± 0.56	−3.34 ± 0.03	1.00 ± 0.00	3

Threshold, slope and y-intercept values were determined with using serial dilutions of cDNA derived from mouse brainstem tissue as qPCR templates (for details, please see methods). (*) Primers did detect but not discriminate between described splice variants.

sample used as template in the individual qPCR reactions (pools of 15 hMNs: 5/17 for Ca^{2+} transporters, and 1/9 for marker genes ChAT, GFAP, and ND1; single hMN: 1/2 for marker genes ChAT and GFAP, and 1/6 for genomic ND1). The $Y_{intercept}$ unit-magnitude corresponds to the respective standard utilized (i.e., pg equivalents of standard cDNA, derived from brainstem tissue mRNA). Single cell cDNA amounts were calculated with a $Y_{intercept}$ of 42 for all Ca^{2+} transporter genes, and with $Y_{intercept}$ from respective standard curves for ChAT, GFAP and ND1. Relative expression data are given as mean ± s.e.m., without and with normalization to ND1 and ChAT DNA/cDNA levels, respectively. Normalization was carried out by dividing respective Ca^{2+} transporter expression values to respective relative ChAT or ND1 expression values (relative to the WT mean values for ChAT and ND1), for each individual hMN pool. For statistical comparison Mann-Whitney-U-Tests were used. Significant differences are indicated by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

RESULTS

To analyze expression levels of mitochondrial as well as plasma membrane Ca^{2+} transporters in individual hMNs from WT and endstage $SOD1^{G93A}$ mice with best possible stratification, we further optimized our established single cell UV-LMD RT-qPCR protocol (Gründemann et al., 2011; Fuchs et al., 2013; Schlaudraff et al., 2014). **Figure 1** summarizes the general work flow.

For cell-specific normalization and stratification of mitochondrial Ca^{2+} transporter mRNA expression-levels, we utilized the NADH dehydrogenase ND1 gene, that is encoded by mitochondrial genomic DNA, and is almost never affected by genomic DNA-degradation (He et al., 2002; Bender et al., 2006). Quantifying ND1 DNA copies in parallel with mitochondrial Ca^{2+} transporter mRNAs in individual hMNs allows the normalization of mitochondrial Ca^{2+} transporter expression levels to the number of mitochondria/mitochondrial genomes in the respective analyzed hMN pools. To probe if the number of mitochondrial genomes is altered in individual hMNs from WT and $SOD1^{G93A}$ mice, we first quantified ND1 genomic DNA copy numbers after isolation of genomic DNA from individual hMNs. We detected no significant difference in ND1 genomic DNA levels in individual hMNs from WT and $SOD1^{G93A}$ mice (**Figure 2A** and **Table 2A**). To probe if this is also the case, when analyzing (intronless) genomic ND1 levels without a distinct genomic DNA isolation step by using our established UV-LMD RT-qPCR protocol (a prerequisite for using this gene and this approach for RT-qPCR data normalization), we quantified ND1

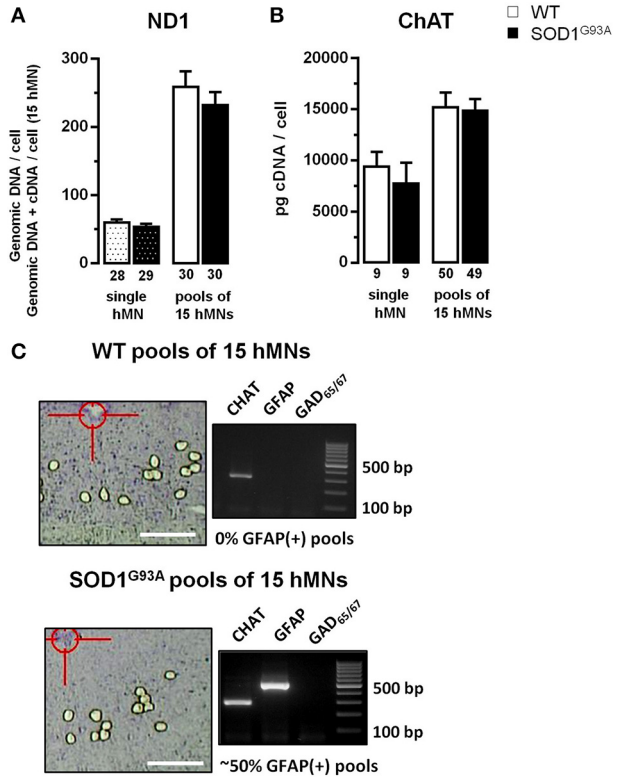

FIGURE 2 | Evaluation of ND1 and ChAT expression, used for normalization of cell-specific hypoglossal motor neurons qPCR data. **(A)** qPCR data for mitochondrially coded NADH dehydrogenase ND1, using genomic DNA from individual hMNs as templates, indicate similar numbers of mitochondrial genomic DNA molecules in hMNs from WT and from endstage SOD1^{G93A} mice. Note that combined mitochondrial genomic DNA and cDNA levels for ND1, determined via RT-qPCR of pools of 15 hMNs each, are also not different between WT and SOD1^{G93A} mice, a prerequisite for using ND1 DNA-levels for normalization of respective mitochondrial Ca^{2+} transporter RT-qPCR results. **(B)** RT-qPCR data for choline-acetyltransferase (ChAT), derived either from single hMNs or from pools of 15 hMNs each, are similar in WT and SOD1^{G93A} mice, a prerequisite for using ChAT cDNA-levels for normalization of respective plasma membrane Ca^{2+} transporter RT-qPCR results. **(C)** WT and SOD1^{G93A} coronal brainstem section after UV-LMD of 15 individual hMNs and agarose gel products of multiplex-nested RT-PCR of the marker genes ChAT, GFAP (glial fibrillary acidic protein), and $GAD_{65/67}$(L-glutamate decarboxylase). Note that most WT pools (or individual hMNs) were GFAP-negative while GFAP was robustly detected in ∼50% of SOD1^{G93A} pools (or via qPCR in 100% of individual hMNs or pools from SOD1^{G93A}, compare **Table 2A**). Scale bars: 250 μm.

DNA levels (genomic DNA + cDNA) in respective pools of 15 hMNs from WT and SOD1^{G93A} mice. Again, we detected no difference between hMNs from WT and SOD1^{G93A} mice, but as expected, about 4-fold higher ND1 DNA levels (genomic DNA + cDNA) per cell (WT: 4.28; SOD1^{G93A}: 4.30) (**Figure 2A** and **Table 2A**).

For normalization and stratification of plasma membrane Ca^{2+} transporter expression levels, we utilized the cytoplasmic key enzyme for acetylcholine synthesis, the choline-acetyltransferase (ChAT). Again, as a prerequisite for using ChAT for cell-specific normalization of hMN expression data, we determined ChAT mRNA-levels of individual hMNs as well as of pools of hMNs in WT and endstage SOD1^{G93A} mice. Similar, as for ND1, we detected no difference in ChAT mRNA-levels in single or pooled individual hMNs from WT and SOD1^{G93A} mice (**Figure 2B** and **Table 2A**). However, as expected, we detected a strong correlation of ChAT mRNA-levels with individual hMN cell sizes (WT: $R^2 = 0.45$; $p < 0.001$; $n = 50$; SOD1^{G93A}: $R^2 = 0.48$; $p < 0.001$; $n = 49$), further indicating the suitability of ChAT for normalization of plasma membrane Ca^{2+} transporter expression data from individual hMNs.

To ensure homogeneity of laser microdissected hMN pools, we analyzed for all hMN cDNA pools from WT and endstage SOD1^{G93A} mice ($n = 32$ and 55, respectively) a respective marker-gene expression profile. We utilized either qualitative RT-multiplex nested PCR for ChAT, the astroglia-marker GFAP (glial fibrilaric acidic protein) and the GABAergic neuron markers GAD_{65} and GAD_{67}(L-glutamate-decarboxylase) (**Figures 1C**, **2C**), or qPCR for ChAT and GFAP (**Figure 1C** and **Table 2A**). Only ChAT positive and $GAD_{65/67}$ negative hMN pools were further analyzed. All ChAT positive hMN pools from WT or SOD1^{G93A} mice were negative for $GAD_{65/67}$. However, as illustrated in **Figure 2C**, while WT pools showed no signal for GFAP, we obtained positive GFAP RT-PCR signals in about 50% of multiplex-nested PCRs of hMN cDNA pools from SOD1^{G93A} mice. To further address this unexpected finding, we quantified GFAP expression levels in pools of 15 hMNs as well as in individual hMN of WT and SOD1^{G93A} mice after UV-LMD. Again, we almost never detected any robust signal for GFAP in individual WT hMNs ($n = 1$ of 9 neurons), but in all 9 tested individual hMNs from SOD1^{G93A} mice, and in 100% ($n = 50$) of hMN pools (GFAP qPCR expression levels are given in **Table 2A**). Given these results, it is very unlikely that GFAP positive hMNs, selectively in SOD1^{G93A} mice are caused artificially due to technical issues. We next compared MCU and MICU1 mRNA-levels between GFAP positive and GFAP negative hMN pools of SOD1^{G93A} mice, and detected no significant difference (SOD1^{G93A}, relative expression; MCU: GFAP pos. 1.71 ± 0.19, $n = 16$; GFAP neg. 1.65 ± 0.16, $n = 23$; $p = 0.96$; MICU1: GFAP pos. 1.98 ± 0.22, $n = 16$; GFAP neg. 1.78 ± 0.15, $n = 23$; $p = 0.51$). Thus, we did not exclude GFAP positive hMN pools from further PCR analysis of Ca^{2+} transporter expression.

Next, via RT-qPCR, we analyzed qualitative mRNA expression of the main described potential mitochondrial and plasma membrane Ca^{2+} transporters (**Figure 3A**) in pools of 15 hMNs from WT and SOD1^{G93A} mice. As illustrated in **Figure 3B**, mRNAs for all tested mitochondrial Ca^{2+} transporters (MCU/MICU1/MCUR1, Letm1, UCP2, MNCX) but UCP3, and for all plasma membrane Ca^{2+} transporters (PMCA1-4, NCX1, NCX3) but NCX2, were detected in ChAT and ND1 positive hMNs ($n = 3$ pools of 15 neurons each) from WT and SOD1^{G93A} mice. Accordingly, we quantified mRNA-levels for all these mitochondrial and plasma membrane Ca^{2+} transporters via qPCR in hMNs from WT and endstage SOD1^{G93A} mice. Results are given in **Figure 4** and **Table 2B**. Without cell-specific normalization, we detected significantly higher mRNA levels of

only MICU1 and UCP2 in hMNs from SOD1^{G93A} mice (each about 1.7-fold higher compared to WT; **Figure 4A** left and **Table 2B**). All other Ca^{2+} transporter mRNA-levels were not significantly altered (although trends were observed in particular for MCU and Letm1, compare **Figure 4A** and **Table 2B**). Accordingly, cell-specific normalization of hMN qPCR data for mitochondrial Ca^{2+} transporters to mitochondrially coded ND1 DNA-levels (and thus to respective hMN mitochondrial genome numbers) revealed significantly higher mRNA levels of not only MICU1 and UCP2, but also of MCU and Letm1 in hMNs from SOD1^{G93A} mice (each about 1.8-fold higher compared to WT), while levels of MCUR1 and MNCX were not altered (**Figure 4B** left and **Table 2B**). In addition, by cell-specific normalization of hMN qPCR data for all analyzed plasma membrane Ca^{2+} transporters to relative ChAT levels (and thus to respective hMN cell-sizes), we detected about 1.7-fold higher mRNA-levels, selectively of NCX1 in hMNs from SOD1^{G93A} mice compared to WT (**Figure 4B** right and **Table 2B**). All other tested plasma membrane Ca^{2+} transporters (PMCA1-4 and NCX3) were not altered in hMNs from SOD1^{G93A} mice compared to those of WT.

In summary (**Figure 4C**), we detected at the mRNA-level a concerted up-regulation of three distinct putative mitochondrial Ca^{2+} transporters, MCU/MICU1, Letm1 and UCP2, in individual hMNs from endstage SOD1^{G93A} mice compared to WT, likely causing altered Ca^{2+} uptake of diseased mitochondria in hMN from SOD1^{G93A} mice. The elevated expression of the plasma membrane Ca^{2+} transporter NCX1 provides a molecular explanation for the described functionally enhanced activity dependent intracellular Ca^{2+} extrusion via the plasma membrane in hMNs from SOD1^{G93A} mice (Fuchs et al., 2013).

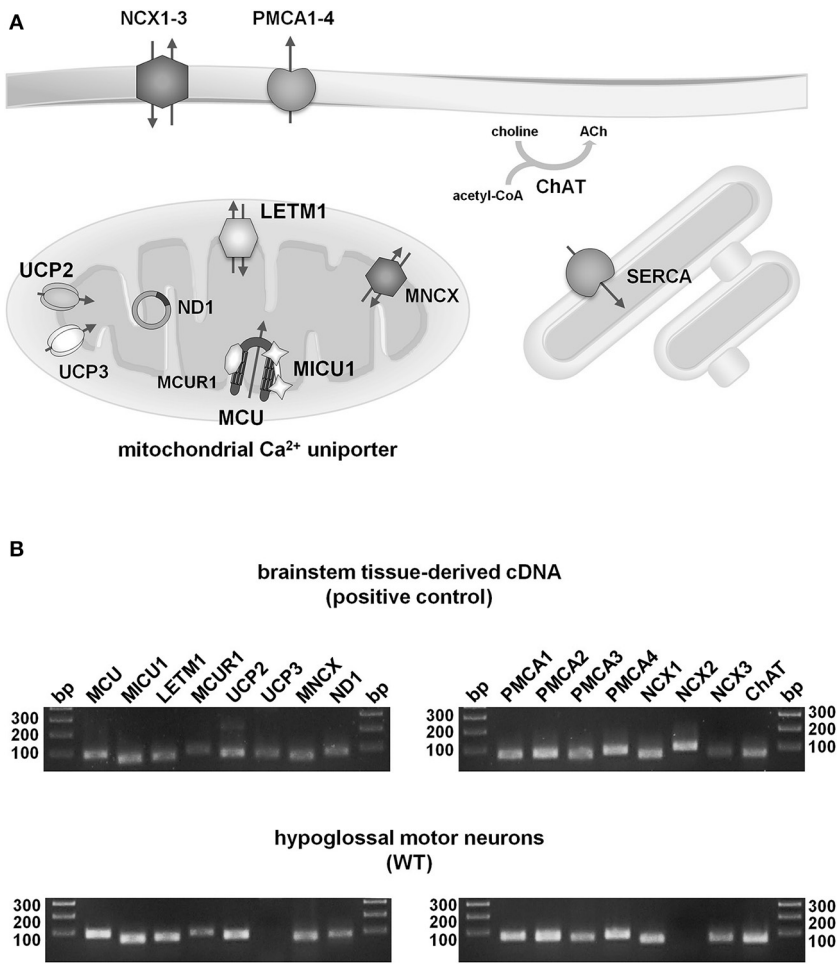

FIGURE 3 | Qualitative expression profiling of putative mitochondrial and plasma membrane Ca^{2+} transporters in individual hypoglossal motor neurons from WT and endstage SOD1^{G93A} mice. (A) Illustration of proteins for main putative mitochondrial (MCU/MICU1/MICUR1, Letm1, UCP2/3, MNCX), plasma membrane (PMCA1-4, NCX1-3) and ER (SERCA) Ca^{2+} transporters in cholinergic (ChAT) hMNs. For details and abbreviations, please see text. **(B)** Upper: Agarose gel (2%) electrophoresis of RT-qPCR products indicates that all putative mitochondrial and plasma membrane Ca^{2+} transporters, as well as ChAT and ND1, are expressed in cDNA, derived from mouse brainstem tissue (positive control). Lower: Agarose gel electrophoresis of RT-qPCR products shows that all putative mitochondrial and plasma membrane Ca^{2+} transporters, but UCP3 and NCX2, are expressed in ChAT and ND1 positive hMNs from WT mice (as well as from SOD1^{G93A} mice, not shown) (bp: base pairs, DNA ladder).

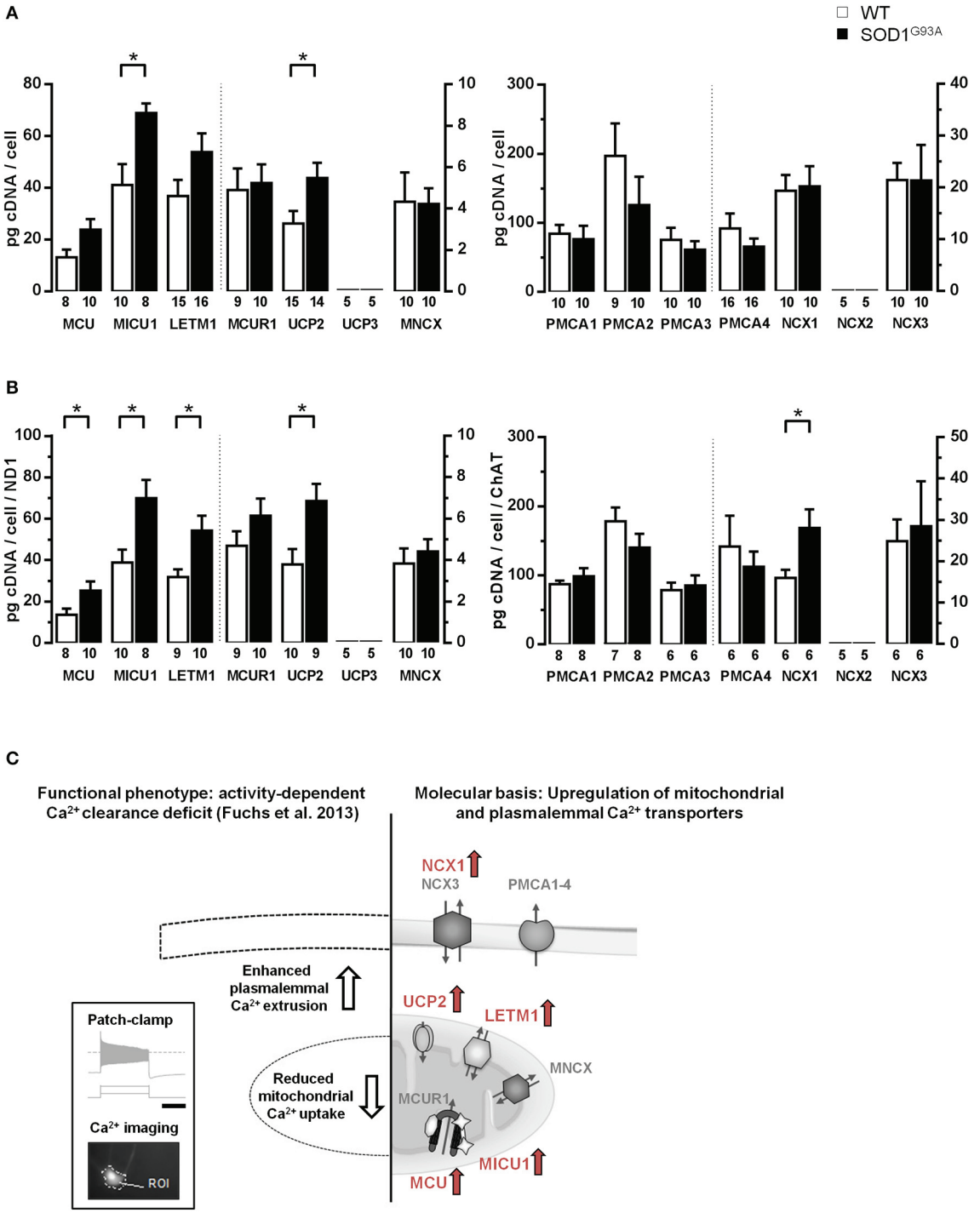

FIGURE 4 | Elevated mRNA-levels of distinct Ca^{2+} transporters in individual hypoglossal motor neurons from $SOD1^{G93A}$ mice compared to WT. (A) Cell-specific RT-qPCR data for mitochondrial and plasma membrane Ca^{2+} transporters, derived from pools of 15 hMNs each from $SOD1^{G93A}$ mice and WT. Data are given as [pg/cell] in respect to a cDNA standard curve, generated from WT mouse brainstem tissue. **(B)** Data from **(A)**, normalized to mitochondrially coded ND1 DNA-levels for mitochondrial Ca^{2+} transporters, and to ChAT cDNA levels for plasma membrane Ca^{2+} transporters. Significant differences according to Mann-Whitney-U-Tests are marked with (*), as defined in methods section. Note that data in **(A,B)** for the respective genes refer to two different axes, as indicated by the dashed line. **(C)** Right: Overview of concerted elevated Ca^{2+} transporter expression in individual hMN from $SOD1^{G93A}$ mice compared to WT (marked in red: MCU/MICU1, Letm1, UCP2, and NCX1). Left: These findings can provide a molecular basis for the recently described, selective functional, activity-dependent Ca^{2+} homeostasis deficit in hMN from endstage $SOD1^{G93A}$ mice. Insert shows stimulation protocol and resulting electrophysiological recordings (scale bar: 1 s), combined with calcium imaging (for details please see text and Fuchs et al., 2013).

DISCUSSION

Here we provide a cell-specific quantitative analysis of mitochondrial and plasma membrane Ca^{2+} transporter mRNA expression in highly vulnerable cholinergic hypoglossal motor neurons from $SOD1^{G93A}$ transgenic mice compared to WT. We identified a selective up-regulation of the mitochondrial Ca^{2+} transporters MCU/MICU1, Letm1 and UCP2 as well as of the plasma membrane Na^{+}/Ca^{2+} exchanger NCX1 in remaining hMNs in endstage $SOD1^{G93A}$ mice, while cDNA and genomic DNA levels for the mitochondrially coded ND1 gene, as well as cDNA levels for ChAT, the key enzyme for acetylcholine-synthesis, were not altered.

$SOD1^{G93A}$ transgenic mice are still the most widely used and most extensively characterized mouse-model for ALS (Vinsant et al., 2013a,b). These mice express a high copy number of the human G93A mutant SOD1, and recapitulate many key features of the human ALS phenotype, like adult disease onset, selective motor neuron degeneration and axonal loss (Gurney et al., 1994; Chiu et al., 1995; Fuchs et al., 2010). The limited clinical efficacy of compounds tested on SOD1 mice (Vucic et al., 2014), as well as the notion that SOD1 mutations account only for a small number (∼2%) of sporadic ALS cases (Renton et al., 2013) prompted the generation of new rodent ALS-models, like mice with mutated or overexpressed TDP-43 or fused in sarcoma (FUS) (Da Cruz and Cleveland, 2011; Van Den Bosch, 2011; Wegorzewska and Baloh, 2011; McGoldrick et al., 2013). However, given our previous functional analysis of activity dependent Ca^{2+} homeostasis in hMNs from $SOD1^{G93A}$ mice (Fuchs et al., 2013) and the fact that none of the recently generated models was shown to recapitulate most aspects of human ALS as convincing as the SOD1 mutant mouse, we continued to focus on the analysis of $SOD1^{G93A}$ mice in this complementary study.

Although mitochondrial dysfunction is present in MNs in human ALS as well as in $SOD1^{G93A}$ mice (Barrett et al., 2011; Martin, 2011), and macroscopic and functional alterations of mitochondria have been characterized extensively (Kawamata and Manfredi, 2010; Cozzolino and Carrì, 2012; Vehviläinen et al., 2014), the number of mitochondria/mitochondrial genomes in highly vulnerable MNs to our knowledge has not yet been addressed. Our cell-specific ND1 data (**Figure 2A**) argue against significant changes in number of mitochondria or mitochondrial genomes (at least in the cell soma) in hMNs from endstage $SOD1^{G93A}$ mice, compared to WT. In accordance with our findings, ND2 genomic DNA levels in human *postmortem* spinal cord motor neurons of ALS patients were also not altered compared to controls, whereas mitochondrial genomic DNA-levels for mitochondrially coded cytochrome oxidase and ND4 were significantly lower in ALS (Keeney and Bennett, 2010)—probably due to mitochondrial DNA deletions that occur preferentially in the ND4 gene (He et al., 2002). These deletions or mutations are likely to occur more frequently in ALS, as DNA repair enzymes in mitochondria have been shown to be impaired (Murakami et al., 2007).

In addition, our cell-specific data identified mitochondrially coded ND1 as a well-suited gene for normalization and further stratification of RT-qPCR data from hMNs. This is particularly useful when analyzing cell-specific expression-levels of targets with gene-products located in mitochondria (like MCU/MICU1/MICUR1, Letm1, UCP2, and MNCX). Furthermore, we identified ChAT as a well-suited gene for normalization of RT-qPCR data for target-genes expressed in the cytoplasm or in the plasma membranes of hMNs (like PMCA1-4 and NCX1/3). Our data show, that this cell- and transporter-class-specific normalization approach allows stratification of single cell hMN expression data, and thus reduces the number of neurons or neuronal pools that need to be analyzed for detection of significant differences (compare **Figures 4A,B**, **Table 2B** and Fuchs et al., 2013); MCU here: $n = 8$ and 8; in Fuchs et al. without normalization $n = 21$ and 39 for WT and $SOD1^{G93A}$ hMN pools, respectively).

GFAP CO-EXPRESSION IN ChAT-POSITIVE hMNs SELECTIVELY FROM $SOD1^{G93A}$ BUT NOT FROM WT MICE?

Surprisingly, with multiplex-nested PCR, as well as with more sensitive qPCR, we detected GFAP co-expression in about 50 or 100% respectively, of analyzed ChAT positive hMN cDNA pools from $SOD1^{G93A}$ mice but not in WT (**Figure 2B** and **Table 2A**). How to interpret these findings? As we can exclude general technical issues (i.e., contaminations), we provide two explanations for the detected GFAP signals selectively in hMNs from $SOD1^{G93A}$ but not WT mice. Either $SOD1^{G93A}$ hMNs might de-differentiate or reprogram their neuronal phenotype due to the disease process, as described for other cell types (Sarthy et al., 1991; Hol et al., 2003; Arendt, 2008; Puri and Hebrok, 2012; Qiang et al., 2013; Gao et al., 2014), and due to this process co-express GFAP. Or alternatively, the well-described reactive atsroglia cell activation and proliferation, present in ALS and other neurodegenerative diseases (Guan et al., 2007; Chen et al., 2012; Parpura et al., 2012; Forsberg et al., 2011; Bi et al., 2013), might change the morphological astroglia—neuron interaction, and thus, dendrites of astroglial cells might reside more closely to hMNs from $SOD1^{G93A}$ compared to WT, and might have been partly laser microdissected together with the hMN cell bodies. However, it is important to note that we gained no morphological evidence for this latter GFAP contamination explanation in our UV-LMD samples from $SOD1^{G93A}$ brains. Furthermore, we never detected respective GFAP co-expression in individual dopamine neurons from, e.g., Parkinson's disease brains, where a respective reactive gliosis also has been described ('Episcopo et al., 2013), or from respective Parkinson's disease mouse models, in over 15 years of UV-LMD RT-qPCR analysis of individual dopamine neurons (Ramirez et al., 2006; Gründemann et al., 2008, 2011; Schiemann et al., 2012; Schlaudraff et al., 2014). Thus, while we can rule out general methodological issues, we cannot for sure conclude that the detected GFAP signal of hMNs selectively from $SOD1^{G93A}$ but not from WT mice is genuinely derived from hMNs, and further immunohistological studies are necessary to address this point and its possible implication for ALS. However, this surprising finding further highlights the emerging crucial role of astroglia cells in ALS and its animal models (Valori et al., 2014). Indeed mutant SOD1 expression has been shown to greatly affect the astroglial functional phenotype, turning astrocytes into neurotoxic cells, more prone to cell death, and altering their vital MN-supportive functions (Valori et al., 2014).

Table 2 | Marker gene and Ca^{2+} transporter mRNA-levels in individual, laser microdissected hMNs from WT and endstage $SOD1^{G93A}$ mice.

A

	Relative mRNA amount [pg/cell]						
	wildtype			$SOD1^{G93A}$			
	Mean	**±s.e.m.**	***n***	**Mean**	**±s.e.m.**	***n***	***p***
ChAT (15 hMNs)	15,196	1452	50	14,853	1156	49	0.80
ChAT (single hMN)	9411	1405	10	7743	2012	10	0.35
GFAP (15 hMNs)	**1.85**	**0.50**	**50**	**80.38**	**8.46**	**50**	**<0.001*** **
GFAP (single hMNs)	**0.49**	**0.49**	**10**	**87.62**	**15.77**	**10**	**<0.001*** **
ND1† (15 hMNs)	258.5	23.22	30	232.0	18.82	30	0.51
ND1# (single hMN)	60.32	3.64	28	53.91	3.73	29	0.23

B

	(1) Relative mRNA amount [pg/cell]							(2) Relative amounts, normalized to ND1 levels [pg/cell]						
	Wildtype			$SOD1^{G93A}$				wildtype			$SOD1^{G93A}$			
	Mean	**±s.e.m.**	***n***	**Mean**	**±s.e.m.**	***n***	***p***	**Mean**	**±s.e.m.**	***n***	**Mean**	**±s.e.m.**	***n***	***p***
MCU	13.17	2.98	8	23.88	4.00	10	0.08	**13.57**	**2.98**	**8**	**25.29**	**4.41**	**10**	**0.04***
MICU1	**41.18**	**8.02**	**10**	**68.90**	**3.80**	**8**	**0.03***	**38.79**	**6.28**	**10**	**69.95**	**8.80**	**8**	**0.02***
MCUR1	4.90	1.03	9	5.24	0.90	10	0.70	4.69	0.70	9	6.13	0.83	10	0.39
LETM1	36.78	6.32	15	53.89	7.13	16	0.08	**31.86**	**3.64**	**9**	**54.25**	**7.26**	**10**	**0.01***
UCP2	**3.27**	**0.61**	**15**	**5.48**	**0.73**	**14**	**0.03***	**3.79**	**0.74**	**10**	**6.85**	**0.82**	**9**	**0.01***
UCP3	–	–	5	–	–	5	–	–	–	5	–	–	5	–
MNCX	4.33	1.42	10	4.22	0.77	10	0.47	3.83	0.74	10	4.41	0.60	10	0.39
								(3) Relative amounts, normalized to ChAT levels [pg/cell]						
PMCA1	84.42	12.52	10	76.55	19.11	10	0.39	87.15	5.32	8	98.53	11.90	8	0.43
PMCA2	197.30	46.89	9	126.0	41.11	9	0.31	178.4	20.07	7	140.2	20.20	7	0.46
PMCA3	75.60	17.34	10	61.33	12.16	10	0.57	78.76	10.82	6	85.28	14.58	6	0.68
PMCA4	12.08	2.88	16	8.58	1.54	16	0.58	23.56	7.45	6	18.69	3.66	6	0.68
NCX1	19.39	3.05	10	20.24	3.89	10	0.95	**15.99**	**1.96**	**6**	**28.07**	**4.48**	**6**	**0.03***
NCX2	–	–	5	–	–	5	–	–	–	5	–	–	5	–
NCX3	21.46	3.31	10	21.39	6.84	10	0.24	24.89	5.24	6	28.47	10.86	6	0.79

(A) *RT-qPCR data for the marker-genes ChAT, GFAP, and ND1, derived from either pools of 15 hMNs, or from one individual hMN, given as pg/cell in respect to cDNA standard curves, generated from WT mouse brainstem tissue. Note that ND1 DNA-levels for individual hMNs (#) were derived from isolated genomic DNA (without RT), while ND1 DNA-levels from pools of 15 hMNs (†) were derived from cDNA + genomic DNA. Note that in contrast to WT, via qPCR, GFAP was robustly detected in about 100% of ChAT positive hMNs of endstage $SOD1^{G93A}$ mice (compare* ***Figure 2C****).* ***(B)*** *RT-qPCR data for mitochondrial and plasma membrane Ca^{2+} transporters, derived from pools of 15 hMN each. Data are given (1) as [pg/cell] in respect to a cDNA standard curve, generated from WT mouse brainstem tissue, and (2) normalized to mitochondrially coded ND1 DNA-levels for mitochondrial Ca^{2+} transporters, or (3) normalized to ChAT cDNA levels for plasma membrane Ca^{2+} transporters. Significant differences according to Mann-Whitney-U-Tests are marked in bold and with (*), as defined in methods section.*

ELEVATED mRNA LEVELS OF THE MITOCHONDRIAL Ca^{2+} TRANSPORTERS MCU/MICU1, LETM1 AND UCP2 AND THE PLASMA MEMBRANE Na^+/Ca^{2+} EXCHANGER NCX1 IN hMNs OF $SOD1^{G93A}$ MICE

The present study was motivated by our recent finding of an activity-dependent Ca^{2+} clearance deficit selectively in individual hMNs, at the endstage of disease in $SOD1^{G93A}$ mice (Fuchs et al., 2013). More precisely, by combining patch-clamp analysis with fura-2 calcium imaging and selective pharmacology (e.g., the MCU-inhibitor RU-360) of individual, highly vulnerable hypoglossal and mostly resistant oculomotor MNs, we identified a remodeling of activity-dependent, intracellular Ca^{2+} clearance, selectively in hMNs in $SOD1^{G93A}$ mice at disease endstage, that was characterized by a reduction of mCU-mediated mitochondrial Ca^{2+} uptake, and an enhanced Ca^{2+} extrusion across the plasma membrane, under high-Ca^{2+} loading conditions (Fuchs et al., 2013). Our preliminary molecular analysis pointed to a complex underlying mechanism, as mRNA-levels of the mCU core components MCU and MICU1 were about 1.7-fold higher in hMNs from $SOD1^{G93A}$ mice (Fuchs et al., 2013). With an improved UV-LMD RT-qPCR protocol (**Figures 1**, **2**), we could reproduce these findings in an independent cohort of mice (**Figures 4A,B**; **Table 1B**). Increased MCU/MICU1 expression might enforce metabolic coupling, as mitochondrial Ca^{2+} activates, e.g., enzymes of the tricarboxylic acid cycle (McCormack and Denton, 1990; Wiederkehr et al., 2011). On the other hand, MICU1 does also act as gatekeeper of mCU that sets a threshold for maximal Ca^{2+} uptake. Thus, a MICU1 up-regulation provides a protective mechanism against mitochondrial calcium overload due to increased cytosolic Ca^{2+} levels (Mallilankaraman et al., 2012b), preventing excessive ROS generation and apoptosis (de Stefani et al., 2011). This mCU repressor-function of elevated MICU1 could explain the described functionally reduced mitochondrial Ca^{2+} uptake, while MICU1 and MCU mRNA levels are elevated. The finding that mRNA-levels of another regulatory mCU subunit MCUR1 (Mallilankaraman et al., 2012a) were not altered in hMNs from $SOD1^{G93A}$ mice, further supports the idea that the stoichiometry and thus general function of mCU might indeed be altered in hMNs from $SOD1^{G93A}$ mice. Additional mCU components have recently been identified (Marchi and Pinton, 2014), like EMRE and MICU2, which regulate MCU/MICU1 activity (Ahuja and Muallem, 2014; Kamer and Mootha, 2014; Kevin Foskett and Madesh, 2014), that need to be analyzed in further studies.

In addition to elevated MCU/MICU1 levels, we detected similarly elevated levels for Letm1 as well as for UCP2 mRNA in hMNs from $SOD1^{G93A}$ mice, while UCP3 mRNA was neither detected in hMNs from WT nor from transgenes (**Figures 4A,B**;

Table 1B). Letm1 is regarded as a highly Ca^{2+} sensitive mitochondrial Ca^{2+}/H^{+} exchanger, operating mainly at cytosolic Ca^{2+} levels below 1 μM (Jiang et al., 2009; Waldeck-Weiermair et al., 2011). As significant mitochondrial Ca^{2+} uptake via the mCU becomes apparent particularly at cytosolic Ca^{2+} concentrations not below 5–10 μm (Rizzuto and Pozzan, 2006; Marchi and Pinton, 2014), a concerted up-regulation of mCU and Letm1 migth counteract pathophysiological activity dependent, altered cytosolic Ca^{2+} levels in $SOD1^{G93A}$ mice, and thereby promote hMN survival. This complex view is further supported by the detected elevated levels of UCP2 in hMNs from $SOD1^{G93A}$ mice. In addition to the proposed mediated direct mitochondrial Ca^{2+} uptake, UCP2 acts as mitochondrial uncoupling protein, and thus decreases the amount of reactive oxygen species produced by a steep proton gradient of the respiratory chain (Donadelli et al., 2014). This mild uncoupling has been shown to be neuroprotective in highly vulnerable dopamine neurons in mouse models of Parkinson's disease (Liss et al., 2005; Guzman et al., 2010). However, overexpression of human UCP2 in $SOD1^{G93A}$ mice paradoxically accelerated disease progression and further reduced mitochondrial Ca^{2+} uptake capacity (Peixoto et al., 2013). The authors conclude, that in bio-energetically defective mitochondria of $SOD1^{G93A}$ mice, UCP2 might have adverse effects, possibly by enhancing sensitivity to Ca^{2+} induced depolarization of mitochondria and/or interacting with other Ca^{2+} uptake pathways.

Mitochondrial Ca^{2+} uptake and Ca^{2+} transporter activity is closely related to ER Ca^{2+} uptake, predominantly mediated by SERCA (Hovnanian, 2007; Chaudhari et al., 2014; Hajnóczky et al., 2014). We did however not analyze mRNA expression levels of SERCA-isoforms, as our previous functional and pharmacological (thapsigargin) analysis gave no hint for altered ER Ca^{2+} uptake in hMNs of endstage $SOD1^{G93A}$ mice, but for enhanced plasma membrane Ca^{2+}extrusion (compare **Figure 4C** and Fuchs et al., 2013). In accordance with this functional phenotype, we detected an about 1.7-fold higher mRNA-level of the plasma membrane Na^{+}/Ca^{2+} exchanger NCX1 in hMNs from $SOD1^{G93A}$ mice, while NCX2 was not expressed, and NCX3 as well as PMCX1-4 mRNA-levels were not altered (**Figure 4** and **Table 2B**). The lack of NCX2 mRNA detection in hMNs from WT as well as from $SOD1^{G93A}$ mice is somewhat surprising, since this isoform was attributed a key role in neuronal Ca^{2+} homeostasis (Brini and Carafoli, 2011). The low-affinity/high-capacity Ca^{2+} transporter NCX is believed to mediate the bulk of plasma membrane Ca^{2+} export after stimulation, rather than the PMCAs (Brini and Carafoli, 2011; Sharma and O'Halloran, 2014). Moreover, NCXs are controlled by cytosolic concentrations of Na^{+} and Ca^{2+} (Brini and Carafoli, 2011), thus the observed increased functional plasma membrane Ca^{2+}-extrusion is likely caused by an enhanced NCX1 expression, accompanied by an enhanced functional NCX transport capacity, due to pathologically elevated cytosolic Ca^{2+} and a reduced mitochondrial Ca^{2+} uptake capacity in hMNs from endstage $SOD1^{G93A}$ mice. Vice versa, overexpression of NCX1 has been shown to reduce not only cytosolic but also mitochondrial Ca^{2+} transients, providing an additional feedback loop to protect mitochondria from an extensive Ca^{2+} overload (Brini et al., 2002). An up-regulation of NCX1 in neurons has been demonstrated to be neuroprotective in other pathologic conditions, e.g., ischemic events (Pignataro et al., 2012). However, PMCAs are also stimulated by Ca^{2+} via calmodulin or phosphorylation (Strehler, 2013; Lopreiato et al., 2014). As specific PMCA splice variants differ, e.g., in their affinity and reaction upon calmodulin binding (Brini et al., 2013; Strehler, 2013), an pathologic shift in PMCA splice variants might also contribute to enhanced plasma membrane Ca^{2+} extrusion in hMNs from $SOD1^{G93A}$ mice. It is noteworthy in this context, that our PMCA TaqMan assays are detecting but not discriminating distinct PMCA splice variants. To date, functional studies of plasma membrane Ca^{2+} transporters are hampered by lack of specific pharmacology which makes it difficult to address the distinct contributions of individual NCX- or PMCA-isoforms (Strehler, 2013).

In conclusion, this study provides novel molecular and cell-specific insights into the complex nature of cytosolic Ca^{2+} dysregulation and mitochondrial dysfunction in highly vulnerable hMNs in the most common ALS mouse model, the endstage $SOD1^{G93A}$ mice. The detected concerted up-regulation of distinct mitochondrial and plasma membrane Ca^{2+} transporters with different locations as well as transport kinetics (mCU, Letm1, UCP2, NCX1) might serve as a complex compensatory response to the disease trigger and to the altered Ca^{2+} homeostasis, and protect hMNs in $SOD1^{G93A}$ mice from mitochondrial Ca^{2+} overload and degeneration. However, as we analyzed endstage $SOD1^{G93A}$ mice, where already about 30% of hMNs are lost (Haenggeli and Kato, 2002), we do not know, if the molecular up-regulation of distinct Ca^{2+} transporters did indeed protect those remaining hMNs from degeneration.

AUTHOR CONTRIBUTIONS

TM and JD carried out all experiments and performed data analysis, TM, JD and BL designed the study and wrote the manuscript. JHW and ACL bred and provided $SOD1^{G93A}$ and WT mice and revised the manuscript.

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MINI REVIEW
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Compensatory mechanisms in genetic models of neurodegeneration: are the mice better than humans?

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Neurodegenerative diseases are one of the main causes of mental and physical disabilities. Neurodegeneration has been estimated to begin many years before the first clinical symptoms manifest, and even a prompt diagnosis at this stage provides very little advantage for a more effective treatment as the currently available pharmacotherapies are based on disease symptomatology. The etiology of the majority of neurodegenerative diseases remains unknown, and even for those diseases caused by identified genetic mutations, the direct pathways from gene alteration to final cell death have not yet been fully elucidated. Advancements in genetic engineering have provided many transgenic mice that are used as an alternative to pharmacological models of neurodegenerative diseases. Surprisingly, even the models reiterating the same causative mutations do not fully recapitulate the inevitable neuronal loss, and some fail to even show phenotypic alterations, which suggests the possible existence of compensatory mechanisms. A better evaluation of these mechanisms may not only help us to explain why neurodegenerative diseases are mostly late-onset disorders in humans but may also provide new markers and targets for novel strategies designed to extend neuronal function and survival. The aim of this mini-review is to draw attention to this under-explored field in which investigations may reasonably contribute to unveiling hidden reserves in the organism.

Keywords: neurodegeneration, compensation, Alzheimer's disease, Parkinson's disease, Huntington's disease, transgenic mice, genetic models

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Introduction

The prevalence of neurodegenerative diseases, currently one of the main causes of mental and physical disabilities, has consistently risen because of the progressive aging of the worldwide population and is especially affecting highly developed societies. Examples of the more well-known neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD), but a myriad of other rare neurodegenerative disorders exist, e.g., Pick's disease, Creutzfeldt-Jakob disease (CJD), progressive supranuclear palsy (PSP), and amyotrophic lateral sclerosis (ALS). However, regardless of the nature of the disease, neural loss in the majority of the cases is estimated to usually begin 10–20 years before the first clinical symptoms appear and even a prompt diagnosis at this stage provides very little advantage for further effective treatment. Moreover, most of

the cases have a sporadic occurrence, and even for those in which the genetic factors have been determined, the distinct molecular pathways leading to final cell death remain unclear. Therefore, the currently available pharmacotherapies are based on disease symptomatology and, apart from alleviating the typical symptoms, they do not restore neuronal function or prevent neuronal loss.

Most of the classic animal models for neurodegeneration are based on applying neurotoxins—an effective strategy for studying phenotype but this generates immediate neuronal death, which severely limits the opportunity to observe the molecular changes associated with the authentic, slow neurodegenerative process. Thus, the statement, "the lack of a good animal model is frustrating in efforts to curb disease progression" (Beal, 2010) appears to still be valid, despite the progress in research focused on neurodegeneration.

Why Can We Not Fully Replicate Genetic Diseases in Transgenic Animals?

Advancements in genetic engineering over the last two decades have provided many transgenic mice that have been exploited as alternative, genetic models for various neurodegenerative diseases. These transgenic mice were created by either precisely targeting the same causative genes involved in the human disorders (e.g., HD, some rare familiar forms of PD and AD) or the genes controlling the subcellular changes and processes affected in the diverse neuropathological conditions, such as oxidative stress, rRNA synthesis, inflammation or mitochondrial dysfunction (Schwab et al., 2010; Parlato and Kreiner, 2013; Pickrell et al., 2013; Ribeiro et al., 2013). Surprisingly, many of these models do not fully recapitulate the inevitable neuronal loss (or at least not to the expected extent), supporting the proposal that different genetic, cellular and environmental factors may contribute to the ultimate cell death. Some transgenic mice fail to even demonstrate the phenotypic alterations associated with the modeled diseases, providing further evidence that humans and primates can be more vulnerable than rodents to the same triggers inducing neurodegeneration, a phenomenon also observed in pharmacological models (Przedborski et al., 2001).

In particular, extensively studied transgenic AD models, such as mice overexpressing β-amyloid precursor protein (APP, disputably but generally accepted contributing factor to AD), PS1 and PS2 (expressing mutated presenilin-1 and presenilin-2, respectively), APP/PS1 (harboring human transgenes for both APP and PS1 together) and Tg2676 (overexpressing a mutant form of APP), do not demonstrate the expected loss of neural cells (Duff et al., 1996; Oyama et al., 1998; Elder et al., 2010). Conversely, there are examples of models in which cognitive functions remain intact despite overexpression of APP (Masliah et al., 2001). Moreover, the so called "tau pathology" —formation of neurofibrillary tangles (NFT) due to hyper-phosphorylation of a microtubule-associated protein, which is another characteristic feature of AD—was not observed in most of the APP overexpressing models (Ribeiro et al., 2013). Another approach of creating AD transgenic mice models was related to tau proteins. Hyperphosphorylation of microtubule-associated protein tau (MAPT) can result in the self-assembly of NFT being involved in the pathogenesis of AD. The first transgenic model designed upon targeting tau protein did come out with any visible neurological phenotype (Gotz et al., 1995). Further attempts revealed only minor motoric impairments and tau protein accumulation (mostly in brain and spinal cord), however classic NFT were not observed or narrowed only to certain neural tissues (Eriksen and Janus, 2007; Wiedlocha et al., 2012). On the other hand, in the Htau mice characterized by expressing six isoforms of human tau without containing any mouse tau protein, the development of NFT was not correlated to the direct phenotype of the mutation and extensive neuronal loss in aged mice (Andorfer et al., 2003, 2005) indicating that the mechanism of Tau-mediated neuronal cell death remains elusive (Andorfer et al., 2005).

These discrepancies between the expected and observed phenotypes were particularly surprising in transgenic models created by directly targeting the identified causative genes. In the case of PD, there are currently recognized up to 20 genetic loci that have been described and implicated in the pathogenesis of PD, in which the mutations contribute to the familial form of PD (Scholz et al., 2012). However, none of the rodent models created by targeting these genes demonstrate profound neurodegeneration of dopaminergic cells, including the dominant mutation in leucine-rich repeat kinase 2 (LRRK2), the most widespread mutation among humans (Chesselet and Richter, 2011; Bezard et al., 2013).

Perhaps the most disappointing results have come from the classic models of HD. Huntington's disease is a progressive autosomal dominant inherited neurodegenerative disease that is characterized by uncontrolled movements (chorea) together with emotional and cognitive symptoms and inevitably leads to death within approximately 20 years. The cause of HD has been known since 1993, when it was identified as a polyglutamine (polyQ) expansion of a stretch of CAG repeats in the amino-terminal region of the huntingtin (HTT) protein (MacDonald, 1993). Therefore, one would expect that an accurate replication of the genetic malfunction directly responsible for HD in humans should result in exactly the same phenotype in mice. In fact, knock-in HD mice with expanded polyQ tracts do not differ from their control littermates in life span or body weight, demonstrating only mild motor deficits and very moderate cell loss (Lin et al., 2001). Moreover, this resistance to the mutation is emphasized by the fact that these mouse models harbor a considerably larger CAG expansion than is necessary to induce the human form of HD. Thus, it may be concluded that mutant HTT (mHTT) appears to be more toxic to primates than to rodent models. Paradoxically, despite knowing the precise mutation responsible for HD for more than 20 years, the most studied transgenic animal model of HD is the R6/2 mouse, which was created by expressing the amino-terminal region of HTT, thus not accurately representing the cause of the disease (Li et al., 2005).

The aforementioned observations provide clues to the potential compensatory mechanisms that protect neurons from death in the evaluated genetic models, which may help us to understand the preclinical deficits observed in neurodegenerative diseases and provide better insight into the pathogenic mechanisms underlying the initial, symptomless phase of their onset. There are several candidate pathways and molecules whose activation can be considered as an effect of the compensatory processes evoked in response to the introduced mutations. The limited content of this mini-review will not allow for a discussion of all the possible dilemmas but will concentrate on a selected intriguing examples based on the very recent literature, listed in alphabetical order.

Autophagy

Autophagy is a self-degradative process that removes unnecessary or dysfunctional cellular components through the actions of lysosomes and is important for balancing sources of energy at critical times during development, and in response to any type of cellular stress. It is generally considered a pro-survival mechanism (Glick et al., 2010). Thus, autophagy may be an essential factor that maintains neuronal homeostasis, and its impairment has been implicated in the development of neurodegenerative pathologies (Bakhoum et al., 2014). Additionally, it has also been proposed that the autophagy-lysosomal activities may play a pivotal role in neurodegenerative diseases by removing damaged or dysfunctional proteins and organelles that are a cause of oxidative stress, such that autophagy may be considered an antioxidant system (Giordano et al., 2013). Induction of autophagy has also been reported to be associated with rescue of the tau pathology, which suggests that formation of autophagosomes may be considered a compensatory mechanism rather than a trigger for neurodegeneration (Bakhoum et al., 2014). Our recent study, exploiting the new model of HD-like neurodegeneration based on the selective removal of transcription factor TIF-IA from medial spiny neurons (MSN), revealed that the transient upregulation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) kinase, a tumor suppressor that inhibits mammalian targeting of rapamycin signaling and induces autophagy, may result in enhanced MSN resistance (Kreiner et al., 2013).

ERK (Extracellular Signal-regulated Kinases) Pathway

Alterations in the ERK pathway have been reported in some neurodegenerative diseases, accompanied by an increase in the protein levels of ribosomal S6 kinases (RSK) in several models of HD (Xifro et al., 2011). RSK are involved in cell growth and survival, and are regulated by phosphorylation controlled by mitogen-activated protein (MAP) kinases, including ERK (Chen et al., 1992). Pharmacological inhibition of RSK, as well as knock-down and overexpression experiments, have indicated that RSK activity exerts a protective effect and may act as a compensatory mechanism with the capacity to prevent cell death in HD (Xifro et al., 2011). Another study reported a role of the regulator of G-protein signaling 2 (RGS2) in controlling the compensatory response in the striatal neurons of HD models, suggesting that RGS2 inhibition may be considered an innovative target for neuroprotection (Seredenina et al., 2011). An investigation of the possible mechanism underlying RGS2-mediated neuroprotection revealed that RGS2 downregulation enhanced activation of the ERK pathway (Seredenina et al., 2011).

Glycolysis

Glycolysis, the well-known metabolic pathway of glucose degradation resulting in formation of the high-energy compounds ATP and NADH, may also be involved in a compensatory response in neurodegenerative diseases. Glycolysis has been shown to compensate for mitochondrial dysfunction at the motor terminals of SOD1 transgenic mice (most widely used animal model of ALS), and this mechanism may help to support metabolism in the presence of dysfunctional mitochondria (Carrasco et al., 2012).

Metalloproteases

Metalloproteases are a group of enzymes that contain a catalytic metal ion at their active site and assist in the hydrolysis of peptides, which ultimately leads to protein degradation. Metalloproteases are important in many developmental processes, including cell proliferation, differentiation and migration (Chang and Werb, 2001). Endogenous metalloproteases have been proposed to regulate mitochondrial activity in degenerating neurons, and their activation may be regarded as an adaptive and compensatory response to stressful stimuli to protect mitochondrial function (de Oca Balderas et al., 2013). Specifically, it has been shown that metalloprotease inhibition stimulates mitochondrial activity impairment induced by 3-nitropropionic acid (3-NP, striatal cell neurotoxin), and metalloproteases may be involved in the cellular reorganization induced by 3-NP (de Oca Balderas et al., 2013).

Neurotrophins and Neurogenesis

Neurotrophins are a family of proteins that stimulate the development, differentiation and survival of neurons and are thus natural candidates for self-defense in case of neuronal loss. Neurotrophins can help to stimulate and control de novo neurogenesis. However it has been debated whether this phenomenon has any functional relevance, neurogenesis in the adult brain has also been proposed as a possible compensatory mechanism activated in response to environmental and genetic cues (Pierce and Xu, 2010).

One of the most studied and well-known triggers of neurogenesis is brain-derived neurotrophic factor (BDNF). It has been suggested that an increase in BDNF expression may reflect a compensatory mechanism against early neurodegeneration

(Faria et al., 2014). Specifically, increased BDNF levels have been reported in early stages of AD; these levels decrease over the course of the disease and are inversely correlated with dementia (Laske et al., 2006). It has also been reported that serum levels of BDNF are significantly lower in PD patients and correlate with the advancement of motor impairment (Scalzo et al., 2010). Several studies have shown that a loss of BDNF protein in the brains of HD patients may contribute to the clinical manifestation of the disease (Zuccato and Cattaneo, 2014).

Turning back to animal models, it was recently shown that neurotrophin treatments used on transgenic mouse models of AD lead to a reduction in Aβ generation that was mostly dependent on the BDNF-mediated decrease in glycogen synthase kinase-3-β (GSK3β) activity, emphasizing the potential of neurotrophins as targets for disease modifying therapy (Kazim et al., 2014). Experiments performed on mice hypomorphic for TrkB tyrosine kinase receptor (mainly activated by BDNF) have revealed a profound loss of dopaminergic neurons in the region of the substantia nigra (SN) together with elevated levels of dopamine in the striatum and yet no alteration in the turnover of this neurotransmitter (Zaman et al., 2004). These findings were associated with increased BDNF levels in the striatum but not the SN, suggesting the existence of a putative compensatory mechanism that follows dopaminergic cell loss in the SN (Zaman et al., 2004). An interesting recent study by the Minichiello group in a novel genetic mouse model showed that the selective removal of BDNF from enkephalinergic striatal neurons results in spontaneous and drug-induced hyperlocomotion associated with dopamine D2 receptor-dependent increased striatal protein kinase C (PKC) and MAP kinase activation, a mechanism that may have impact on striatal neuron vulnerability in the early-stage of HD (Besusso et al., 2013).

Noradrenaline

Noradrenaline (NA) is one of the most important neurotransmitter in the brain and the projections of noradrenergic neurons originating in the locus ceruleus penetrate virtually all brain structures. Degeneration of noradrenergic neurons is observed both in PD and AD to even greater extent and exacerbate the loss of dopaminergic and cholinergic neurons, respectively (Zarow et al., 2003). Experimental data indicate the important involvement of NA associated with PD brain damage i.e., the loss of NA in PD can worsen the dopamine nigrostriatal damage and, in opposite—an enhanced level of NA may have a neuroprotective effect (Srinivasan and Schmidt, 2003; Rommelfanger et al., 2004). These data prompt a statement that NA may serve as a compensatory mechanism in PD dopaminergic neurodegeneration (Rommelfanger and Weinshenker, 2007).

In a genetic mouse model of PD based on damaged mitochondrial DNA in dopaminergic neurons it was proven that NA and serotonin were increased after the dopaminergic cell loss (Pickrell et al., 2011). Recently, it has been shown that mirtazapine, an noradrenergic and serotonergic antidepressant drug, has a therapeutic potency in a classic pharmacological model of PD, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated mice (Kadoguchi et al., 2014).

The activation of NA in human neuronal cultures and rat primary hippocampal neurons protects against neuronal amyloid toxicity by stimulating neurotrophic pathways (Counts and Mufson, 2010). In rats with the lesion of medial septum cholinergic neurons, sprouting of noradrenergic sympathetic fibers triggered by neurotrophins was noted, contributing to cholinergic reinnervation what experimentally reiterated the clinical implications of sprouting as an innate compensatory mechanism (Nelson et al., 2014).

PKCδ (Protein Kinase Cδ)

PKC belongs to the group of enzymes that regulate the function of other proteins at the intracellular level and thus influence important cellular functions, including proliferation and apoptosis (Griner and Kazanietz, 2007). A balance between cell survival and apoptosis is crucial for avoiding neurodegeneration, and alterations in PKC activity have been associated with various neurodegenerative disorders, including AD, PD and HD. Recently, it was shown that an increase in the degradation of the PKCδ isoform is related to the compensatory pro-survival mechanism activated in response to mHTT-induced toxicity and is responsible for a delay in neuronal loss in HD (Rué et al., 2014). Consistent with this observation, overexpression of the PKCδ isoform *in vitro* enhances the negative effects of mHTT (Rué et al., 2014).

Conclusions

Lack of a desired broad-spectrum phenotype and content validity of some transgenic mice used as models of various neurodegenerative diseases is often regarded as a caveat of further practical exploiting of these models and prompts researchers pursuing for alternative models, neglecting the ones which did not fully meet their expectations. However, this failure can be turned into a feature considering that behind the simple resistance to the introduced mutation, there may be a huge variety of compensatory processes delaying or attenuating the expected phenotype in the rodent genetic models. A better evaluation and understanding of these mechanisms may help us to not only explain why neurodegenerative diseases are mostly late-onset disorders in humans but may also provide new disease markers and targets for novel strategies designed to extend neuronal function and survival. Such attempts are apparently rare. Surprisingly, only very few studies describing the observed phenotypes of different genetic models of neurodegenerative diseases have focused on this problem, and these mechanisms were barely investigated in these papers.

The aim of this mini-review is to focus attention on this under-explored field in which investigations may reasonably contribute to unveiling hidden reserves within the organism, particularly important in a preclinical stages of neurodegenerative diseases. Perhaps this is the time to reevaluate the initial descriptive characteristics of the transgenic

mice created upon targeting the same causative genes as in human neurodegenerative diseases, and pursue the potential compensatory mechanisms underlying introduced mutations that do not result in the expected phenotype.

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