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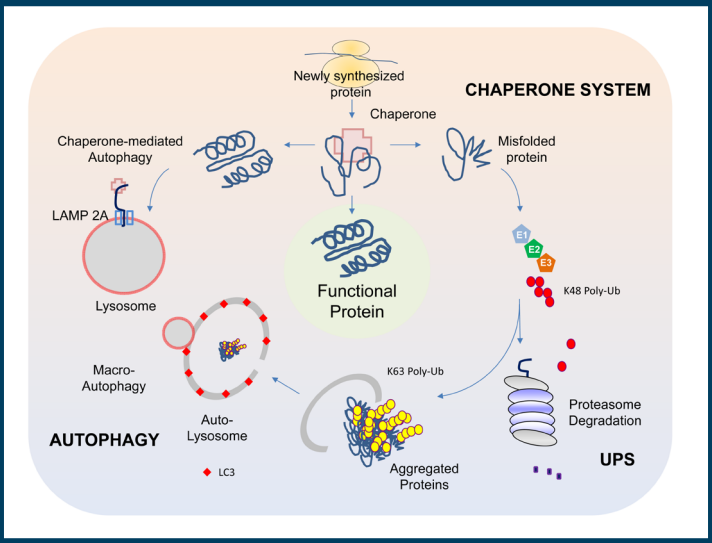

THE CELL AND MOLECULAR BIOLOGY OF NEURODEGENERATIVE DISEASES

Topic Editors
Thomas M. Durcan and Heather L. Montie

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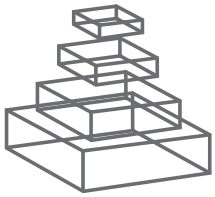

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THE CELL AND MOLECULAR BIOLOGY OF NEURODEGENERATIVE DISEASES

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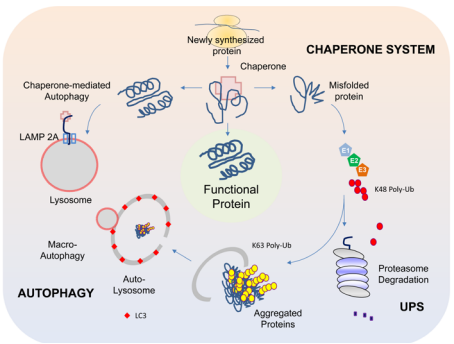

Protein QC systems. The chaperone, ubiquitin-proteasome and autophagy systems function in tandem to maintain intracellular protein homeostasis. The chaperones, represent the first line of defense, ensuring the correct folding and refolding of proteins. Chaperones also direct misfolded protein for degradation by the proteasome. Proteins destined for proteasome-mediated degradation usually have a chain of ubiquitin (Ub) added via a combination of enzymes in the Ub pathway. In some cases, ubiquitinated proteins can be directed for subsequent removal by autophagy. Figure taken from Lim K-L and Zhang CW (2013) Molecular events underlying Parkinson's disease – an interwoven tapestry. *Front. Neurol.* 4:33. doi: 10.3389/fneur.2013.00033.

Neurodegenerative diseases are complex, age-related disorders that are a growing health problem, exerting a tremendous burden on both affected individuals and society as a whole. Moreover, with the rising life expectancy in industrialized countries, and thus the increasing incidences of these disorders, there is now, more than ever, an urgent need for novel therapies to either halt and/or reverse the progression of these disorders. Central to the development of more efficient therapies, has been the extensive research over the past two decades into the molecular and cell biology of many of these disorders, that is now beginning to come to fruition with the development of mechanism-based therapies and biomarkers that can help treat affected patients at earlier stages in the disease.

To date, numerous molecular and cellular events contributing to these disorders have been revealed. Furthermore, increasing evidence implies that mechanisms underlying neuronal demise in these disorders may be shared. For example, compromised mitochondria function, although prominent in Parkinson's disease, has been linked to Alzheimer's disease and amyotrophic lateral sclerosis. Misfolding of proteins has also been demonstrated in many of disorders, and recent studies have demonstrated how misfolded proteins implicated in several neurodegenerative disease are able to propagate themselves.

In this research topic, our emphasis is on outlining progress made in understanding the basic molecular and cell biology of Alzheimer's disease (AD), Parkinson's disease (PD), triple repeat diseases, and other age-related neurodegenerative diseases.

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The cell and molecular biology of neurodegenerative diseases: an overview

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Keywords: neurodegeneration, aggregation, polyglutamine, Parkinson's disease, Alzheimer disease, ALS

In this research topic, the primary focus is on understanding the cellular and molecular mechanisms in the pathogenesis of different neurodegenerative disorders. These include Alzheimer's disease (AD), Parkinson's disease (PD), and polyglutamine (polyQ) expansion diseases. To date, no cure exists for these disorders and it is paramount that research efforts continue to focus on understanding the molecular underpinnings behind these disorders. This will enable better symptom-directed therapeutics and perhaps even curative treatments to be developed.

Throughout this topic, it becomes evident that there are common cellular pathways that are altered in these disorders, including protein, mitochondrial, and transcriptional homeostasis. In the case of AD, it has become widely accepted that AD is a synaptopathy, meaning that there is a loss or damage of synapses. This damage to synapses leads to altered neuronal circuitry. The neuron-specific, post-synaptic protein, Arc, has gained recent attention for its contribution in the regulation of memory consolidation. Kerrigan and Randall (1) discuss how alterations of Arc protein in the brains of AD patients and animal models of AD may be a clue as to how synaptic transmission is altered in AD, and how this cellular pathway may be of interest for therapeutic development.

The next three reviews discuss the molecular events underlying PD and how the normal function of specific proteins associated with PD can help shed light on the causes of familial and sporadic PD. Lim and Zhang (2) outline a range of studies that implicate aberrations in mitochondrial function and protein homeostasis, with oxidative stress as the possible link between these two. A review from Dr. Edward A. Fon's group complements this discussion by focusing on the structure and function of Parkin, PINK1, and DJ-1 as they relate to PD (3). The second review from Dr. Fon's group digs even deeper into the role of Parkin and PINK1 in mitophagy in neurons. They discuss the importance of research initiatives to better define the roles of these two proteins in mitophagy and, in particular, within the context of a neuronal setting (4).

The next five reviews focus on polyQ expansion diseases. Almeida et al. (5) provide a structural and functional view of trinucleotide repeats and encoded homopeptide expansions, emphasizing polyQ expansions and their role in inducing the self-assembly, aggregation, and functional alterations of the protein, leading to neuronal toxicity and cell death. These authors focus on ataxin-3 and huntingtin (Htt), the main protein implicated in Machado–Joseph Disease (MJD) and Huntington's disease (HD) respectively. Drs. Durcan and Fon, also focus on ataxin-3 and its function as a deubiquitinating enzyme (6). These authors have recently identified ataxin-3's E3 ubiquitin ligase partners to be parkin and CHIP. As MJD patients often present with PD symptoms, the fact that parkin's activity is regulated by ataxin-3-mediated deubiquitination is a critical link for this phenotype. As mutant, but not wild-type ataxin-3 promotes clearance of parkin via the autophagy pathway, there seems to be a possibility that increased turnover of parkin contributes to pathogenesis in MJD. Moreover, ataxin-3 also induces a reduction in CHIP levels. In light of these findings, the authors discuss the implications for the role of mutant ataxin-3's effect upon Parkin and CHIP levels in understanding the molecular processes involved in SCA3 and perhaps other neurodegenerative disorders.

Moumné et al. (7) discuss the role of transcriptional disruption in HD, specifically, as it relates to the transcriptional repressor, R element-1 silencing transcription factor (REST). REST is a transcriptional repressor of neuronal survival factors and normally associates with wild-type Htt. However, there is an aberrant alteration of cytoplasmic retention of the transcriptional repressor REST by mutant Htt. The authors go on to describe studies that have implicated this aberrant effect of mutant Htt on REST function to regulate neuronal genes and how this may impact pathogenesis in HD. Du et al. (8) focus on depression, the major psychiatric manifestation of HD. They discuss potential mechanisms of pathogenesis identified from animal models and compare depression in HD patients with non-HD persons, asking the question; Does HD-related depression differ from non-HD persons? They also go on to discuss some molecular and cellular mechanisms which may contribute to depression in HD.

Beitel et al. (9) discuss spinal and bulbar muscular atrophy (SBMA), a polyQ expansion disease caused by the expansion of a CAG tract in the androgen receptor (AR) gene. This review summarizes all of the aspects of AR metabolism, from posttranslational modifications, to protein degradation and transcriptional function that have been implicated in SBMA pathogenesis.

Spencer et al. (10) offer a commentary on Western Pacific Amyotrophic lateral sclerosis (ALS) – parkinsonism-dementia complex (PDC) that plagues the island populations of Chamorros on Guam, Japanese in Honshu Island's Kii Peninsula, and Papuan New Guineans in Irian Jaya, Indonesia. It is a spectrum disorder believed to be to be triggered by a toxin in the seed of the neurotoxic cycad plant. This toxin is thought to induce a prototypical

neurodegenerative disorder linked to DNA damage and aberrant proteogenesis.

The final review discusses how cellular surfaces modulate protein aggregation related to neurodegeneration. The interaction of proteins with liquid/surface interfaces is a fundamental phenomenon with potential implications for protein-misfolding diseases. Burke et al. (11) provide an overview of what is known about the influence of (sub) cellular surfaces in driving protein aggregation and/or stabilizing specific aggregate forms and how further understanding of such could provide new insights into toxic mechanisms associated with these diseases.

Lastly, the methods paper by Lange et al. (12) describes a detailed method for culturing embryonic dorsal root ganglion neurons for Seahorse Extracellular Flux XF24 analysis. This is a procedure used to measure the relative state of glycolytic and aerobic metabolism in live cells, in order to assess mitochondrial function. As changes in mitochondrial dynamics and function contribute to multiple neurodegenerative diseases, this method outlined herein is of significant interest to this topic.

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A new player in the "synaptopathy" of Alzheimer's disease – Arc/Arg 3.1

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Alzheimer's disease (AD) is increasingly referred to as a "synaptopathy." This moniker reflects the loss or damage of synapses that occurs as the disease progresses, which in turn produces functional degeneration of specific neuronal circuits and consequent aberrant activity in neural networks. Accumulating evidence supports the functional importance of the early-expression activity-regulated cytoskeletal (*Arc*) gene in regulating memory consolidation. Interestingly, AD patients express anomalously high levels of Arc protein. Arc physically associates with presenilin1, a pivotal protease for the generation of Amyloid β (Aβ) peptides. Arc expression itself is disrupted in the vicinity of Aβ oligomers and plaques. Such alterations result in the interruption of neuronal network integration *in vivo*. It is not clear what the impacts of these alterations are on the functional neurophysiology of transgenic mouse models of AD-associated amyloidopathy. Our group and others have described alterations to neuronal excitability and thus intrinsic firing within these transgenic mice models. This brief review will emphasize the rising role of Arc and its involvement in neurophysiological alterations of current AD models.

Keywords: Arc, amyloid beta protein, Alzheimer's disease, neurophysiology, AMPA receptor trafficking, intrinsic plasticity

INTRODUCTION

Alzheimer's disease (AD) appears to be primarily a disorder of synaptic failure (Selkoe, 2002) which is becoming one of the most predictable features in the pathophysiology of the disease. This "synaptopathy" is associated with disruptions in synaptic structure and function, leading to aberrant neural processing and network disruptions.

Early research on AD pathology focused attention on the involvement of the amyloid precursor protein (APP) pathway and the plaques formed by its proteolytic cleavage product Amyloid β (Aβ; Glenner and Wong, 1984; Hardy and Higgins, 1992; Hardy and Selkoe, 2002). Evidence accumulated in recent years has led to the emergence of the soluble, oligomeric Aβ peptide playing a pivotal role in the disruption of synaptic function and thus neuronal network activity (Walsh and Selkoe, 2004, 2007). Although picomolar concentrations of Aβ may play critical physiological roles in synaptic plasticity (Puzzo et al., 2008) and activity-dependent regulation of synaptic vesicle release (Abramov et al., 2009), abnormal accumulations lead to the self-assembly of neurotoxic Aβ oligomers, which interfere with synaptic function and cause neurodegeneration. These alterations in classic neurophysiological processes are believed to be the main substrates of cognitive decline in AD.

Indeed, levels of soluble Aβ oligomers are highly correlated with synaptic dysfunction in AD. It has been possible to monitor the targeting of Aβ oligomers to synapses (Lacor et al., 2004; Deshpande et al., 2009), and to follow the changes in spine morphology and density (Lacor et al., 2007). These synaptic alterations correspond to the best pathological correlate of memory deficits in AD (DeKosky and Scheff, 1990; Terry et al., 1991; Selkoe, 2002), although the exact mechanisms are still unknown. The intriguing targeting of Aβ oligomers to synapses and their disruption brought our focus to one of the genes shown to be vital for memory consolidation and synaptic plasticity, namely the immediate-early gene Arc/Arg3.1 (early-expression activity-regulated cytoskeletal gene, here on referred to as Arc).

Arc is a neuron-specific, post-synaptic protein that is selectively expressed in Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII)-positive neurons (Vazdarjanova et al., 2006). Upon activation, Arc is targeted to the post-synaptic density of synaptically active dendritic spines (Lyford et al., 1995; Steward and Worley, 2001; Moga et al., 2004) where it associates with polysomes (Bagni et al., 2000). Arc interacts with endophilin 2/3 and dynamin, contributing to alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) type glutamate receptor (AMPAR) modulation, by enhanced receptor endocytosis (Chowdhury et al., 2006). The Arc-endosome also traffics APP and physically associates with presenilin (PS1), thereby increasing the amount of activity-dependent Aβ generated (Wu et al., 2011). Interruption of the Arc-PS1 interaction prevents activity-dependent increases of Aβ (Wu et al., 2011). The precise signaling cascades involved in Arc transcription are not well defined. For a more comprehensive review on Arc function and signaling, the reader is referred to: Tzingounis and Nicoll, 2006; Miyashita et al., 2008; Bramham et al., 2010; Shepherd and Bear, 2011.

Arc-mediated endocytosis of AMPARs dampens the activity of neuronal networks, enhancing the activity-dependent generation of Aβ (Wu et al., 2011). If Arc-endosome trafficking and resultant activity-dependent generation of Aβ remained unchecked, it will create a positive feedback mechanism in which the synaptic

removal of AMPAR will produce a significant loss of dendritic spines and synaptic activity, resulting in synaptic failure, similar to that observed in AD (Hsieh et al., 2006; Shankar et al., 2007; Li et al., 2010).

This brief review will provide an overview of the importance of "synaptopathy" in the pathogenesis of AD, with particular emphasis being placed on the rising role of Arc, in neurophysiology. We aim to provide a critical assessment of the current literature, to address the impact of altered Arc expression on the molecular and cellular mechanisms underlying the functional neurophysiology in transgenic mouse models of AD-associated amyloidopathy.

ARC AND SYNAPTIC TRANSMISSION

Processing of information for memory storage requires specific patterns of activity that lead to the modification of synapse structure and eventually to changes in neural connectivity (Lamprecht and LeDoux, 2004; Marrone, 2007). These modifications can be defined as synaptic plasticity, of which, long-term potentiation (LTP) and long-term depression (LTD) are the two main cellular mechanisms that are associated with learning and memory (Bliss and Collingridge, 1993; Kandel, 2001; Malenka, 2003).

Arc was first identified as a hippocampal transcript strongly induced by epileptic seizures and synaptic plasticity-inducing electrical stimulation in the rat hippocampus (Link et al., 1995; Lyford et al., 1995). Arc is not expressed in presynaptic terminals or axons, but it is notable that its mRNA and protein accumulate in dendrites at sites of recent synaptic activity (Steward et al., 1998). The induction of Arc synthesis upon neuronal activation and its localization to active dendrites, make it a prime candidate for investigating the mechanisms underlying learning and memory. The importance of Arc in learning and memory is corroborated in Arc knock-out (KO) animals where loss of the Arc gene results in unusual phenotypic behavior, wherein the animals are able to retain short term memory formation, however long-term memories cannot be formed (Plath et al., 2006). Reduced Arc expression in the hippocampus by infusion of antisense oligodeoxynucleotides interferes with synaptic plasticity and hippocampus-dependent learning and memory (Guzowski et al., 2000).

It has been demonstrated that Arc transcripts are also induced during certain behaviors. The exploration of a novel environment induces Arc expression in a subset of context-activated pyramidal neurons, and can therefore be associated with experience-dependent forms of plasticity (Guzowski et al., 1999; Vazdarjanova and Guzowski, 2004; Gao et al., 2010; Wibrand et al., 2012). The specificity and characteristic time course of Arc mRNA induction can be used to monitor neural circuit activation following behavior episodes, as initially demonstrated by Guzowski et al. (1999, 2001). They were able to detect from Arc RNA *in situ* hybridization studies that in CA1 neurons, it is possible to distinguish between populations of neurons that responded to two different environments and were able to reveal whether the same neuron was activated twice (Guzowski et al., 1999). This unique correlation between RNA expression and neuronal activity levels, allows for Arc mRNA to be used as a tool for the detection of when and where activity in response to learning is being altered. Arc expression itself differs between brain regions and cell types (e.g., CA1 from CA3), suggesting that it plays an important role in detecting changes in neuronal activity in an experience-dependent manner (Kelly and Deadwyler, 2003; Daberkow et al., 2007; Miyashita et al., 2009). Arc is also heavily involved in different forms of synaptic plasticity, however to cover these is beyond the context of this article. We therefore suggest the following reviews (Bramham et al., 2010; Korb and Finkbeiner, 2011; Shepherd and Bear, 2011).

Episodic hippocampal-dependent memory loss, is the earliest clinical sign of AD, and is thought to be a result of changes in synaptic function rather than neuronal loss (Morrison and Hof, 1997; Arendt, 2009). *In vivo* brain imaging studies, using functional magnetic resonance imaging (fMRI) have revealed aberrant networking in brain regions linked to memory function (Sperling et al., 2009). High levels of amyloid deposition are associated with this aberrant default network, suggesting that amyloid pathology in early stages of AD is linked to neural dysfunction, memory loss, and aberrant synaptic plasticity (Sperling et al., 2009). Although still unclear, a general picture is emerging in which Aβ oligomers seem to highjack the molecular machinery necessary to induce synaptic plasticity. It appears that Aβ induces aberrant synaptic plasticity by inhibiting LTP, and more interestingly, by facilitating LTD, causing AMPAR endocytosis (Hsieh et al., 2006; Shankar et al., 2007; Li et al., 2010).

To prevent these imbalances in synaptic plasticity from developing in normal physiology, neurons have developed a unique mechanism which modulates their global levels of post-synaptic AMPAR in response to the level of activity seen in the cells, as expressed by the rate of action potential firing. This process is known as synaptic scaling and is thought to maintain post-synaptic action potential firing rates within certain bounds (average firing rate; Turrigiano et al., 1998). Synaptic scaling is a cell-wide mechanism of plasticity, and is thus referred to as a form of homeostatic plasticity (Fregnac, 1998; Galante et al., 2001). This form of plasticity is particularly sensitive to the levels of Arc (Shepherd et al., 2006; Turrigiano, 2007). Indeed, Arc KO animals as well as neurons overexpressing Arc are not capable of maintaining this negative feedback mechanism (Shepherd et al., 2006).

Although the detailed mechanisms through which Arc affects hippocampal functions are still under investigation, recent evidence has demonstrated a role of Arc in AMPAR trafficking, with evidence pointing to regulation of AMPAR endocytosis (Chowdhury et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006). Arc directly interacts with the endocytic machinery by binding to endophilin 1 and dynamin 2, and selectively increasing the rate of AMPAR recycling (Chowdhury et al., 2006; Shepherd et al., 2006). These studies suggest that when Arc expression is low the steady state of AMPAR trafficking will shift to increase the distribution of AMPAR to the membrane (Shepherd et al., 2006). The inverse is true under conditions of high Arc expression (see **Figure 1**). Arc is therefore likely important for limiting the level of neuronal excitation since Arc-mediated endocytosis of AMPARs will dampen activity of neuronal networks.

Arc activity is capable of regulating AMPAR endocytosis and both spine size and type. These distinct actions are all means to modulate synaptic strength (Chowdhury et al., 2006; Peebles et al., 2010). As outlined above it appears that Arc controls surface expression of AMPAR in a homeostatic manner and acts to

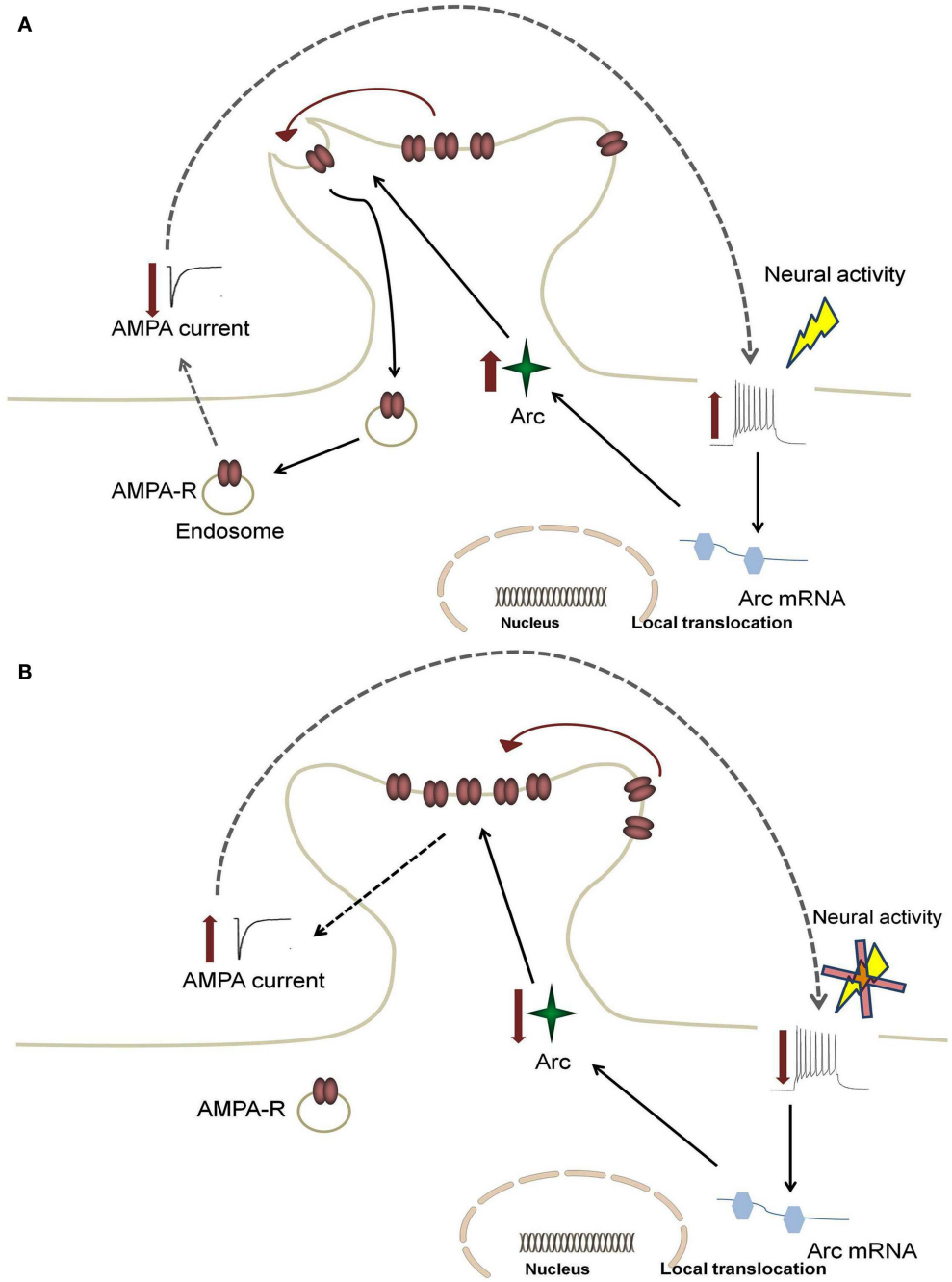

FIGURE 1 | Feedback mechanism for Arc-mediated AMPA receptor endocytosis. An increase in neural firing leads to somatic alterations in calcium levels, which causes increased activation in the nucleus to enhance local translocation of Arc mRNA. This increase results in enhanced Arc protein levels and increased AMPA receptor (AMPA-R) endocytosis **(A)**. This leads to a decrease in synaptic strength, which subsequently returns firing rates back to target levels **(B)**. In AD, enhanced Arc expression due to hyperexcitability leads to a positive feedback mechanism in which the dampening response of Arc is limited.

keep surface levels and subunit composition optimal for Hebbian plasticity in normal physiology (Shepherd et al., 2006; Gao et al., 2010).

If Arc-mediated endocytosis remain unchecked, excessive modifications of synaptic strength might generate instability or altered synchrony in neuronal networks, leading in turn to disease states characterized by network imbalances, as observed in AD and epilepsy (Driver et al., 2007; Bragin et al., 2009; Palop and Mucke, 2009). The AD-associated peptide, Aβ, depresses AMPA receptor currents in brain slices and induces AMPAR endocytosis

via a mechanism which is similar to Group 1 metabotropic glutamate receptor (mGluR-LTD; Hsieh et al., 2006). There is also further evidence that AMPAR trafficking is reduced in certain transgenic mouse models overexpressing APP (Almeida et al., 2005; Chang et al., 2006). Is it therefore plausible that AD may follow a similar mechanism in which a disruption of the Arc protein and its expression results in aberrant AMPAR trafficking?

FUNCTIONAL NEUROPHYSIOLOGY IN AD MODELS – A RISING ROLE FOR Arc?

A number of compelling findings suggest that Arc may contribute to the cognitive deficits and Aβ-dependent alterations in synaptic plasticity experienced in AD. In fact, oligomeric forms of Aβ have been shown to induce Arc expression itself (Lacor et al., 2004). The synaptic binding of Aβ was able to induce a sustained Arc expression within minutes, leading to ectopic protein diffusion throughout the dendrite (Lacor et al., 2004).

Intriguingly, some AD mouse models show a decrease in the number of Arc-expressing cells in the cortex (Wegenast-Braun et al., 2009), a reduced Arc mRNA expression following explorative behavior (Palop et al., 2005; Wegenast-Braun et al., 2009), and lower levels of Arc mRNA in Aβ-containing brain regions (Dickey et al., 2004). Taken together, these data suggest that the widespread Arc expression from acute Aβ exposure might be paralleled by down-regulated Arc signaling in transgenic animals which continuously over-produce Aβ. Early-stage synaptic deterioration may be explained by the age-dependent decreases in Arc mRNA and therefore altered dendritic transport in some transgenic mice (Dickey et al., 2003).

Along with the disruption in AMPAR trafficking, there may be structural modifications in the architecture of the neurons taking place, which could account for some of the neurophysiological alterations experienced in AD (Jacobsen et al., 2006; Middei et al., 2008). Indeed, post-mortem tissues from AD patients reveal reduced spine density (Scheff et al., 1996). Several *in vitro* studies have demonstrated that oligomeric Aβ causes a reduction in the number and/or length of dendritic spines in hippocampal neurons (Calabrese et al., 2007; Lacor et al., 2007; Shankar et al., 2007). Similar to Arc KO mice, mouse AD models display similar decreases in spine density and impairment of long-term memory (Jacobsen et al., 2006; Peebles et al., 2010; Perez-Cruz et al., 2011).

A number of studies in mutant APP-expressing transgenic models have indicated increased Arc activation in response to neuronal activity (Grinevich et al., 2009; Perez-Cruz et al., 2011), as shown in **Table 1**. In these studies the APP-based transgenic lines were studied prior to plaque deposition, suggesting that soluble oligomeric, rather than deposited fibrillar Aβ is responsible for the enhanced Arc expression (Perez-Cruz et al., 2011). In fact, in the Tg2576 mouse line, the observed loss in dendritic spines were said to be attributed to a loss of inhibitory interneurons, which resulted in hyperexcitability caused by enhanced glutamate and calcium-mediated excitation, subsequently causing the enhanced expression of Arc (Perez-Cruz et al., 2011). We have confirmed the hippocampal network hyperexcitability described in this particular mouse line, along with a double mutant $APP_{SWE}/PS1_{M146L}$ (PSAPP) mutation and have found that hippocampal CA1 pyramidal cells have enhanced "burstiness" (Brown et al., 2011). Such alterations in excitability could lead to the early disruption in synchronous network activity (Brown et al., 2005; Driver et al., 2007).

Although the increase in cellular excitability described above occurs in the absence of changes to resting potential, it seems to arise from alterations to voltage-gated Na^+ channels (Brown et al., 2011; Randall et al., 2012). These alterations in Na^+ channels are an age-dependent event that was absent from the early preplaque Tg2576 mice (used in Perez-Cruz et al., 2011; Brown et al., 2011), however present in the more aggressive Aβ generating PSAPP mouse (Brown et al., 2011). We cannot rule out the involvement of a possible bidirectional control of intrinsic excitability (Fan et al., 2005; Brager and Johnston, 2007), as a result of enhanced neuronal activity due to increased Aβ burden.

Interestingly, a recent study revealed that Arc increases the association of presenilin/γ-secretase with endosomes that traffic APP (Wu et al., 2011). When binding of Arc to PS1 was interrupted activity-dependent increases in Aβ ceased. These workers also revealed that the level of Arc expression determines the burden of Aβ in the APP-based model used. This was the first study to reveal that Arc protein was capable of increasing the generation of Aβ *in vivo*. Wu et al. (2011) further explored the role of Arc in AD by examining the medial frontal cortex of post-mortem human tissue of patients with AD. The levels of Arc protein were significantly increased in the medial frontal cortex of patients with late stage advanced AD (Braak Stage V and VI) when compared to their non-demented age-matched controls. Based on this the authors concluded that Arc expression could contribute to Aβ generation and pathology in AD (Wu et al., 2011).

Another *in vivo* study revealed that amyloid plaques act locally to aberrantly increase Arc expression in active neurons (Rudinskiy et al., 2012). However, the proportion of neurons that were active in the location of amyloid plaques was significantly decreased. This variation in active neurons near pathological plaques could provide an explanation to the discrepancies that exist in current literature involving Arc expression in AD (**Table 1**). Moreover this study provides valuable insight into the underlying mechanisms which could contribute to the alterations experienced in the "default mode network, DMN" (Raichle et al., 2001) of patients with AD, which is currently being explored in clinical neurophysiology. fMRI studies and position emission tomography (PET) of AD patients have revealed a distinct correlation between default activity patterns in cortical regions in young adults prior to the development of AD and topography of Aβ deposition in early AD cases (Buckner et al., 2005; Sperling et al., 2009). This correspondence raises the possibility of a relationship between activity patterns in early adulthood and later Aβ deposition, providing valuable information and possible insight into the development of AD. Indeed, Rudinskiy et al. (2012) suggest that the pattern of Arc expression reflects the nervous system responses to, and physiological consolidation of, behavioral experience. They conclude that disruptions in Arc patterns reveal plaque-associated interference with neural network integration, which could ultimately lead to the synaptopathy of AD.

Table 1 | Summary of effects of AD-related pathologies on *Arc* expression.

Species	AD related model	Level of Arc expression	Brain region	Reference
Mouse	APP/PS1, APPDutch, and APP23 (aged and young)	Decreased	Hippocampus and neocortex	Wegenast-Braun et al. (2009)
Mouse	APP/PS1	Decreased	Hippocampus	Dickey et al. (2003, 2004)
Mouse	hAPP (preplaque, FAD)	Decreased	Hippocampus (DG) and cortex	Palop et al. (2005)
Human	Neurons with NFT	Decreased	Hippocampus	Ginsberg et al. (2000)
Mouse	Synthetic Aβ	Decreased	Cortical primary cultures	Echeverria et al. (2007)
Rat	Synthetic Aβ	Decreased	Cortical primary cultures	Wang et al. (2006), Chen et al. (2009)
Mouse	TG2576 and APP/Lo (preplaque)	Increase	Hippocampus	Perez-Cruz et al. (2011)
Mouse	APP/PS1 (preplaque)	Increase	Hippocampus	Grinevich et al. (2009)
Mouse	Synthetic Aβ	Increase	Cortical primary cultures	Wu et al. (2011)
Rat	Synthetic Aβ	Increase	Hippocampal primary neurons	Lacor et al. (2004)
Mouse	4–7 Month old hAPP-J20 (preplaque)	Both increase and decrease	Hippocampus	Palop et al. (2005)
Mouse	TG2576 (aged)	No change	Hippocampus	Cuadrado-Tejedor et al. (2011)
Mouse	CRND8 (aged)	No change	Hippocampus	Herring et al. (2012)

CONCLUDING REMARKS

Despite its well characterized role in synaptic plasticity, Arc's involvement in disease is less well understood. It appears that this enigmatic protein plays an imperative role in the maintenance of homeostatic neuronal activity, which if disrupted, presents itself in pathology. In this review, we focused on some of the most valuable findings, providing a unique insight into the mechanisms underlying cognitive decline associated with AD.

We have explored the role of Arc in different models of AD-associated pathology, and experienced a range of fluctuations in neurophysiological properties. Differences *in vitro* can be interpreted as the result of a number of variables that differ between each of the reported studies, including differences in behavioral assays performed. Not only do they include different brain regions, they also include different strains and ages of mice studied. Differences in genetic background and gene inclusions can themselves have profound effects on neurophysiology. Although a detailed morphological analysis of changes in synaptic connectivity in the AD brain over time is difficult and limited to the use of post-mortem material, the generation of APP transgenic mice that over-produce Aβ has enabled a better understanding of the functional and morphological consequences of Aβ overproduction. These studies further highlight the need to understand precisely how Arc expression is affected in AD, and its consequent alterations to neurophysiological function. Valuable evidence now emerging from *in vivo* models can directly be correlated to clinical studies. This provides further support for the continuation of exploring the role of Arc in the synaptopathy of AD. Finally, improving our understanding of the molecular mechanisms contributing to maintaining or strengthening synapses may be an interesting entry point for novel therapeutic intervention in many neurodegenerative diseases.

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Molecular events underlying Parkinson's disease – an interwoven tapestry

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Although a subject of intense research, the mechanisms underlying dopaminergic neurodegeneration in Parkinson's disease (PD) remains poorly understood. However, a broad range of studies conducted over the past few decades, including epidemiological, genetic, and post-mortem analysis, as well as *in vitro* and *in vivo* modeling, have contributed significantly to our understanding of the pathogenesis of the disease. In particular, the recent identification and functional characterization of several genes, including α-*synuclein*, *parkin*, *DJ-1*, *PINK1*, and *LRRK2*, whose mutations are causative of rare familial forms of PD have provided tremendous insights into the molecular pathways underlying dopaminergic neurodegeneration. Collectively, these studies implicate aberrant mitochondrial and protein homeostasis as key contributors to the development of PD, with oxidative stress likely acting as an important nexus between the two pathogenic events. Aberrations in homeostatic processes leading to protein aggregation and mitochondrial dysfunction may arise intrinsically in substantia nigra pars compacta dopaminergic neurons as a result of impairments in the ubiquitin-proteasome system, failure in autophagy-mediated clearance, alterations of mitochondrial dynamics, redox imbalance, iron mishandling, dopamine dysregulation, or simply from the chronic pace-making activity of nigra-localized L-type calcium channels, or extrinsically from non-autonomous sources of stress. Given the myriad of culprits implicated, the pathogenesis of PD necessarily involves an intricate network of interwoven pathways rather than a linear sequence of events. Obviously, understanding how the various disease-associated pathways interact with and influence each other is of mechanistic and therapeutic importance. Here, we shall discuss some key PD-related pathways and how they are interwoven together into a tapestry of events.

Keywords: Parkinson disease, mitophagy, autophagy, proteasome, oxidative stress, protein aggregation

INTRODUCTION

Parkinson's disease (PD) is a prevalent neurodegenerative movement disorder affecting 1–2% of the worldwide population above the age of 65 (Dorsey et al., 2007). Clinically, the disease is attended by a constellation of disabling motoric deficits including bradykinesia (slowness in movements), rigidity, and tremor that progressively worsen with age, ultimately leading to near total immobility. Although pathological changes are distributed in the PD brain (Braak et al., 2003), the principal lesion that underlies the characteristic motor phenotype of PD patients is unequivocally the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain, which normally innervates the striatum. This specific pattern of neurodegeneration in PD is often accompanied by the presence of eosinophilic intracytoplasmic inclusions known as Lewy bodies (LBs) in surviving neurons in the SN and other affected brain areas (Braak et al., 2003). The depletion of striatal dopamine (DA) as a result of SNpc dopaminergic neuronal loss leads to an impaired nigro-striatal system that otherwise allows an individual to execute proper, coordinated movements. Accordingly, pharmacological replacement of brain DA via L-DOPA administration represents an effective symptomatic recourse for the patient (especially during the initial stages of the disease) and remains a clinical gold standard treatment for PD. However, neither L-DOPA nor any currently available therapies can slow or stop the insidious degenerative process in the PD brain. Thus, PD remains an incurable disease. Invariably, the debilitating nature and morbidity of the disease present significant healthcare, social, emotional, and economic problems. As the world population rapidly ages, these problems undoubtedly will also increase. This is definitely a worrying trend, and one that aptly emphasizes the urgency to develop more effective treatment modalities for the PD patient. Toward this endeavor, a better understanding of the molecular mechanism(s) that underlies the pathogenesis of PD would certainly be helpful, as the illumination of which would allow the identification and therapeutic exploitation of key molecules/events involved in the pathogenic process.

Although a subject of intense research, the mechanisms underlying PD pathogenesis remain incompletely understood. However, a broad range of studies conducted over the past few decades,

including epidemiological, genetic, and post-mortem analysis, as well as *in vitro* and *in vivo* modeling, have contributed significantly to our understanding of the pathogenesis of the disease. In particular, the recent identification and functional characterization of several genes, including α-*synuclein*, *parkin*, *DJ-1*, *PINK1*, and *LRRK2*, whose mutations are causative of rare familial forms of PD have provided tremendous insights into the molecular pathways underlying dopaminergic neurodegeneration (Martin et al., 2011). Notably, the clinical manifestations and neuro-pathology of familial parkinsonism can often be quite indistinguishable from sporadic cases, which fueled the widely held assumption that the two forms of PD are likely to have shared pathogenic mechanisms. Collectively, these studies consistently implicate aberrant protein and mitochondrial homeostasis as key contributors to the development of PD, with oxidative stress likely acting as an important nexus between the two pathogenic events. Further, emerging evidence also implicates a host of new pathways, including impairments in vesicular dynamics, calcium homeostasis, and lipid metabolism that might contribute to disease pathogenesis in a manner that is not necessarily uncoupled from one another or from protein and mitochondrial homeostatic processes. Thus the molecular events underlying dopaminergic neurodegeneration in PD appears interwoven and complex. Here, we shall review some key PD-related events and discuss their reciprocal effects on each other.

ABERRANT PROTEIN HOMEOSTASIS AND PD

The presence of LBs in affected regions of the PD brain in numbers that far exceed their occasional presence in the normal brain arguably provides the most glaring evidence suggesting that protein homeostasis has gone awry during disease pathogenesis. As the cell is endowed with several complex surveillance machineries (including the chaperone, ubiquitin-proteasome, and autophagy systems) that rapidly detect and repair faulty proteins, and also destroy those that are beyond repair (**Figure 1**) (for a recent review, please refer to Tan et al., 2009), the presence of LBs suggests that these homeostatic response systems have failed in one way or another in the PD brain. Support for this came from various groups following the identification of α-synuclein, a presynaptic terminal-enriched protein that is prone to misfolding and

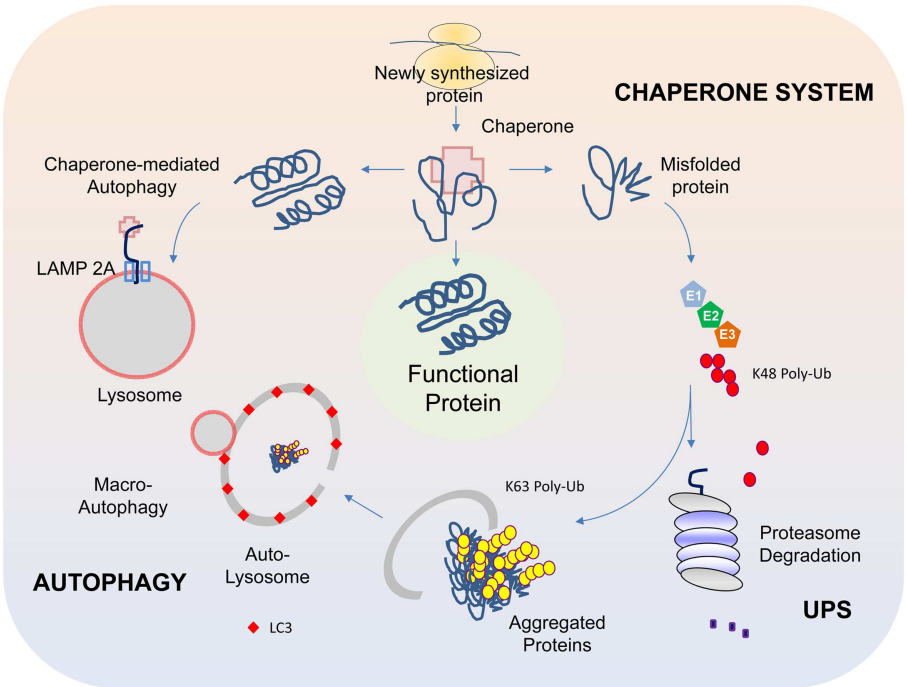

FIGURE 1 | Protein QC systems. The chaperone, ubiquitin-proteasome, and autophagy systems function co-ordinately to maintain intracellular protein homeostasis. The chaperones, comprising of members of the heat-shock proteins, represent the first line of defense in ensuring the correct folding and refolding of proteins. When a native folding state cannot be attained, the chaperones will direct the misfolded protein for degradation by the proteasome. Proteins that are destined for proteasome-mediated degradation usually have a chain of ubiquitin added via a reaction cascade that involves the ubiquitin-activating (E1), -conjugating (E2), and -ligating (E3) enzymes, whereby successive iso-peptide linkages are formed between the terminal residue (G76) of one ubiquitin molecule and a lysine (K) residue (most commonly K48) within another. The (G76-K48) polyubiquitinated substrate is then recognized by the 26S proteasome as a target for degradation. In some cases, proteins may be modified by K63-linked polyubiquitination, which can promote their aggregation into inclusion bodies and their subsequent removal by autophagy. The autophagy process involves the sequestration of substrates by a phagophore that expands into a double-membrane structure called autophagosome that engulfs the substrate. The autophagosome then fuses with a lysosome to form autolysosomes, within which the inner membrane of the autophagosome is broken down and the cargo degraded by acidic lysosomal hydrolases. Another form of autophagy is chaperone-mediated autophagy (CMA), which involves the direct translocation of unfolded substrate proteins across the lysosomal membrane through the actions of a cytosolic chaperone hsc70, and an integral lysosomal membrane receptor LACMP2A (lysosome-associated membrane protein type 2A).

aggregation, as a major component of LBs (Spillantini et al., 1997). Accordingly, elucidating how α-synuclein interacts with the various protein quality control (QC) systems to result in LB biogenesis would be an important cornerstone upon which a better understanding of PD pathogenesis could be built.

We now know that all the three protein QC systems (i.e., chaperone, UPS, and autophagy) are actively involved in the cellular management of α-synuclein. Not surprisingly, the respective inhibition of chaperone, proteasome, or autophagy function enhances the accumulation of α-synuclein and simultaneous inhibition of these systems promotes a synergic formation of α-synuclein-positive inclusions (Rott et al., 2008). In turn, the accumulation of α-synuclein can exert reciprocal effects on the various QC systems. For example, components of the Hsp70 chaperone system (Hsp70 and Hsp40) can be depleted through their sequestration within α-synuclein-positive aggregates (Auluck et al., 2002). Further, aggregated α-synuclein species can selectively interact with the components of the proteasome complex and concomitantly inhibit its function (Snyder et al., 2003). Finally, α-synuclein can also inhibit macroautophagy (otherwise referred to as "autophagy"). This was exemplified in a mouse model of PD where α-synuclein is over-expressed. These mice exhibit signs of autophagy inhibition that apparently occurs at a very early stage of autophagosome formation (Winslow et al., 2010). Supporting this, another group recently reported that α-synuclein over-expression can result in disrupted localization and mobilization of Atg9, a multi-spanning membrane protein whose associated vesicles are important sources of membranes for the synthesis of early autophagosomes, thereby providing a mechanism for α-synuclein-induced inhibition of autophagy (Yamamoto et al., 2012). Besides macroautophagy, α-synuclein can also affect the function of chaperone-mediated autophagy (CMA), a specialized form of lysosomal degradation where proteins like α-synuclein containing a particular penta-peptide motif related to KFERQ are transported across the lysosomal membrane via the action of the integral membrane protein LAMP-2A that is assisted by both cytosolic and lumenal hsc70 (Klionsky et al., 2011) (**Figure 1**). Membrane bound α-synuclein species harboring disease-associated mutations or those modified by DA bind to the CMA lysosomal receptor with high affinity but are poorly translocated, which allow them time to seed the formation of oligomeric complexes on the membrane surface that consequently places the translocation complex under siege (Cuervo et al., 2004; Martinez-Vicente et al., 2008). The resulting blockage of uptake and degradation of CMA substrates further amplifies the burden of misfolded protein load (including α-synuclein) for the cell and perpetuates a vicious cycle of protein aggregation that can lead to the demise of neurons, especially dopaminergic neurons. Consistent with this, CMA inhibition following L-DOPA treatment is more pronounced in ventral midbrain cultures containing dopaminergic neurons than in non-DA producing cortical neurons (Martinez-Vicente et al., 2008).

It is apparent from the above that the chaperone, ubiquitin-proteasome, and autophagy pathways all have a role to play in the biogenesis of α-synuclein-positive LBs and thereby PD. Accordingly, pharmacological or genetic inhibition of these protein QC pathways (particularly those involved in protein degradation) in animal models should in theory be able to recapitulate the disease process. Although controversial, several groups have indeed reported evidence of SN dopaminergic neurodegeneration and associated locomotion deficits as well as the presence of neuronal inclusions in rodents subjected to subcutaneous injections of either naturally occurring or synthetic proteasome inhibitor (Lim, 2007). Using a genetic approach, Bedford et al. (2008) made similar observations in mice that are selectively depleted of functional proteasomes in their SN, which exhibit extensive nigro-striatal degeneration that is accompanied by the presence of α-synuclein-positive LB-like inclusions. Likewise, targeted genetic ablation of essential autophagy components (i.e., Atg5 or Atg7) in neural cells of mice also results in extensive neurodegeneration and widespread inclusion pathology (Hara et al., 2006; Komatsu et al., 2006). Moreover, when autophagy is selectively disrupted in midbrain dopaminergic neurons, it results in abnormal presynaptic accumulation of α-synuclein that is accompanied by dendritic and axonal dystrophy, reduced striatal DA content, and the formation of somatic and dendritic ubiquitinated inclusions (Friedman et al., 2012). Importantly, these conditionally knockout mice exhibit significant age-dependent loss of nigral dopaminergic neurons that is accompanied by markedly decreased spontaneous motor activity and coordination relative to controls (Ahmed et al., 2012; Friedman et al., 2012). Together, these studies strongly support a role for proteasomal and lysosomal dysfunction in disease pathogenesis.

Perhaps the most direct evidence to date linking lysosomal impairments to PD is the demonstration that loss-of-function mutations in a gene encoding for the lysosomal P-type ATPase named *ATP13A2* cause a juvenile and early-onset form of parkinsonism (albeit one that is also characterized by pyramidal degeneration and dementia) (Ramirez et al., 2006). What is particularly noteworthy is that the expression and toxicity of α-synuclein is enhanced in patient-derived fibroblasts as well as in ATP13A2-silenced primary mouse neurons as a result of impaired lysosomal degradation capacity arising from deficient ATPase function (Usenovic et al., 2012). Importantly, silencing of endogenous α-synuclein ameliorated the toxicity in neurons depleted of ATP13A2, suggesting that ATP13A2-induced parkinsonism may be contributed by α-synuclein accumulation as a result of functional impairments of the lysosome. Supporting this, over-expression of wild type ATP13A2 suppresses α-synuclein-mediated toxicity in *C. elegans* while knockdown of ATP13A2 expression promotes the accumulation of misfolded α-synuclein in the animal (Rappley et al., 2009). Collectively, these studies further emphasize a patho-physiological link between lysosomal dysfunction and α-synuclein in dopaminergic neurodegeneration.

Besides α-*synuclein* and *ATP13A2*, several other PD-linked genes have also been associated directly or indirectly to either the ubiquitin-proteasome and/or autophagy-lysosome systems. Among these is parkin, a ubiquitin ligase (E3) that several groups including ours have shown to be involved in both the proteasomal and autophagy QC systems. Parkin is a unique multifunctional E3 member capable of mediating multiple forms of ubiquitin modifications including mono-ubiquitination, K48-linked (proteasome-associated) and K63-linked (proteasome-independent) polyubiquitination (Dawson and Dawson, 2010). The fate of a parkin substrate thus depends on the ubiquitin topology it receives. For

example, while parkin-mediated (presumably K48-linked) ubiquitination of the substrates AIMP2 and PARIS coupled them to proteasome-mediated degradation (Ko et al., 2005; Shin et al., 2011), we and others have shown that parkin-mediated K63-linked ubiquitination of synphilin and mutant DJ-1 promotes their aggregation into inclusion bodies and their subsequent removal via autophagy (Lim et al., 2005; Olzmann et al., 2007). Thus, parkin-mediated protein QC appears to involve both the proteasome and lysosome degradation machineries. Accordingly, one could envision that disease-associated parkin mutations that result in the functional disruption of its activity can lead to the toxic accumulation of both soluble (that would otherwise be cleared by the proteasome) and/or aggregated forms (that would otherwise be cleared by autophagy) of its broad spectrum of substrates. Not surprisingly, the functional assignment of parkin as a ubiquitin ligase at the turn of the century had fueled intense research into the role of the ubiquitin-proteasome system (UPS) in PD pathogenesis, which arguably has become less "trendy" now. In recent years, the attention of parkin-UPS axis has shifted toward its ability to remove damaged mitochondria via a specialized form of autophagy known as "mitophagy" (Narendra et al., 2008), a term originally coined by Lemasters (2005). Accordingly, impairment in mitochondrial QC due to failed mitophagy in parkin-deficient neurons is now thought to be a key mechanism that predisposes them to degeneration. Understanding precisely how parkin regulates mitochondrial QC and how disruptions in this process contribute to PD pathogenesis is a current "hot" topic amongst PD researchers that has helped rekindle widespread interest in an "old" pathogenic culprit.

MITOCHONDRIAL DYSFUNCTION AND PD

A role for mitochondria dysfunction in the pathogenesis of PD has long been appreciated. The idea that mitochondrial dysfunction could contribute to the development of PD originates from the observation by Langston et al. (1983) in the early eighties that drug abusers exposed to 1-methyl-4-phenyl-1,2,3,4-tertahydropyridine (MPTP), an inhibitor of mitochondrial complex I function, display motoric features that bear uncanny resemblance to those exhibited by sporadic PD patients. Further, through post-mortem analysis performed as early as 1989, several groups have recorded a significant reduction in the activity of mitochondrial complex I as well as ubiquinone (co-enzyme Q10) in the SN of PD brains (Schapira et al., 1989; Shults et al., 1997; Keeney et al., 2006). Consistent with the proposed role of mitochondrial dysfunction as a pathogenic driver of PD, mitochondrial poisoning through the administration of toxins such as MPTP and rotenone recapitulates PD-related features in animals and represents a popular strategy to model the disease (Dauer and Przedborski, 2003). Interesting, whereas dopaminergic neurodegeneration induced by MPTP can be explained by the fact its conversion into its toxic principle MPP+ endow it with the selectivity for dopaminergic neurons (by virtue of the exquisite affinity MPP+ has for DA transporters), rotenone by comparison is more broadly distributed in the brain following its administration into animals (Betarbet et al., 2000). Despite the more systemic distribution of rotenone in treated animals, its toxicity is mostly confined to dopaminergic neurons, suggesting that dopaminergic neurons are uniquely susceptible to complex I inhibition (Betarbet et al., 2000). Similarly, impairment of mitochondrial homeostasis via genetic ablation of TFAM, a mitochondrial transcription factor that plays a critical role in maintaining mitochondrial DNA, in dopaminergic neurons of mice results in an energy crisis and neurodegeneration (Sterky et al., 2011). Moreover, the neuronal loss is progressive and accompanied by intraneuronal cytoplasmic inclusions (albeit not α-synuclein-positive). This interesting mouse model that rather faithfully recapitulates the salient features of PD is popularly known as the "MitoPark" mouse, although critics maintain that this model is of limited therapeutic utility as the mutation is not based on human PD genetics. Notwithstanding this, these above studies when taken together provide compelling support for a role of mitochondrial dysfunction in PD pathogenesis.

Less is however known about how mitochondria become defective in PD. It is important to recognize that mitochondria are not solitary and static structures as depicted in many textbooks but rather are dynamic and mobile organelles that constantly undergo membrane re-modeling through repeated cycles of fusion and fission. In addition, the organelle also undergoes regulated turnover via mitophagy when it is damaged beyond repair. It follows that mitochondrial dysfunction can occur at different levels ranging from organelle biogenesis, fusion/fission to mitophagy. Indeed, genetic mutations that disrupt the function of mitochondrial fusion/fission regulators leads to neurodegenerative diseases such as Charcot–Marie–Tooth type 2A (Zuchner et al., 2004) and autosomal dominant optic atrophy (Alexander et al., 2000; Delettre et al., 2000) although not PD *per se*. At least for parkin-related cases, a mechanism underlying mitochondrial dysfunction has recently emerged (**Figure 2**). Briefly, the proposed model posits that parkin collaborates closely with another PD-linked gene known as PINK1, a mitochondrial serine/threonine kinase, to initiate the removal of depolarized/damaged mitochondria. A key initial event for mitophagy to occur is the selective accumulation of PINK1 on the outer membrane of the damaged organelle, which is otherwise prevented by a series of sequential proteolytic events in healthy mitochondria (Becker et al., 2012; Greene et al., 2012). In depolarized mitochondria, PINK1 stabilization on the outer membrane enables the protein to recruit parkin to the organelle, a process that is apparently dependent on PINK1 autophosphorylation at Ser228 and Ser402 (Okatsu et al., 2012). This event some how triggers parkin self-association (Lazarou et al., 2013), which is likely to unmask its latent activity, the consequence of which is the ubiquitination and subsequent degradation of several outer membrane protein members (Chan et al., 2011; Yoshii et al., 2011) including the pro-fusion mitofusin proteins (Poole et al., 2010; Ziviani et al., 2010). The degradation of mitofusins is probably critical to prevent unintended fusion events involving damaged mitochondria and thereby their re-entry into the undamaged mitochondrial network from occurring. Mitophagy induction then occurs, which likely involves parkin-mediated K63 ubiquitination that will help recruit the autophagy adaptors HDAC6 and p62, subsequently leading to mitochondrial clustering around the peri-nucleus region. By virtue of their association with the autophagy process, the concerted actions of p62 and HDAC6 will presumably facilitate the final removal of damaged mitochondria by the lysosome (Ding et al., 2010; Geisler et al., 2010; Lee et al., 2010). Interestingly,

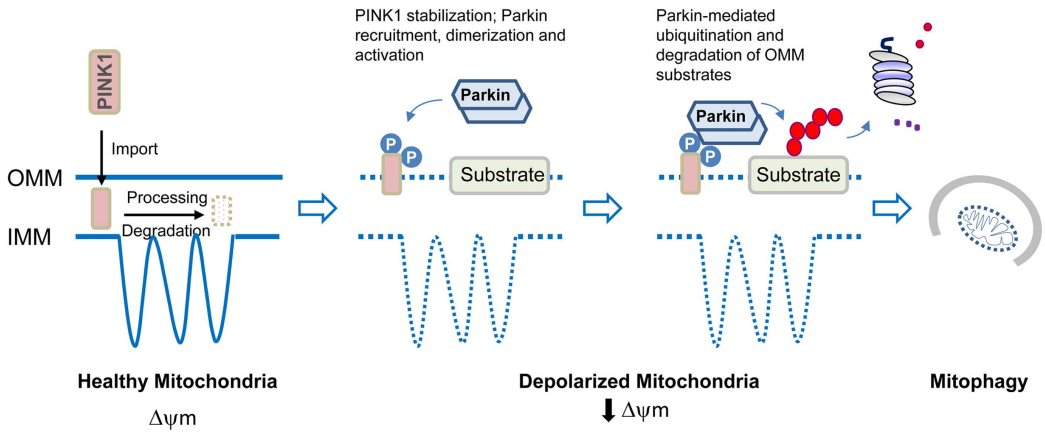

FIGURE 2 | Model of parkin/PINK1-mediated mitophagy. In healthy mitochondria, PINK1 imported through the outer mitochondrial membrane (OMM) is rapidly processed and degraded. Upon mitochondrial depolarization, PINK1 stabilization on the OMM leads to its dualautophosphorylation on Ser228 and Ser402. This event somehow triggers parkin recruitment, self-association, and catalytic activation. Parkin ubiquitinates several proteins on the OMM that results in their degradation by the proteasome. Mitophagy induction then occurs.

according to a recent report from Mizushima's lab, mitophagosomes may be generated in a *de novo* fashion on damaged mitochondria to initiate their removal. The authors demonstrated that parkin recruitment on the mitochondria induces the formation of ULK1 (Atg1) puncta (an upstream nucleation step of the hierarchical autophagy cascade) and Atg9 structures (Itakura et al., 2012), although it remains unclear mechanistically how parkin participates in the *de novo* synthesis of isolation membrane.

Although several groups have demonstrated that PD-associated parkin mutants are defective in supporting mitophagy due to distinct problems at recognition, transportation, or ubiquitination of impaired mitochondria (Lee et al., 2010; Matsuda et al., 2010), a pertinent question to ask is whether deficient mitochondrial QC is relevant at all to the large number sporadic PD cases where parkin is not mutated. Although this remains to be established, it is noteworthy to mention that we and others have previously shown that parkin dysfunction could arise in the PD brain in the absence of apparent mutations. This could be a result of stress-induced biochemical alterations including oxidation and nitrosylation, post-translational modifications or aberrant protein–protein interaction that can either alter the catalytic function of the E3 ligase directly, or indirectly through promoting its aggregation or degradation (LaVoie et al., 2005; Wang et al., 2005). Interestingly, normal parkin in the brain also becomes progressively more detergent-insoluble (and therefore non-functional) with aging (Pawlyk et al., 2003), which may provide an explanation to why age represents a risk factor for PD. In all these cases, the loss of parkin function is expected to compromise the efficiency of parkin-mediated mitophagy, amongst other parkin-regulated events. Thus deficient mitochondrial QC may not necessarily be restricted to cases where parkin (or PINK1) is overtly mutated. Moreover, it is also becoming increasingly clear that various other PD-linked proteins that may appear to have disparate functions could all influence mitochondrial homeostasis, directly or indirectly (Lim et al., 2012). For example, several groups including ours have recently found that the disease-associated LRRK2 G2019S mutant can trigger marked mitochondrial abnormalities when over-expressed in cultured cells and *in vivo* (Ng et al., 2012; Niu et al., 2012; Wang et al., 2012). Corroborating these findings, Mortiboys et al. (2010) demonstrated that cells derived from LRRK2 G2019S patient exhibited similar mitochondrial abnormalities, a phenotype that was also shared by neural cells derived from LRRK2 patients via induced pluripotent stem cell technology (Cooper et al., 2012). More recently, investigators from Chu Lab reported that LRRK2 elicited calcium imbalance and depletion of dendritic mitochondria in neurons (Cherra et al., 2013). Thus, mitochondrial dysfunction seems to be a common denominator underlying both familial and sporadic forms of PD.

CROSSTALK BETWEEN THE PROTEIN AND MITOCHONDRIAL QC SYSTEMS

What is clear from the above description is that the crosstalk between mitochondrial QC sensor and the autophagy apparatus needs to be tightly regulated to ensure that the pool of organelles available to energy-demanding cells such as neurons are bioenergetically competent. Failure in autophagy will therefore be expected to affect mitochondrial QC as much as it will affect protein QC. Given this, it is perhaps not surprising to note the frequent co-occurrence of mitochondrial abnormalities and inclusion bodies, the latter appearance is arguably an indication of failed autophagy function. At the same time, mitochondrial QC is also intimately intertwined with UPS machinery. After all, the initial stages of mitophagy involves the ubiquitination and consequent degradation of mitofusin of several mitochondrial outer membrane proteins such as Tom 20, Tom 40, Tom 70, and Omp 25 (Chan et al., 2011; Yoshii et al., 2011). To facilitate this en-bloc removal of mitochondrial proteins, parkin activates the UPS upon translocation to the mitochondria. This occurs by means of the enrichment of the proteasome-linked K48-linked ubiquitination of targeted proteins and recruitment of the proteasome to the mitochondria, the process of which is thought to lead to the rupturing of the mitochondrial outer membrane in preparation for

mitophagy induction (Chan et al., 2011; Yoshii et al., 2011). Besides mitophagy regulation, a recent study revealed that parkin can also potentially regulate mitochondrial biogenesis by regulating PGC-1α expression indirectly through its ability to down-regulate PARIS (Shin et al., 2011), which otherwise represses PGC-1α expression transcriptionally (Scarpulla, 2008). Again, proteasome function is at play here as the degradation of PARIS occurs via the proteasome machinery following its ubiquitination by parkin (Shin et al., 2011).

Besides parkin, other UPS-associated enzymes also participate in mitochondrial homeostasis. For example, the E3 members, MARCH-V and MULAN, as well as a deubiquitinating enzyme, USP30 are active regulators of mitochondrial dynamics (Livnat-Levanon and Glickman, 2011). All of these UPS-associated members reside on the mitochondrial outer membrane and collectively, they provide a link between the organelle and the proteasome. Interestingly, inclusion bodies formed in mice depleted of functional proteasomes contain mitochondria, suggesting a potential (albeit intriguing) role for the organelle in the biogenesis of protein inclusions (Bedford et al., 2008). In a reciprocal fashion, mitochondrial dysfunction can also impact proteasome function. Notably, energy in the form of ATP is required to assemble the proteasome complex as well as to drive the UPS machinery. Conceivably, in times of energy crisis, the UPS may not function optimally, which in turn may affect mitochondrial QC. Furthermore, an inevitable consequence of aerobic respiration is the generation of reactive oxygen species (ROS), which can modify components of the UPS itself, including E3 ligases that utilize active thiol groups. As alluded earlier, parkin is particularly susceptible to oxidative modification, which alters its solubility and promotes its aggregation in a manner analogous to that brought about by several of its missense mutations (LaVoie et al., 2005; Wang et al., 2005). Similarly, the 19S regulatory cap of the proteasome also appears to be especially sensitive to oxidation. Indeed, the S6/Rpt3 subunit of the 19S cap has been identified to be a major target of carbonylation in cells exposed to inducers of ROS (Ishii et al., 2005). That UPS components are prone to oxidative modification is somewhat paradoxical, as they are ones in the first place responsible for the efficient clearance of proteins damaged by oxidation. Interestingly, a very recent report demonstrated that chronic mitochondrial impairment results in the disassembly of 26S proteasome via calpain-mediated cleavage of Rpn10 that is accompanied by a concomitant increase in 20S proteasome level and activity (Huang et al., 2013). The authors posit that the increased function of 20S proteasomes, which can degrade proteins in an unregulated and energy-independent manner, may help the cell clear randomly unfolded oxidized proteins that would otherwise build up as a result of mitochondrial dysfunction. Obviously, this strategy is beneficial only for the short-term, i.e., if chronic, unregulated protein degradation will be detrimental to cellular survival. Taken together, it is apparent that the UPS, autophagy, and mitochondrial systems interact with, and exert reciprocal effects on one another, and that ROS generated by mitochondrial respiration can modify the function of these systems thereby adding another layer of complexity to an already complex relationship.

OXIDATIVE STRESS AND PD

The production of ROS is intimately associated with mitochondrial function as well as with its dysfunction. As mentioned earlier, ROS generation represents an inevitable consequence of mitochondrial respiration. During the process of aerobic respiration, partial reduction of molecular oxygen to superoxide anion (O_2^-) occurs when electrons leak from the electron transport chain or ETC (particularly at complex I). This free radical can be converted to the highly reactive hydroxyl radical ($OH^\bullet$) via an iron-catalyzed reaction known as Fenton reaction, or to peroxynitrite ($ONOO^-$) upon reaction with nitric oxide (NO). Both hydroxyl radical and peroxynitrite are potent oxidants that can cause marked cellular damage by reacting with proteins, lipids, and nucleic acids. Further, these reactive species may also target the ETC, which results in a feed forward cycle of increasing oxidative stress and injury.

The brain is often thought to be particularly susceptible to oxidation-induced damage because of its high metabolic rate and its relatively reduced capacity to replenish its post-mitotic neuronal populations compared with other organs. For SN dopaminergic neurons, the vulnerability toward oxidative stress is further enhanced by the abundance of redox-active iron in this region of the brain, as well as by the presence of DA, whose oxidation products are potentially cytotoxic (Graham, 1978). Notably, several groups have reported that markers for lipid peroxidation (including 4-hydroxynonenal and malondialdehyde), protein carbonyl modifications and even DNA and RNA oxidation are markedly elevated in the SN of post-mortem PD brains (Alam et al., 1997a,b; Zhang et al., 1999), and that these ROS-induced events are accompanied by a dramatic depletion of reduced glutathione (presumably leading to a considerably weakened antioxidant defense system) (Sian et al., 1994). As mentioned earlier, mitochondrial poisons that recapitulates PD features in humans and animals alike often target complex I, the impairment of which enhances superoxide production and thereby the formation of highly reactive free radicals that can initiate neuronal death. Importantly, oxidative damage and nigral dopaminergic neurodegeneration appears to correlate in a temporal manner in these models, suggesting a causal role of oxidation-induced stress in PD pathogenesis (McCormack et al., 2002; Peng et al., 2005).

Although the Redox chemistry of DA and the abundance of iron in SN dopaminergic neurons may underlie their heightened level of oxidative stress compared to other neuronal subtypes, another tantalizing culprit may be a unique channel type that resides on nigral dopaminergic neurons known as L-type Ca^{2+} channels. Unlike their counterparts in the Ventral Tegmental Area (VTA), SN dopaminergic neurons use L-type Ca^{2+} channels to help maintain autonomous pace-making (Chan et al., 2007). Because L-type Ca^{2+} channels are open most of the time (as they are open at relatively hyperpolarized state), the nigral neurons would experience a significantly larger magnitude and spatial extent of Ca^{2+} influx with time, which obviously comes with a price. Normally, the level of intracellular Ca^{2+} is under very tight homeostatic control by the actions of ATP-dependent pumps whose operations are metabolically expensive. A sustained entry of Ca^{2+} intonigral neurons would presumably work the mitochondria machinery

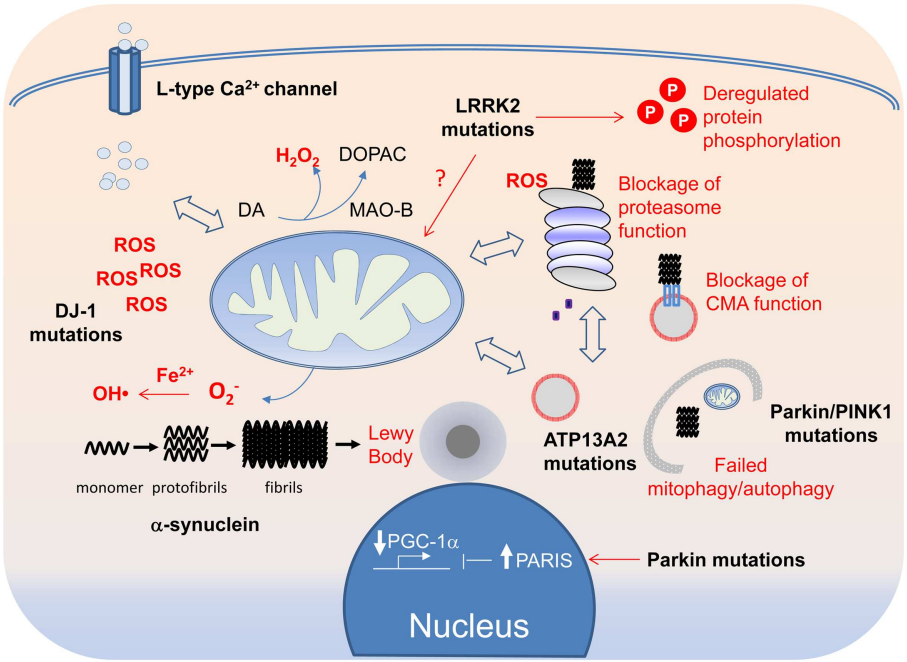

FIGURE 3 | A tapestry of molecular events in PD pathogenesis. Disruption of mitochondrial and protein QCs can arise from overt PD-linked genetic mutations or through oxidative modifications of their components by ROS, the levels of which can be elevated by DJ-1 mutations, Fe^{2+}-mediated Fenton reaction or increased Ca^{2+} influx through the L-type Ca^{2+} channel. Because of the crosstalk that exists between the QC systems, each can in turn affect the other in a reciprocal fashion. Aberrant mitochondrial and protein QCs and redox imbalance all can promote the formation of α-synuclein protofibrils and fibrils, which in turn can block the function of the proteasome and CMA. Aggregated α-synuclein species, if not cleared in a timely fashion, can also seed the formation of LBs, which can be physically obstructive to neuronal function if allowed to grow.

harder and concomitantly raise the level of ROS that would predispose them to oxidative stress-induced degeneration. Indeed, in mice engineered to carry a mitochondrial-localized redox-sensitive form of GFP, the basal oxidation as measured by this reporter is significantly higher in SN dopaminergic neurons relative to their VTA counterparts. Importantly, the enhancement of which can be lowered simply by the administration of L-type Ca^{2+} channel antagonists into these transgenic mice (Guzman et al., 2010). The "L-type Ca^{2+} hypothesis" is certainly an attractive proposition to explain the unique vulnerability of SN dopaminergic neurons toward degeneration. Moreover, neurons in the locus ceruleus region that are also lost in the PD brain are similarly autonomous pacemaker dependent on the activity of L-type Ca^{2+} channels (Williams et al., 1984). However, the hypothesis is not an adequate explanation for all the susceptible sites in the PD brain, which extend beyond the dopaminergic and noradrenergic systems (Braak et al., 2003).

If the autophagy and UPS models of PD are supported by disease-linked ATP13A2 and parkin mutations respectively, then mutations in the redox-sensitive protein, DJ-1, which causes an early-onset form of PD, would provide the genetic support for the role of oxidative stress in PD pathogenesis. DJ-1 is thought to operate as an atypical peroxiredoxin-like peroxidase that is capable of scavenging mitochondrial H_2O_2 (Canet-Aviles et al., 2004). Consistent with this, increased levels of H_2O_2 in mitochondria can be isolated from DJ-1 knockout mice (Andres-Mateos et al., 2007). Notably, a pool of DJ-1 is known to be localized to the mitochondria (Canet-Aviles et al., 2004; Zhang et al., 2005), suggesting a functional link between DJ-1 and the organelle. Moreover, loss of DJ-1 function promotes mitochondrial fragmentation in a variety of cells including lymphoblast cells derived from DJ-1 patients and sensitizes them toward oxidative stress-induced death (Irrcher et al., 2010; Krebiehl et al., 2010; Thomas et al., 2011), a phenotype that can be rescued by restoration of functional DJ-1 expression or by scavengers of ROS (Irrcher et al., 2010; Thomas et al., 2011). Interestingly, a recent study suggest that DJ-1 enhances ERK-dependent mitophagy in the presence of the parkinsonian neurotoxin rotenone and in so doing protects dopaminergic neurons against toxin-induced apoptosis (Gao et al., 2012). Accordingly, the absence of DJ-1 may predispose dopaminergic neurons to mitochondrial dysfunction and oxidative stress-induced degeneration. Indeed, DJ-1-deficient animals are hypersensitive to pharmacological inducers of oxidative stress (Kim et al., 2005; Menzies et al., 2005; Meulener et al., 2005; Park et al., 2005; Yang et al., 2005; Manning-Bog et al., 2007). Consistent with this, dopaminergic neurons derived from *in vitro* differentiated DJ-1-deficient embryonic stem cells display decreased survival and increased sensitivity to oxidative stress (Martinat et al., 2004). Importantly, the ablation of DJ-1 expression results in the amplification of basal oxidant stress in SN dopaminergic neurons (Guzman et al., 2010).

Finally, it is important to recognize that besides intrinsic sources of ROS, oxidative radicals can also come extracellularly from

activated glial cells, which is well documented in affected regions of the PD brain, as well as in genetic and toxin-induced models of PD (Hald and Lotharius, 2005). Although glia-mediated inflammatory events are often perceived as secondary to intrinsic events happening in susceptible neurons, they can aggravate and/or perpetuate the pathogenic outcomes and as such may play an instrumental role in promoting neuronal cell death. Indeed, the role of neuro-inflammation is regaining its prominence in the field as more and more researchers are now focusing on non-cell autonomous forms of death in neurodegenerative diseases (for a recent review, please refer to Hirsch et al., 2012).

CONCLUDING REMARKS – MAKING SENSE OUT OF THE APPARENT CHAOS?

Most readers would agree after reading the description above that the molecular events underlying PD pathogenesis is really complex (**Figure 3**). Indeed, even the genes associated with PD, which otherwise give the disease a tractable etiology, are so disparate in function that at first sight, they seem to have little things (if at all) in common. This is quite unlike the situation in Huntington's disease, which can be traced to a single genetic defect (i.e., mutations in the *Huntingtin* gene) or familial Alzheimer's disease where the majority of the disease-linked genes are clustered around the amyloid precursor protein processing pathway. Although disruptions in protein and mitochondrial QC are consistently implicated in PD pathogenesis and are generally accepted to be the key pathogenic drivers, additional pathogenic events that have recently emerged (or are emerging) include aberrant protein phosphorylation, endosome recycling, and lipid metabolism look set to complicate the picture. Furthermore, as we have discussed, the implicated pathways often act in a reciprocal fashion to influence one another. Moreover, it is also becoming increasingly clear that each of the PD-linked gene products, when dysfunctional, can exert effects on multiple pathways either directly or indirectly. Thus, no matter how upbeat one can be for a favored PD-related pathway, it is highly unlikely to be the only pathway involved in disease pathogenesis. To use an analogy regarding our current knowledge about the molecular events underlying PD pathogenesis – it seems like we are looking at a tapestry but on its reverse side where all the different colored threads are interwoven in a seemingly chaotic fashion. Undeniably, it is a significant challenge to make sense out of the apparent chaos. Nonetheless, we certainly have a better grasp of the pathogenic events happening in the PD brain these days than we have before as a result of concerted efforts in the past decade or so by many investigators around the world in unraveling the molecular causes of the disease. Although we remain uncertain about the initiating event, it is worthy to note that the myriad of pathways proposed to be involved in disease pathogenesis appears to be converging rather than diverging from each other. It is therefore perhaps not surprising to see that various PD-linked genes with apparently different function can directly or indirectly affect the same event (e.g., mitochondrial dysfunction). Paradoxically, recognizing that PD pathogenesis is a complex process may be the first step toward understanding how the tapestry of pathogenic events is weaved together.

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Structure and function of Parkin, PINK1, and DJ-1, the three musketeers of neuroprotection

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Autosomal recessive forms of Parkinson's disease are caused by mutations in three genes: *Parkin*, *PINK1*, and *DJ-1*. These genes encode for proteins with distinct enzymatic activities that may work together to confer neuroprotection. Parkin is an E3 ubiquitin ligase that has been shown to ubiquitinate substrates and to trigger proteasome-dependent degradation or autophagy, two crucial homeostatic processes in neurons. PINK1 is a mitochondrial protein kinase whose activity is required for Parkin-dependent mitophagy, a process that has been linked to neurodegeneration. Finally, DJ-1 is a protein homologous to a broad class of bacterial enzymes that may function as a sensor and modulator of reactive oxygen species, which have been implicated in neurodegenerative diseases. Here, we review the literature on the structure and biochemical functions of these three proteins.

Keywords: Parkinson's disease, Parkin, PINK1, DJ-1, ubiquitin, phosphorylation, mitochondria, oxidative stress

INTRODUCTION

Parkinson's disease (PD) is a degenerative movement disorder characterized by motor symptoms such as slowness of movement, tremor, rigidity, and postural instability. The motor symptoms are caused by the loss of dopaminergic neurons in the substantia nigra (Schapira and Jenner, 2011; Venderova and Park, 2012). Non-motor symptoms such as loss of olfaction, constipation, and rapid eye movement (REM) sleep disorder are also central to PD and can precede the motor symptoms. Although most cases of PD are idiopathic, there are rare familial forms of the disease that follow Mendelian inheritance patterns that can be traced to single gene mutations (Martin et al., 2011). In particular, autosomal recessive PD is caused primarily by mutations in one of three genes that encode proteins with distinct enzymatic activities: Parkin, PINK1, and DJ-1. Pathogenic mutations in these genes include exonic rearrangements and missense or frameshift mutations (Mata et al., 2004; Tan and Skipper, 2007). Since these mutations lead to a loss of function, i.e., a reduced or abolished activity in the corresponding protein (Martin et al., 2011), we can infer that their normal function(s) prevent cell death. Thus a better understanding of their biochemical activities will help decipher the molecular mechanisms underlying neuronal cell death that causes PD. Here we review recently published studies that significantly advanced our understanding of the structure and biochemical mechanisms employed by Parkin, PINK1, and DJ-1, and highlight the knowledge gaps that need to be filled.

PARKIN

Parkin (*PARK2*) was the first gene associated with autosomal recessive PD (Kitada et al., 1998; Lücking et al., 2000). Parkin is a 52 kDa protein with an N-terminal ubiquitin-like (Ubl) domain followed by a 60 amino acid (a.a.) linker and four zinc-finger domains (**Figure 1**). Early studies showed that Parkin is an E2-dependent E3 ubiquitin ligase that binds UbcH7 and UbcH8 (Shimura et al., 2000; Zhang et al., 2000). An E3 ligase is an enzyme that catalyzes the transfer of ubiquitin, a small 76 a.a. protein, from an E2 ubiquitin-conjugating enzyme to a protein substrate. Ubiquitinated substrates then undergo different fates depending on the site and type of ubiquitination (described in more details below). However, even today, its cellular function and associated substrates remain controversial. Over the last decade, Parkin has been implicated in numerous seemingly unrelated cellular processes for which many substrates have been suggested (reviewed in Dawson and Dawson, 2010). However, in recent years much attention has converged on Parkin's role in bioenergetics and mitochondrial quality control pathways (reviewed in Exner et al., 2012). Importantly, Parkin is recruited to depolarized mitochondria where it drives their elimination by autophagy (mitophagy) (Narendra et al., 2008). Interestingly, early studies in Drosophila lacking Parkin revealed its crucial role in mitochondrial homeostasis, and that proteomics studies in Parkin knockout mice showed variations primarily in proteins involved in energy metabolism, mitochondrial function and oxidative stress (Palacino et al., 2004; Periquet et al., 2005). Parkin null mice were recently shown to be acutely resistant to weight gain when fed on a high fat diet due to reduced lipid uptake (Kim et al., 2011). Parkin was also shown to be a p53-target gene that mediates the well-known role of p53 in regulating glucose metabolism (Zhang et al., 2011), and regulates the levels of PGC-1α, an important regulator of mitochondrial biogenesis (Shin et al., 2011). Finally, Parkin was suggested to play a role in the clearance of proteins damaged as a result of dopamine oxidation, as well as in the metabolism of dopamine (Jiang et al., 2004, 2012). These processes all require the ligase activity of Parkin, but the precise molecular mechanisms that underlie these cellular processes remain obscure.

Elucidating the mechanism of ubiquitination by Parkin is crucial for understanding its biological function. The last three zinc-finger domains form an RING1, In-Between, RING2 (RBR) module, which is found in many other E3 ubiquitin ligases such as HHARI, HOIP, HOIL, and Dorfin (Wenzel and Klevit, 2012). A

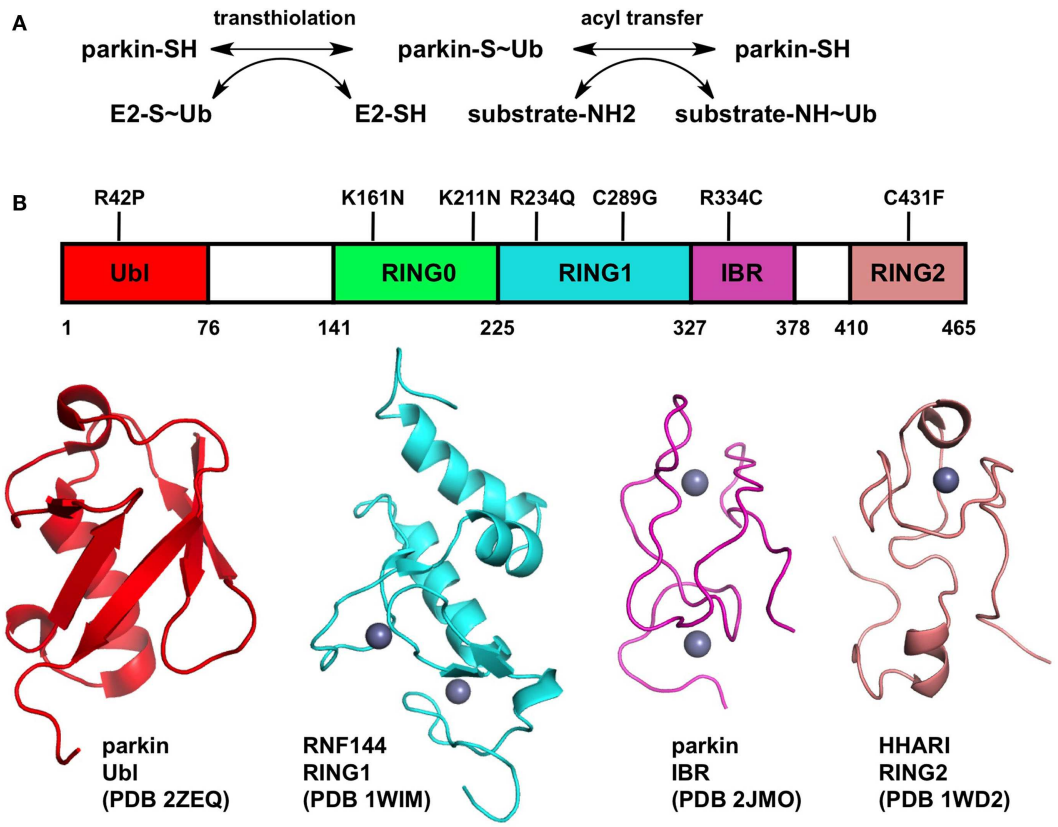

FIGURE 1 | Biochemical mechanism and structure of Parkin. **(A)** Two-step mechanism for substrate ubiquitination by Parkin. The first step (transthiolation) is the transfer of ubiquitin from a thioester bond on a cysteine on an E2 enzyme to a thioester bond on Parkin Cys431. The second step (acyl transfer) is the formation of an isopeptide bond on a substrate amino group, typically a lysine side-chain. **(B)** Structure of Parkin. The domain boundaries of full-length human Parkin and selected PD mutations are displayed at the top. The structures of individual domains from Parkin or RBR homologs are shown below. Zinc atoms are displayed as gray spheres.

major breakthrough in our understanding of Parkin function was the discovery that RBR proteins use a two-step catalytic mechanism similar to that used by HECT ubiquitin ligases (Wenzel et al., 2011). The discovery stemmed from the observation that the E2 enzyme UbcH7 can only discharge ubiquitin onto cysteine, as opposed to other E2s like UbcH5C that can discharge on cysteine or lysine. Consequently, E3 ligases that are able to use UbcH7 as a conjugating enzyme, like HECT ligases, must bear an acceptor cysteine. In RBR ligases, this cysteine is located in the RING2 domain. Thus in the first reaction (transthiolation), the C-terminus of ubiquitin is transferred from an E2 enzyme's cysteine to the acceptor cysteine, resulting in the formation of a thioester intermediate (**Figure 1**). In Parkin, the acceptor cysteine Cys431 is required for its ubiquitin ligase activity (Zhang et al., 2000; Wenzel et al., 2011) and the mutation C431F causes PD (Maruyama et al., 2000), in agreement with its proposed role in catalysis. The second step of the reaction (acyl transfer) involves the transfer of ubiquitin C-terminus from the acceptor cysteine to an amino group on a substrate, forming an isopeptide bond. This HECT-type catalytic model has recently been confirmed for the E3 ligase HOIP, which synthesizes linear polyubiquitin chains (Smit et al., 2012; Stieglitz et al., 2012). Moreover, activation of Parkin upon mitochondrial depolarization induces the HECT-like activity of Parkin, resulting in the formation of an oxyester Parkin∼Ub adduct for the C431S mutant of Parkin (Lazarou et al., 2013). This model has important functional implications for Parkin and may help resolve apparent contradictions in the plethora of biochemical studies on Parkin. For example, binding of E2 enzyme has been reported to require an intact RBR module in cells: deletion of either RING1 or RING2 in Parkin abolished binding (Shimura et al., 2000). Recently, the RING1 domain of HOIP was shown to be required for its strong UbcH7-dependent activity, but not its weaker E2-independent activity, as opposed to RING2 which was required for both functions (Smit et al., 2012). Moreover, the sequence similarity of the RBRs RING1 domain to other E2-binding RING domains, which adopt the cross-brace zinc-binding topology, suggest that it could be the initial docking site of an E2∼Ub complex. Whether this implies that the E2 enzyme binds both RING1 and RING2 simultaneously remains to be investigated.

The structure of an entire RBR module would shine light on our understanding of how E2 enzymes bind Parkin and transfer ubiquitin. Although the structure of full-length Parkin has yet to be determined, structures of individual zinc-finger domains of Parkin and RBR homologs are available (**Figure 1**). Parkin binds a

total of eight zinc atoms and presumably each zinc-finger domain binds two zinc atoms (Hristova et al., 2009). Indeed the structures of the RBR protein RNF144A RING1 (pdb code 1wim, unpublished) and the Parkin IBR (Beasley et al., 2007) have two bound zinc atoms. However, the structure of HHARI RING2 binds only one zinc atom (Capili et al., 2004). To resolve this apparent contradiction, a recent study suggested a novel mode of zinc coordination by Parkin RING2, which comprises two zinc atoms: one bound by Cys418, Cys421, Cys436, Cys441, and the other by Cys446, Cys449, Cys457, and His461 (Rankin et al., 2011). The strong conservation of these eight residues across Parkin orthologs and cysteine mutagenesis studies (Wong et al., 2007) are consistent with this model, which remains to be confirmed by structure determination. The structure of Parkin RING2 could reveal how Cys431 can form a thioester bond with ubiquitin and enable transfer to a substrate amino group.

If the functions of RING1 and RING2 can be inferred from structural and biochemical studies, the functions of the RING0 and IBR domains remain mysterious. The structure of the Parkin IBR reveals a zinc-finger domain with an unusual zinc coordination topology (Beasley et al., 2007). The proximity of its N- and C-termini led Beasley et al. to suggest a role in positioning RING1 and RING2 in proximity, which would be required for the transfer of ubiquitin from a RING1-bound E2∼Ub complex to the acceptor cysteine in RING2. Mutagenesis studies in the zinc-binding cysteines of HOIP IBR domain have indeed shown that the IBR domain is required for its E2-dependent ubiquitin ligase activity (Smit et al., 2012). In this respect, the structure of an entire RBR module would give tremendous insights into the role of the IBR domain. In Parkin, the RBR module is preceded by the RING0 domain, a non-classical zinc-finger that was suggested to adopt a unusual hairpin topology based on weak sequence homology with the cysteine-rich domain of bacterial DnaJ (Rankin et al., 2011). The RING0 domain is unique to Parkin among RBR proteins and is conserved in all its orthologs, suggesting it plays a conserved function specific to Parkin. Future biochemical studies on Parkin should specifically address the role of the IBR and RING0 domain in its ubiquitin ligase activity.

One aspect of Parkin's function that requires thorough investigation is the type of polyubiquitin chains that it forms. E3 ubiquitin ligases transfer ubiquitin carboxy-terminus from an E2 cysteine thiol onto a protein primary amino group, typically a lysine side-chain ε-amino group or an amino-terminus. The acceptor protein can either be a substrate, or another ubiquitin molecule. The latter give rises to polyubiquitin chains, which can be linked through any of ubiquitin's seven lysines (K6, K11, K27, K29, K33, K48, or K63-linked chains) or its amino-terminus (linear chains). Using methylated or lysine-free (K0) ubiquitin, two studies on Parkin's *in vitro* ubiquitin ligase activity claimed that Parkin mediates multiple monoubiquitination, both on itself and on substrates (Hampe et al., 2006; Matsuda et al., 2006). This activity was shown to require only the RING2 domain, as pathogenic mutation in RING1 and IBR, as well as deletions containing only IBR and RING2, did not significantly affect its ubiquitin ligase activity. This contradicted earlier studies that showed both RING1 and RING2 were required for E2-dependent ubiquitination with immunoprecipitated Parkin (Shimura et al., 2000), and that Parkin can make K48 and K63 polyubiquitin chains in cells using one lysine-only ubiquitin variants (Doss-Pepe et al., 2005; Lim et al., 2005). More recently, Durcan et al. (2011) showed that Parkin can form K6, K27, K29, and K63 chains in cells, and that it can form polyubiquitin chains that are deconjugated by ataxin-3 *in vitro* (Durcan et al., 2012). Upon recruitment of Parkin to mitochondria depolarized with the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), K27, and K63 lysine-only ubiquitin variants were shown to accumulate on mitochondria (Geisler et al., 2010). However, in a separate study, K48 and K63-linked chains were directly detected by mass spectrometry on CCCP-depolarized mitochondria in a Parkin-dependent manner (Chan et al., 2011), and the same chains were also detected on Mfn1 using linkage-specific antibodies (Lazarou et al., 2013). Overall these studies strongly suggest that Parkin has the capacity to synthesize polyubiquitin chains. In the light of the HECT-type model recently proposed for RBR ligases (Wenzel et al., 2011), Parkin could have intrinsic chain type specificity independently of which E2 is used, as was shown for HECT E3 ligases (Kim and Huibregtse, 2009) and the RBR-containing LUBAC complex (Kirisako et al., 2006; Stieglitz et al., 2012). Recently, the IBR-RING2 domains of Parkin were indeed shown *in vitro* to make K48 chains in an E2-independent manner, although the assays were carried out at a high pH (8.8), which can considerably affect the reactivity of the nucleophiles involved in the ubiquitin transfer reactions (Chew et al., 2011). The type of chains and the factors involved in imparting linkage specificity to Parkin thus remain to be determined.

A number of substrates of Parkin have been proposed, but how most of them are recruited to Parkin is unknown. The prime suspect for that function is the Ubl domain, a protein-protein interaction domain that has been shown to bind ubiquitin-interacting motifs (UIM) (Sakata et al., 2003; Fallon et al., 2006), SH3 domains (Trempe et al., 2009) as well as its own C-terminus (Chaugule et al., 2011). A comparison of affinity constants shows that binding to the SH3 domain of endophilin-A and C-terminal of Parkin is in the 1–10 μM range (Trempe et al., 2009; Chaugule et al., 2011), whereas binding to UIMs is >100 μM (Safadi and Shaw, 2010). Structurally, the Ubl interacts with all its ligands via the same surface centered on Ile44. A number of PD mutations are found in the Ubl domain, implying that it is essential to the function of Parkin, although some mutations such as R42P unfold the domain, which can lead to its aggregation and degradation (Henn et al., 2005; Safadi et al., 2011). The Ubl domain is required for some proposed functions of Parkin, such as endophilin-A ubiquitination (Trempe et al., 2009) and the regulation of lipid uptake through ubiquitin-mediated stabilization of the CD36 lipid transporter (Kim et al., 2011). Although it is not known whether the Ubl binds a mitochondrial ligand, it has been shown to be required for efficient mitochondrial recruitment and mitophagy in two reports (Narendra et al., 2010; Shiba-Fukushima et al., 2012), but not in others (Geisler et al., 2010; Matsuda et al., 2010). This contradiction can be resolved by considering that Parkin without the Ubl domain has slower recruitment kinetics, as shown recently by Shiba-Fukushima et al. (2012). But since Parkin can be recruited to the mitochondria without the Ubl, it seems unlikely that recruitment to mitochondrial substrates such as mitofusin and Miro is

mediated by the Ubl domain. However, PD mutants in the RING0 domain (K161N, K211N, C212Y) have strongly impaired mitochondrial recruitment and clearance activity (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010), raising the possibility that the RING0 domain could mediate substrate recruitment on mitochondria.

The activity of Parkin appears to be regulated at multiple levels. The first is that Parkin appears to be auto-inhibited in its basal state. Deletion or mutations in the Ubl domain, as well as addition of Ubl-binding ligands or N-terminal tags, increase substantially the autoubiquitination activity of Parkin (Chaugule et al., 2011). Chaugule et al. proposed that Parkin auto-inhibition is maintained by the interaction of the Ubl with the C-terminal domains of Parkin, although exactly how this is achieved is unclear. ΔUbl Parkin does not bind E2 enzymes more strongly, but it exhibits slightly faster E2∼Ub discharging kinetics (Chaugule et al., 2011). Identifying the Ubl-binding site on Parkin's C-terminal domains will help resolve the mechanism of auto-inhibition.

The second level of Parkin regulation is through post-translational modifications. There is strong evidence that phosphorylation plays a key role in the regulation of Parkin. First, PINK1 kinase activity is required for the recruitment of Parkin to depolarized mitochondria and for the activation of its ubiquitin ligase activity (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010; Lazarou et al., 2013). FLIM studies suggest that Parkin and PINK1 are in close proximity on depolarized mitochondria (Vives-Bauza et al., 2010). Immunoprecipitations experiments have shown that Parkin and PINK1 are part of the same complex (Xiong et al., 2009; Sha et al., 2010; Vives-Bauza et al., 2010), but recent blue native PAGE (Lazarou et al., 2012) and size-exclusion chromatography (Thomas et al., 2011) studies showed that PINK1 and Parkin do not form a complex. Thus, whether PINK1 and Parkin bind each other remains controversial. However, *in vitro* experiments have demonstrated that PINK1 phosphorylates Ser65 in the Ubl domain, which increases its ubiquitin ligase activity (Kondapalli et al., 2012). Mutation of Ser65 to Ala significantly impaired, but did not inhibit, translocation of Parkin to depolarized mitochondria, suggesting that Ser65 phosphorylation primes Parkin for recruitment (Shiba-Fukushima et al., 2012). This effect could be mediated by the disruption of the autoinhibitory interaction of the Ubl domain (Chaugule et al., 2011). Phosphorylation can also negatively regulate Parkin: two groups found that Tyr143 phosphorylation by the protein tyrosine kinase c-Abl reduces Parkin ubiquitination activity *in vitro* and in cells (Ko et al., 2010; Imam et al., 2011). Parkin inactivation can also be brought upon oxidative stress (Winklhofer et al., 2003) and covalent modifications of its essential cysteines, either through S-nitrosylation or dopamine quinone adducts formation (Chung et al., 2004; LaVoie et al., 2005). Finally, Parkin levels may be regulated by autoubiquitination, and indeed its coupling to the deubiquitinating enzyme ataxin-3 appears to regulate its stability in cells (Durcan et al., 2011, 2012). Moreover, activation of Parkin upon mitochondrial membrane depolarization induces its degradation through the proteasome, suggesting that the auto-inhibition of Parkin may protect itself from ubiquitin-mediated degradation (Rakovic et al., 2012).

PINK1

The *PINK1* (*PARK6*) gene was first described as PTEN-induced putative kinase 1, a ubiquitous gene product whose expression was abolished in ovarian tumor tissues due to defect in PTEN signaling (Unoki and Nakamura, 2001). Later, mutations in *PINK1* were found to cause autosomal recessive PD (Valente et al., 2004). Mammalian PINK1 is a 581-residue protein, with an N-terminal mitochondrial targeting sequence, a transmembrane helix, a serine/threonine kinase domain, and a C-terminal domain of unknown function (**Figure 2**) (Beilina et al., 2005). Studies in Drosophila showed that PINK1 has a role in the maintenance of mitochondria, and this role is intimately linked to Parkin (Clark et al., 2006; Park et al., 2006). PINK1 was shown to regulate HtrA2, a mitochondria protease which plays a role in mitochondrial homeostasis (Plun-Favreau et al., 2007). PINK1 also regulates mitochondrial morphology in mammalian cells (Poole et al., 2008), and is essential for the recruitment of Parkin to mitochondria in cultured immortalized cells (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010; Lazarou et al., 2013) as well as in neurons (Wang et al., 2011; Joselin et al., 2012). Physiologically, PINK1 deficiency leads to an altered mitochondrial calcium buffering capacity and impaired respiration caused by a reduced provision of electron transport chain substrates (Gandhi et al., 2009). Recently, vitamin K2 was found to rescue PINK1 deficiency through its capacity to carry electrons (Vos et al., 2012). Thus PINK1 appears to have a clear role in mitochondrial maintenance, which contributes to neuronal survival.

Many of the mitochondrial functions carried by PINK1 depend on its kinase activity, which has been the focus of a number of studies. For example, whereas wild-type PINK1 can rescue Parkin mitochondrial localization in *PINK1*$^{-/-}$ mouse embryonic fibroblasts (MEFs), a kinase-dead mutant of PINK1 cannot (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). Therefore a better understanding of PINK1 kinase activity will give much insight into the function of PINK1. *In vitro*, recombinant PINK1 from different species can phosphorylate itself as well as artificial substrates such as α-casein or the myelin basic protein (Beilina et al., 2005; Silvestri et al., 2005; Woodroof et al., 2011). However, the degree of activity appears to be dependent on the PINK1 construction used, the expression system and the species. In one instance, recombinant human PINK1 (112–496), which was expressed in *Escherichia coli* and lacked the C-terminal domain, was found to have increased activity compared to a construct that contained the entire C-terminus (112–581) (Silvestri et al., 2005). The human PINK1 kinase domain (112–496) was also shown to be active in two earlier reports (Nakajima et al., 2003; Beilina et al., 2005). However, later studies obtained opposite results, although the construct boundaries were different: human PINK1 (148–581) expressed in Sf9 insect cells was more active than the isolated kinase domain (148–515) (Sim et al., 2006). In a survey of kinase activity across different PINK1 orthologs, *Tribolium castaneum* (Tc) PINK1 was the most active and human PINK1 was completely inactive (Woodroof et al., 2011). Moreover, TcPINK1 128–570 was more active than 155–570, and 155–486 had no activity. Overall, these studies suggest that the segments located at the

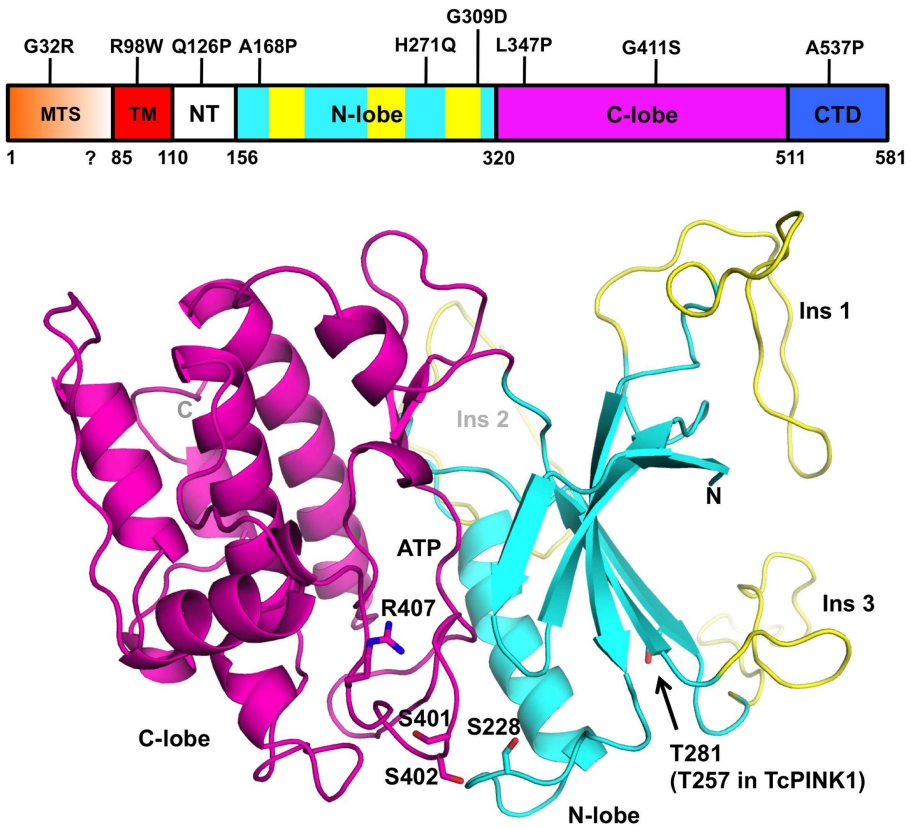

FIGURE 2 | Structural model of PINK1. The domain boundaries of full-length human PINK1 and PD mutations are displayed at the top. The domains are colored as follows: mitochondrial targeting sequence (MTS, orange), transmembrane helix (TM, red), N-terminal regulatory region (NT, white), N- and C-terminal lobes (cyan and magenta), C-terminal domain (CTD, blue). The three PINK1-specific insertions are colored in yellow. The coordinates for human PINK1 156–511 (bottom cartoon) were obtained from the Protein Model DataBase (PM0077187). The N- and C-terminal lobes are colored in cyan and magenta, respectively. The ATP binding site is located in the cleft between the two lobes. The three phosphorylation sites (Ser228, Ser402, Thr281) and activation loop residues (Ser401, Ser402, and Arg407) are shown as sticks. The model does not comprise the N-terminal (112–155) and C-terminal (512–581) regions of the soluble domain, which cannot be modeled by homology.

N- and C-termini of the kinase domain have regulatory functions, and that the human PINK1 may be auto-inhibited. The importance of these regions in regulating PINK1 is supported by the clustering of several PD mutations within these regions (Sim et al., 2012).

Although there is no crystal structure of PINK1 available, the similarity of its kinase domain to other serine/threonine kinases led a number of groups to perform homology modeling (Beilina et al., 2005; Mills et al., 2008; Cardona et al., 2011; Sim et al., 2012). The protein is most similar to the calmodulin-dependent kinase family, with which it shares a number of structural features (**Figure 2**). Overall, the kinase domain consists of N- and C-terminals lobes, which can be further subdivided into smaller subdomains found in most kinases. The cleft between the two lobes harbors the catalytic and ATP:Mg^{2+} binding sites (Cardona et al., 2011). Three loop insertions in the N-terminal lobe are unique to PINK1 and contain PD mutations, but the function of these inserts is unknown. The PINK1 kinase domain also contains an activation loop with two serine residues (Ser401-Ser402) whose phosphorylation was shown to be activating in other kinases (Nolen et al., 2004). The activation loop also contains Arg407, a PD mutation site and a potential site of interaction with a phosphorylated serine.

Similarly to Parkin, PINK1 is activated in cells upon mitochondrial membrane depolarization, but the mechanism of activation remains unknown. Activation appears to be linked to its abundance, localization and processing. Indeed, endogenous PINK1 is found at low levels in cells as a result of its high turnover rate (Lin and Kang, 2008; Narendra et al., 2010). PINK1 is normally imported through the mitochondrial outer and inner membranes, where it is successively cleaved by the mitochondrial processing peptidase (MPP) and PARL/AFG3L2 (Jin et al., 2010; Deas et al., 2011; Meissner et al., 2011; Greene et al., 2012). The MPP cleavage site in PINK1 is unknown but is probably located in the residue range amino acids 20–70, and PARL cleaves PINK1 between Ala103-Phe104 in the transmembrane helix (Jin et al., 2010; Deas et al., 2011; Kondapalli et al., 2012). This hydrophobic transmembrane helix was also suggested to act as a stop signal that prevents further translocation into the matrix of the mitochondria (Zhou et al., 2008; Lin and Kang, 2010; Becker et al., 2012). The resulting 52 kDa processing fragment may then undergo multiple fates: in

its soluble form it is exported to the cytosol where it is degraded by the proteasome (Lin and Kang, 2008; Narendra et al., 2010; Greene et al., 2012). However, a recent study showed using an *in vitro* import assay that the 52 kDa form can also be retained at the outer mitochondrial membrane (OMM) via its C-terminal fragment (Becker et al., 2012). Because mitochondrial import of PINK1 is driven by the inner membrane proton gradient, membrane depolarization leads to the accumulation of unprocessed PINK1 at the OMM (Narendra et al., 2010; Becker et al., 2012; Greene et al., 2012). Prior studies had shown that the kinase domain of PINK1 faces the cytoplasm in its unprocessed mitochondria-tethered form, which would enable PINK1 to phosphorylate both cytosolic and OMM proteins (Zhou et al., 2008; Lin and Kang, 2010). The complex membrane potential-dependence of PINK1 processing and localization would thus serve to link PINK1's activity to the state of a mitochondrion, thereby suggesting a mechanism for the detection of damaged mitochondria.

Recent studies point to the role of oligomerization and autophosphorylation in the activation of PINK1. Indeed, upon accumulation on the OMM, PINK1 forms a large 700 kDa multimeric complex with the translocase of the outer membrane (TOM) complex (Becker et al., 2012; Lazarou et al., 2012). This was proposed to allow for the rapid reimport and processing of PINK1 upon reestablishment of the membrane potential. Moreover, PINK1 autophosphorylates when it accumulates on mitochondria (Okatsu et al., 2012). In this study, Okatsu et al. showed that Ser402 in the activation loop was required for autophosphorylation, although the authors could not detect direct phosphorylation of this residue, but detected Ser228 phosphorylation. The double mutant S228A/S402A failed to rescue GFP-Parkin mitochondrial translocation in *PINK1*$^{-/-}$ MEFs, but S228D/S402D did rescue, suggesting that PINK1 autophosphorylation is required for Parkin activation. In a separate study, Kondapalli et al. (2012) found that Thr257 in TcPINK1 (Thr282 in human) is phosphorylated upon mitochondrial membrane depolarization, but mutation of that residue did not affect Parkin activation. The ability of PINK1 to multimerize and autophosphorylate is reminiscent of the activation mode of the homologous calmodulin-dependent kinases, which form dodecameric ring structures where the monomers phosphorylate each other upon binding Ca^{2+}-bound calmodulin (Rellos et al., 2010; Chao et al., 2011). Interestingly, Sha et al. (2010) observed that PINK1, affinity-purified from mammalian cells, could only phosphorylate Parkin in the presence of Ca^{2+}. Considering the role of PINK1 in regulating Ca^{2+} buffering by mitochondria (Gandhi et al., 2009), future studies should address how Ca^{2+} influences the activation of PINK1.

The identity of PINK1 substrate(s) remains a subject of much debate. Studies using artificial peptide substrates indicated that proline was the preferred amino acid at the +1 position (Woodroof et al., 2011), and PREDIKIN predicts a weak consensus phosphorylation site (Sim et al., 2012). Early studies identified the mitochondrial chaperone TRAP1 as a ligand and phosphorylation substrate of PINK1 (Pridgeon et al., 2007). More recently, Miro, a protein involved in the axonal transport of mitochondria, was also found to be phosphorylated by PINK1, resulting in Parkin-mediated ubiquitination and degradation of Miro (Wang et al., 2011). Although an earlier study failed to detect Parkin phosphorylation by PINK1 *in vitro* (Vives-Bauza et al., 2010), Parkin was later reported to be phosphorylated by PINK1 *in vitro* and *in vivo* (Sha et al., 2010). More recently, *in vitro* assays with recombinant proteins failed to detect significant phosphorylation of either TRAP1 or Miro, but detected significant phosphorylation of Parkin on Ser65 in the Ubl (Kondapalli et al., 2012). Parkin Ser65 phosphorylation was later confirmed and showed to be required for efficient Parkin mitochondrial translocation (Shiba-Fukushima et al., 2012). Therefore it appears that Parkin could be the main substrate of PINK1, but the structural basis for PINK1 kinase activity targeting the Parkin Ubl remains unknown.

DJ-1

The *DJ-1* (*PARK7*) gene was first identified as an oncogene (Nagakubo et al., 1997), and similarly to PINK1, was later associated with familial PD (Bonifati et al., 2003). The protein has neuroprotective activity and affects sensitivity to oxidative stress (Canet-Aviles et al., 2004; Martinat et al., 2004). The effect could be mediated through localization to mitochondria, where it can reduce the oxidative stress induced by inhibitors of the respiratory chain such as rotenone (Canet-Aviles et al., 2004; Blackinton et al., 2009). DJ-1 deficiency leads to altered mitochondrial morphology and increases the production of reactive oxygen species (ROS) as a results of altered mitochondrial dynamics (Irrcher et al., 2010). DJ-1-dependent mitochondrial defects can be rescued by addition of a cell-permeable glutathione precursor or Parkin/PINK1 overexpression (Thomas et al., 2011). Recently, DJ-1 was found to negatively regulate PINK1-dependent Parkin translocation to depolarized mitochondria in neurons, as a result of its ability to control ROS generation (Joselin et al., 2012). Overall, functional data indicate that DJ-1 protects cells from oxidative stress caused by ROS, but how this is achieved at the molecular level is unclear.

The DJ-1 protein forms a single 20 kDa domain homologous to a number of bacterial enzyme families such as ThiJ, a protein involved in thiamine biosynthesis, and the Pfp1 protease (Bonifati et al., 2003), as well as the YajL redox-sensitive chaperone (Gautier et al., 2012). Its three-dimensional structure has been determined by several groups nearly 10 years ago (Honbou et al., 2003; Lee et al., 2003; Tao and Tong, 2003; Wilson et al., 2003), and shows a compact globular domain with an active site Cys106 particularly sensitive to oxidation (**Figure 3**). In solution, DJ-1 forms a stable dimer (Wilson et al., 2003). The PD mutation L166P, which abolishes dimerization, also abrogates its neuroprotective activity, suggesting that dimerization is essential to its function (Olzmann et al., 2004).

In spite of this wealth of structural and homology data, the precise biochemical function of DJ-1 has surprisingly not yet been ascribed. Its closest homolog in bacteria, ThiJ, catalyses the phosphorylation of hydroxymethylpyrimidine (HMP) to HMP-phosphate, a thiamine derivative (Mizote et al., 1996). Under oxidative conditions the mammalian DJ-1 forms a cysteine-sulfinic acid adduct *in vitro* and in cells, which has been proposed to drive mitochondrial localization and neuroprotection (Canet-Aviles et al., 2004; Blackinton et al., 2009). The molecular consequences of adduct formation remain to be investigated, but it likely modifies one of its many proposed enzymatic activities. DJ-1 bears homology to bacterial proteases and although its Cys/His/Glu

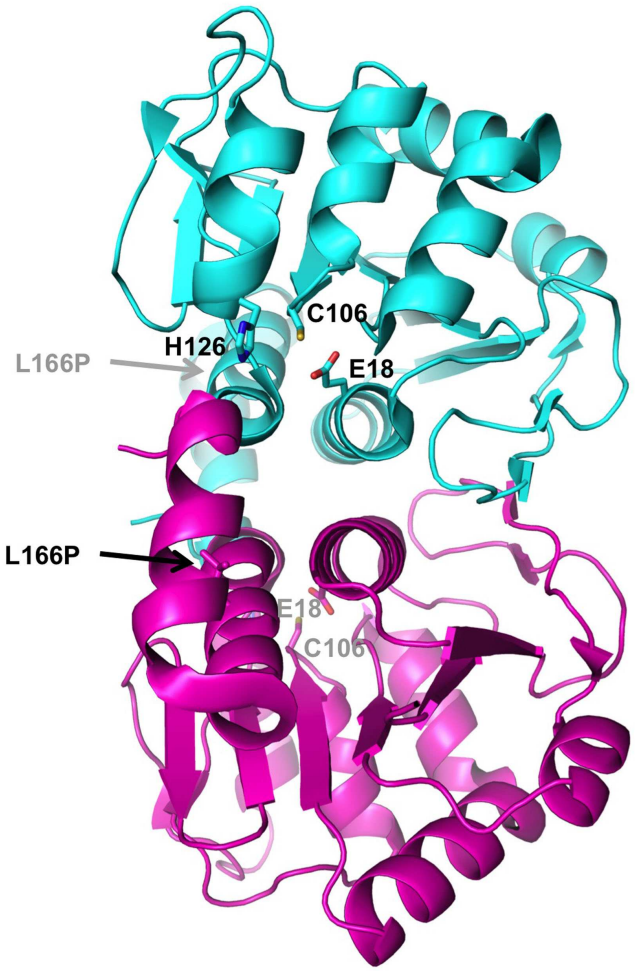

FIGURE 3 | Structure of DJ-1. The crystal structure of human DJ-1 (PDB 1P5F) was used to generate this dimer, which represents the biologically active unit. The two subunits are colored in cyan and magenta. Residues of the active site catalytic triad (Glu18, Cys106, His126) are shown as sticks. The location of the PD mutation that disrupts dimerization (L166P) is indicated by arrow.

catalytic triad is not optimally positioned for catalysis (Wilson et al., 2003), DJ-1 was nonetheless shown to have proteolytic activity upon removal of its inhibitory C-terminal helix (Chen et al., 2010). DJ-1 also prevents thermal aggregation of citrate synthase *in vitro* as well as α-synuclein fibril formation *in vitro* and *in vivo* (Shendelman et al., 2004), suggesting that DJ-1 could have chaperone activity similarly to the YajL bacterial chaperone (Gautier et al., 2012). Interestingly, this chaperone activity is mediated only by DJ-1 with an oxidized Cys106, thus providing a potential molecular mechanism for the detection of ROS. An interesting new lead for DJ-1 comes from the recent discovery of its glyoxalase activity (Lee et al., 2012). Glyoxals are small α-oxoaldehyde molecules produced notably as a result of glucose oxidation in conditions of rapid glycolysis. Glyoxals are toxic to the cell because they react with proteins to form advanced glycation end-products (AGEs), which have been implicated in a number of neurodegenerative diseases including PD (Castellani et al., 1996). DJ-1 was shown to protect MEFs and SH-SY5Y cells, as well as *C. elegans*, against glyoxals treatment. However, cells being already equipped with two glutathione-dependent glyoxalases that remove reactive glyoxals, it is unclear what additional roles could be played by DJ-1.

In addition to its proposed enzymatic activities, DJ-1 was also found to bind many biological macromolecules, including RNA (van der Brug et al., 2008), Cezanne (McNally et al., 2011), and Bcl-XL (Ren et al., 2011). All of these interactions were shown to depend on the oxidation state and/or the presence of Cys106. However, considering the high reactivity of the active site cysteine, it is possible that these interactions are mediated through non-specific covalent bond formation between the ligands and Cys106 in DJ-1.

At present, it is difficult to assign a specific biochemical activity to its function *in vivo* because all enzymatic activities described for DJ-1 rely on the active site Cys106 reactivity. Indeed, Cys106 has a depressed pKa as a result of the proximity of Glu18, which makes it a strong nucleophile (Witt et al., 2008). However, the different activities proposed for DJ-1 are not necessarily exclusive: both protease and glyoxalase activities would be diminished upon oxidation of Cys106 or formation of a sulfinic acid derivative. An altered enzymatic activity even provides a molecular mechanism for the proposed role of DJ-1 as a cellular redox sensor. Finally, dopamine-derived quinones were recently shown to covalently modify Cys106, which could potentially impair any of its biological activities (Girotto et al., 2012). This is especially relevant to PD, as oxidized dopamine side-products have been suggested to play a role in the degeneration of dopaminergic neurons (Hastings et al., 1996). Future work should therefore aim to identify the biochemical function of DJ-1 most relevant to its neuroprotective role.

CONCLUDING REMARKS

Over the past 15 years, deciphering the biochemical basis for the function of Parkin, PINK1, and DJ-1 has led to great advances in our understanding of the pathways and mechanisms involved in PD. One challenge for the future will be to integrate the functions of these three enzymes into a single coherent model of neuroprotection. In recent years, a picture has emerged wherein the hub of all three enzymes appears to be mitochondrial quality control through regulation of fusion/fission and ROS generation. Indeed, deficiency in any of these three genes has been shown to affect mitochondrial morphology and dynamics (Deng et al., 2008; Poole et al., 2008; Irrcher et al., 2010), and these defects can be rescued by overexpression of any of the other three genes (Thomas et al., 2011). Mitochondrial fragmentation caused by a-synuclein overexpression can also be rescued by overexpression of wild-type Parkin, PINK1, or DJ-1, but not their functionally deficient mutants (Kamp et al., 2010). Overexpression of either PINK1 or Parkin lead to mitochondrial arrest in the axons of cultured neurons, an effect which depends on the kinase activitiy of PINK1 and the presence of Parkin in the case of PINK1 overexpression (Wang et al., 2011). Finally, the recruitment of Parkin to depolarized mitochondria in neurons is influenced by oxidative stress levels and the presence of wild-type, but not C106A, DJ-1 (Joselin et al., 2012). These cross-talks

between Parkin, PINK1, and DJ-1 activities are most likely mediated through OMM proteins involved in fusion/fission such as Mfn1/2 or Fis1 (Yang et al., 2008; Cui et al., 2010; Rakovic et al., 2011; Zhang et al., 2012), or regulators of motility such as Miro1/2 (Wang et al., 2011). Future studies should examine in more details the functional relationships between these three proteins at the biochemical level.

Another important goal is to gain more detailed structural insight for Parkin and PINK1, which will lead to a better understanding of their biochemical functions and regulatory mechanisms. Taken together, the structure and function of this trio of enzymes will lead to the design of new therapies that will enhance and/or correct the function of these proteins, when they are defective in PD.

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Parkin- and PINK1-dependent mitophagy in neurons: will the real pathway please stand up?

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Parkinson's disease (PD) is characterized by massive degeneration of dopaminergic neurons in the *substantia nigra*. Whereas the majority of PD cases are sporadic, about 5–10% of cases are familial and associated with genetic factors. The loss of parkin or PINK1, two such factors, leads to an early onset form of PD. Importantly, recent studies have shown that parkin functions downstream of PINK1 in a common genetic pathway affecting mitochondrial homeostasis. More precisely, parkin has been shown to mediate the autophagy of damaged mitochondria (mitophagy) in a PINK1-dependent manner. However, much of the work characterizing this pathway has been carried out in immortalized cell lines overexpressing high levels of parkin. In contrast, whether or how endogenous parkin and PINK1 contribute to mitophagy in neurons is much less clear. Here we review recent work addressing the role of parkin/PINK1-dependent mitophagy in neurons. Clearly, it appears that mitophagy pathways differ spatially and kinetically in neurons and immortalized cells, and therefore might diverge in their ultimate outcome and function. While evidence suggests that parkin can translocate to mitochondria in neurons, the function and mechanism of mitophagy downstream of parkin recruitment in neurons remains to be clarified. Moreover, it is noteworthy that most work has focused on the downstream signaling events in parkin/PINK1 mitophagy, whereas the upstream signaling pathways remain comparatively poorly characterized. Identifying the upstream signaling mechanisms that trigger parkin/PINK1 mitophagy will help to explain the nature of the insults affecting mitochondrial function in PD, and a better understanding of these pathways in neurons will be the key in identifying new therapeutic targets in PD.

Keywords: autophagy, Mitochondria, mitophagy, neurons, parkin, Parkinson's disease, PINK1

INTRODUCTION

Over the past two decades, the identification of genes responsible for complex neurodegenerative disorders has profoundly changed our understanding of pathogenic mechanisms leading to neuronal cell death. Whereas previous research focused on post-mortem studies, analysis of the physiological function of causative genes now allows us to focus directly on key cellular pathways involved in pathology (1).

Among these pathways, dysregulation of mitochondrial quality control has emerged as a common theme for many neurological diseases and in particular for Parkinson's disease (PD) (2). Mitochondrial dysfunction has been a longstanding theme in PD following observations that mitochondrial toxins such as MPTP and rotenone could induce acute parkinsonism (3, 4) and that mitochondrial respiration was defective in the *substantia nigra pars compacta* (SNpc) of post-mortem PD patients (5). Furthermore, the SNpc of patient brains has been shown to have a higher occurrence of mtDNA deletions compared to aged-matched controls (6, 7).

More recently, two PD-linked genes – parkin and PINK1 – have been implicated in mitochondrial quality control, via the degradation of dysfunctional mitochondria by autophagy (a process termed mitophagy). This suggests that the mitochondrial dysfunction observed in PD may be the result of compromised mitochondrial quality control mechanisms. Most studies examining this process, however, have employed immortalized cell lines in place of primary cell cultures, and few have studied it in neurons. In this review, we summarize the evidence for a physiological role for mitophagy in neurons, discussing the possible role of parkin and PINK1 in such a pathway and its relevance to PD.

MITOCHONDRIAL DYNAMICS AND QUALITY CONTROL IN HEALTH AND DISEASE

Mitochondria – double membrane-bound organelles originating from the symbiosis between an early eukaryotic cell and a prokaryotic cell – are essential for generating energy through the process of oxidative phosphorylation (OXPHOS) and also play important roles in fatty acid metabolism, apoptosis, and calcium-buffering (8).

Long regarded as individual, "bean-shaped" organelles, mitochondria are now understood as a dynamic, inter-connected network, linked to other organelles and important players in a myriad of cellular signaling pathways (9). By regulating the connectivity and the size of the mitochondrial network, the cell can regulate energy production and most other mitochondrial processes (9). While the shape of the mitochondrial network is

controlled by fusion- and fission-specific GTPases, the size of the network is controlled by *de novo* mitochondrial biogenesis and macroautophagy.

Mitochondria are the cellular site of OXPHOS, as well as many other biosynthetic reactions. These essential processes generate, as by-products, reactive intermediates, and oxidizing agents, which in turn damage mitochondrial proteins and lipids (10). To this end, distinct mitochondrial quality control mechanisms – the degradation of unfolded mitochondrial proteins by mitochondrial proteases, the elimination of selective cargo by mitochondria-derived vesicles and the elimination of whole mitochondria by mitophagy – function in response to the degree of mitochondrial damage present (10–14).

Nowhere is the requirement for effective mitochondrial quality control systems more important than in neurons, where high energetic demands and need for high calcium-buffering capacity due to action potential-driven calcium influxes rely heavily on proper mitochondrial function (15). This mitochondrial dependence renders neurons especially vulnerable to mitochondrial damage, and, in turn, efficient and properly functioning mitochondrial quality control pathways are paramount to neuronal survival. Highlighting this are genetic studies demonstrating the involvement of genes regulating mitochondrial morphology – MFN2 and GDAP-1 in Charcot-Marie-Tooth type2A, as well as OPA1in Optic Atrophy – and mitochondrial quality control – AFG3L2 in Spinocerebellar Ataxia type 28, parkin and PINK1 in PD – in neurodegenerative disease (16–23).

THE PINK1/PARKIN PATHWAY: A LINK BETWEEN MITOCHONDRIAL QUALITY CONTROL AND PARKINSON'S DISEASE

It has been hypothesized recently that the decline in mitochondrial function observed in PD may stem from the rapid deregulation of mitochondrial quality control mechanisms in patients affected by the disease (10, 24). Importantly, recent studies have implicated two genes linked to autosomal-recessive juvenile parkinsonism (AR-JP) in humans – PINK1, a mitochondrially targeted serine/threonine kinase, and parkin, an E3 ubiquitin ligase – in a mitochondrial quality control pathway involving the degradation of damaged mitochondria by autophagy.

Initial genetic evidence from *Drosophila* had suggested that both parkin and PINK1 function in a common pathway regulating mitochondrial morphology by promoting mitochondrial fission – either by inhibiting the pro-fusion protein Fzo1 (the major *Drosophila* mitofusin homolog) or by activating the pro-fission protein Drp1 (25, 26). However, a clear consensus of how these genes affect morphology in mammalian cells has yet to be established (27–29). In regulating mitochondrial function, however, PINK1 has been shown to promote mitochondrial respiration and increase mitochondrial membrane potential ($\Delta\psi_m$) (30–32), with a specific link to complex I (33, 34), as well as proper calcium homeostasis in mammalian cell lines (35–38).

Overwhelming evidence in mammalian cell lines has implicated parkin and PINK1 in the mitophagic degradation of dysfunctional, depolarized mitochondria. Upon ablation of $\Delta\psi_m$ by the chemical uncoupler CCCP, the $\Delta\psi_m$-dependent mitochondrial import of PINK1 – a polypeptide that, basally, is rapidly turned over by proteases once inside mitochondria – is halted, allowing PINK1, bound to the TOM complex, to build up on the outer mitochondrial membrane (39–42). Here, PINK1 recruits parkin from the cytosol, in a manner dependent on functional PINK1 kinase activity, and promotes parkin's E3 ubiquitin ligase activity, possibly through direct phosphorylation of parkin by PINK1 (43–47). Once recruited to depolarized mitochondria, parkin-dependent ubiquitination and proteasomal degradation of outer membrane proteins – notably the mitofusins, VDACs, and Miro – ultimately lead to autophagy, a step that possibly involves the rupture of the outer mitochondrial membrane (43, 48–55). PINK1-/parkin-dependent mitophagy enlists the canonical ATG (*aut*ophag*y*-related gene) pathway, originally identified in yeast (56). The ubiquitination of mitochondrial proteins by parkin has been suggested to recruit ubiquitin-binding adaptor proteins, such as p62/SQSTM1, to depolarized mitochondria (43, 57, 58). This in turn was shown to induce mitochondrial clustering around the nucleus (43, 57, 58), possibly facilitating the autophagy of mitochondria by increasing their proximity to the endoplasmic reticulum, a possible source of autophagic membranes. Although PINK1/parkin mitophagy has not been fully characterized with respect to the canonical ATG pathway, the requirement for LC3, p62, and ATG5 suggests that depolarization-induced, PINK1-/parkin-dependent mitophagy indeed makes use of the conserved ATG pathway to remove damaged mitochondria.

Clearly, the ability to pharmacologically disrupt $\Delta\psi_m$ has enabled the study of the PINK1/parkin pathway using a robust and effective paradigm, although parkin-dependent mitophagy has also been observed under less severe conditions. For example, in fusion-deficient cells, parkin recruitment to depolarized mitochondria (arising from uneven fission) has been demonstrated at the steady-state (50). Furthermore, in cells harboring severe mtDNA mutations, parkin has been shown to selectively remove dysfunctional mitochondria over time (59). However, a truly robust, physiological assay with which to determine the effectiveness of PINK1- and parkin-dependent mitophagy has remained elusive.

TYPES OF MITOPHAGY UNDER PHYSIOLOGICAL CONDITIONS

Selective mitophagy (depicted in **Figure 1**) is critical during the development of cells that specifically degrade their mitochondria as they mature. The most-studied example concerns red blood cells (RBCs), which lose their mitochondria in order to transport oxygen instead of consuming it (60). While it was long known that RBCs are devoid of nuclei and organelles such as mitochondria and Golgi apparatus (61), only recently have studies identified mitophagy as the mechanism by which mitochondria are removed (62). Mitophagy in RBCs occurs canonically, according to the conserved ATG protein pathway (56), as well as through a redundant ATG7-independent mechanism involving NIX/BNIP3L, a protein related to Bcl-2 (63–65). Moreover, NIX/BNIP3L has been shown to be an essential mediator of mitochondrial depolarization prior to autophagy (66, 67).

The observation that both NIX- and parkin-/PINK1-dependent mitophagy seem to rely on mitochondrial depolarization as an upstream mechanism prior to autophagy suggests

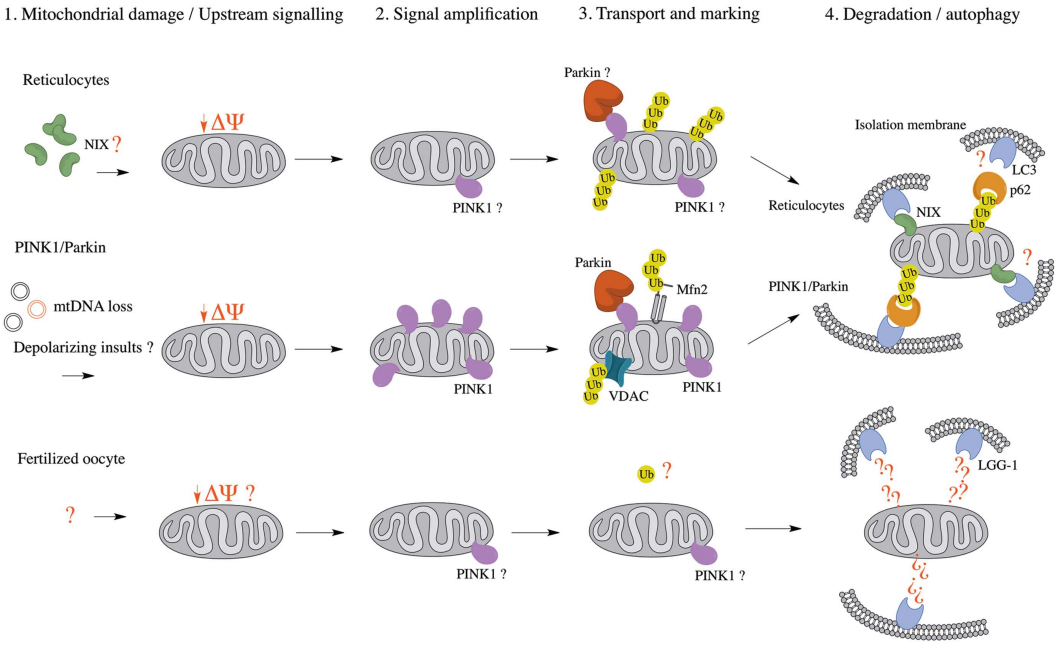

FIGURE 1 | Comparison of known mitophagy pathways (Reticulocytes, PINK1/Parkin, and Fertilized oocyte) in four major steps. (1) Upstream signaling or mitochondrial damage activates mitophagy pathways. (2) The initial mitochondrial signaling or mitochondrial damage converges at a flagship protein, which amplifies the signal. (3) Mitochondria are transported to isolation membrane-rich sites and marked for either proteasomal or autophagic degradation. (4) Mitochondria are enveloped by isolation membranes and delivered to autophagosomes. $\Delta\psi$, mitochondrial membrane potential; LC3, microtubule-associated protein 1 light chain 3; LGG-1, LC3, GABARAP, and GATE16 family 1; Mfn2, mitofusin 2; mtDNA, mitochondrial DNA; NIX, NIP3-like protein X; PINK1, PTEN-induced putative kinase 1; Ub, ubiquitin; VDAC, voltage-dependent anion channel.

Table 1 | Comparison of mitophagy pathways.

	Parkin/PINK1	Reticulocytes	Fertilized oocyte
Dependence on ATG family proteins	Yes (LC3, ATG5)	Yes (LC3)	Yes (LGG-1)
Ubiquitination of mitochondria prior to autophagy	Yes	N/A	No
Known, essential pathway components	Parkin, PINK1, HDAC6 (88), Ubiquitin-proteasome system (UPS) (48), VDAC1, 2, and 3 (89), Ambra1 (90), NIX (63)	NIX	N/A
Loss of $\Delta\Psi_m$	Yes	Yes	N/A
Complete loss of mitochondria	Yes	Yes	Yes
Type of mitophagy	Reactionary (?)	Programed	Programed

a conserved mechanism among pathways (**Table 1**), whether they are programed (reticulocyte differentiation) or reactionary (parkin/PINK1). Indeed, NIX was found to be necessary for LC3 activation following $\Delta\psi_m$ loss and parkin recruitment to the mitochondria, suggesting it might be essential to the parkin/PINK1 mitophagy pathway (63). This also raises the possibility that parkin and PINK1 function in a programed mitophagy pathway, although it would be unlikely to involve RBCs, as one would then expect parkin- or PINK1-associated PD patients to present with anemia (68).

A second type of programed mitophagy consists of the elimination of paternal mitochondria in the fertilized oocyte (69). While the notion that mitochondrial DNA is inherited uniquely from the mother has been long established, only recently have studies in *C. elegans* found that the degradation of paternal mitochondria, as well as its mtDNA, occurs through mitophagy (70–72). Whereas autophagy of paternal mitochondria was demonstrated to rely on the ATG-associated machinery for mitochondrial degradation, it was also shown that mitochondrial ubiquitination does not appear prior to engulfment, as opposed to mitophagy occurring during reticulocyte maturation and the parkin/PINK1 pathway (**Table 1**). However, further work on autophagy of paternal mitochondria, clarifying whether mitochondria are depolarized prior to engulfment, remains to be done; it would be interesting to see if parkin and PINK1 are implicated in this pathway, possibly by examining the occurrence of autophagy of paternal mitochondria in

fertilized ova of parkin- or PINK1-null mice. Interestingly, parkin- and PINK1-null flies both show mitochondrial abnormalities in embryonic development, characterized by swollen or vacuolated nebenkern – spheres encompassing two giant mitochondria in the developing fly spermatid – which result in male sterility (73–75). These findings suggest that, in spermatogenesis, parkin and PINK1 may have a role in mitophagy-related events.

In essence, it seems that many mitophagic pathways utilize the canonical ATG-associated machinery, yet differ both in the manner through which mitochondria are signaled for autophagosomal engulfment and in upstream signaling mechanisms. In the case of parkin-/PINK1-dependent mitophagy, it is the latter that is poorly defined, and it is plausible that a programed mitophagic pathway, utilizing parkin and PINK1, exists in mammals and contributes to PD pathogenesis. In addition, we have already described that PINK1 can accumulate on mitochondria upon disruption of mitochondrial import in the absence of depolarization (39), suggesting that several upstream mechanisms may impinge on a canonical, programed parkin/PINK1 mitophagic pathway.

MITOPHAGY IN PARKINSON'S DISEASE

Although studies examining parkin-/PINK1-dependent mitophagy as a quality control mechanism have relied heavily on the use of chemical uncouplers in heterologous cell culture (42, 43, 45, 47, 50), the existence and relevance of such a pathway in neurons has remained elusive, based on a handful of studies relying predominantly on parkin overexpression (76–78). Alarmingly, other groups were unable to show recruitment of overexpressed parkin in neurons following mitochondrial depolarization (79), or showed that endogenous parkin failed to mediate mitophagy in neurons and cultured cells (80).

As described previously, complete autophagy of the mitochondrial network can occur in many cell types in response to intrinsic or extrinsic signals. Neurons, however, cannot switch to glycolytic metabolism (as an ATP-generating mechanism) during acute mitochondrial stress, and hence are utterly dependent on mitochondria for energy production (81, 82). Therefore, it is unlikely that molecular pathways have evolved to remove the whole mitochondrial network following mitochondrial damage in neurons. Moreover, it has been shown that neurons divert glucose away from glycolysis to the pentose phosphate pathway – in order to maintain a high level of reduced glutathione – and instead generate ATP predominantly through OXPHOS (83, 84). As such, disruption of the OXPHOS process by uncouplers or other mitochondrial toxins results in a bioenergetic crisis inherent to neurons, and may contribute to the ambiguity surrounding findings concerning the PINK1/parkin pathway obtained from this cell type.

Thus, two important questions arise from the controversy surrounding the relevance of the PINK1/parkin pathway in neurons: (1) whether or not parkin is recruited to depolarized mitochondria in these cells and, if so, (2) what is the physiological role of this recruitment in neurons? While parkin recruitment was shown to be robust and reproducible in immortalized cells, the data indicate that this is more variable in neurons. This is not so surprising since neuronal culture protocols carry many more variables than those for immortalized cells. When analyzing data from the five studies on parkin-/PINK1-dependent mitophagy in neurons (**Table 2**), we find that many components of neuronal media could influence parkin translocation. Cai and colleagues used inhibitors of apoptosis in their neuronal culture (the caspase inhibitor Z-VAD-FMK) to counter the effects of high doses of chemical uncouplers triggering apoptosis in an environment devoid of protective glia. While these conditions do lead to parkin translocation in neurons, they may also mask the normal physiological reaction of neurons to gross depolarization of the mitochondrial network. It is unlikely that neurons have evolved to

Table 2 | Comparison of data on Parkin/PINK1-dependent mitophagy in neurons.

	Cai et al. (76)	Joselin et al. (77)	Seibler et al. (78)	Van Laar et al. (79)	Rakovic et al. (80)
Neuronal type	Cortical	Cortical	IPS-derived dopaminergic neurons	Cortical	IPS-derived dopaminergic neurons
Glial bed	Yes	No	No	No	No
Days *in vitro*	8–10	8	–	9	–
Apoptotic inhibitors	Z-VAD-FMK	No	No	No	No
B-27	Yes	No	–	Yes	Yes
Uncoupling agent + time of exposure	10 μM CCCP 24 h	5 μM CCCP 4 h	1 μM Valinomycin 12 h	10 μM CCCP 6 h	1 μM Valinomycin 12 h
% Cells with parkin recruitment	30%	70%	N/A (increased colocalization)	No CCCP effect 25% basal	N/A
Quantified parkin-dependent mitophagy	No	No	No (reduced mtDNA copy numbers)	N/A	Yes (no parkin-dependent mitophagy)
Endogenous parkin recruitment	Yes	N/A	N/A	No	N/A
PINK1 dependence	N/A	Yes	Yes	N/A	Yes

adapt to this type of insult, and apoptosis may be the physiological response. Interestingly, in the study (77) that showed the highest percentage of parkin translocation (about 70%), it was found that recruitment was dependent on the absence of antioxidants (in the form of the B-27 supplement) in the media. Taken together, these factors might explain why some groups (79) were not able to detect significant parkin translocation upon mitochondrial depolarization in neurons. Importantly, parkin translocation was also much slower (12–24 h) in the study that used B-27 than in studies without it (4 h). This indicates that neuronal cultures containing antioxidant supplements may counteract the action of chemical uncouplers. It would however be important to determine if, under growth conditions lacking antioxidants, neurons can survive over long time periods following mitochondrial depolarization. In light of these recent studies, we conclude that parkin can translocate to mitochondria in neurons following depolarization, given the proper culture conditions. However, most of these studies did not quantify mitophagy following parkin translocation (76–78). While Seibler and colleagues found that parkin-positive cells have reduced mitochondrial DNA copy numbers after exposure to CCCP, they did not rule out decreased mitochondrial biogenesis as a possible mechanism. This raises the question of whether parkin translocation proceeds to mitophagy in neurons, or plays another role. Rakovic and colleagues addressed this question by looking at the degradation of a number of mitochondrial proteins both at the outer membrane, the inner membrane, and the matrix. Surprisingly, they found that even when overexpressing parkin in induced pluripotent stem (iPS) cell-derived dopaminergic neurons, parkin does not promote mitophagy upon mitochondria depolarization. However, given that the kinetics of parkin recruitment to mitochondria seem considerably slower in neurons (70% recruitment at 4 h) than in immortalized cells (100% at 2 h) (**Table 3**), it is plausible that mitophagy may proceed more slowly and may not be detectable at 16 h with the 1 μM valinomycin used by Rakovic and colleagues. Again, one obvious issue may be that incubating neuronal cultures with chemical uncouplers for an extended period may induce apoptosis. Adding apoptotic inhibitors may circumvent this limitation, allowing for the study of parkin-dependent mitophagy in neurons on a longer time scale, as shown by Cai and colleagues. Whereas this allowed them to show colocalization between autophagic markers and mitochondria in isolated events, Cai and colleagues did not quantify them in parkin-overexpressing versus mock-transfected neurons.

To overcome the limitations of *in vitro* neuronal cultures, Sterky and colleagues crossed MitoPark mice – which develop progressive parkinsonism and mitochondrial abnormalities stemming from the ablated expression of mitochondrial transcription factor A in dopaminergic neurons (85) – with parkin knockout mice. These mice did not show increased neurodegeneration or accumulation of damaged mitochondria, suggesting that parkin had no role in degrading damaged mitochondria (86). Moreover, they overexpressed parkin in the MitoPark mouse and found that parkin was not recruited to mitochondria at the steady-state. While these data suggest that parkin may not be involved in mitophagy in the brain *in vivo*, it is noteworthy that the mitochondrial defects of MitoPark mice have not been fully characterized, and, as such, the mitochondria of these mice may not be sufficiently depolarized to stabilize PINK1 levels and trigger parkin recruitment. Interestingly, a recent study by Vincow and colleagues demonstrated that parkin null flies exhibit a slower turnover of mitochondrial proteins (87). Moreover, they showed that electron transport chain (ETC) protein turnover is especially affected in both parkin and PINK1 single-null flies, suggesting that, under physiological conditions, parkin and PINK1 might have a specific role in regulating the levels of ETC proteins, as opposed to the complete removal of mitochondria following depolarization in cell lines. In light of these results, it is clear that further studies will be required to test whether parkin promotes mitophagy in neurons and what are its consequences *in vivo*.

Table 3 | Comparison of parkin recruitment in immortalized cells versus neurons.

	Immortalized Cells (HeLa, Hek293T, SH-SY5Y, MEFs)	Neurons (primary, IPS-derived)
Mean time of parkin recruitment upon $\Delta\Psi_m$ depolarization (more than 30% cell with parkin on mitochondria)	30 min (50)	4 h (77); 6 h (79); 12 h (78); 24 h (76)
Dependence on PINK1	Yes	Yes
Survival after long-term exposure to chemical uncouplers	Yes	N/A; use of apoptotic inhibitors (76)
Complete removal of mitochondria	Yes	N/A

CONCLUSION

Parkin and PINK1 are the first two PD-associated genes to be implicated in a common genetic pathway. More specifically, the association of parkin and PINK1 in a common mitochondrial quality control pathway has consolidated the hypothesis that mitochondrial defects are central to PD pathogenesis. However, the physiological relevance of such a pathway in neurons requires further investigation. Moreover, neuronal parkin and PINK1 may play roles in mitochondrial homeostasis other than degrading damaged mitochondria, such as regulating ETC protein turnover. Upon review of the few, pioneering studies that have aimed to clarify these questions, we conclude that, although it is robust and implicates many other players subsequent to parkin recruitment, the parkin/PINK1 mitophagy pathway still lacks proper upstream signaling characterization. This is reflected in the inability to find a consensus on the proper conditions with which to study this pathway in a more disease-relevant cell type. We also conclude that, while redistribution of parkin to depolarized mitochondria has now been shown in neurons, the physiological role of such recruitment – specifically, whether or not this proceeds to mitophagy – remains elusive. By understanding the physiological function of parkin and PINK1 in neurons, future studies will undoubtedly reveal key molecular mechanisms underlying neurodegeneration and hence novel therapeutic targets for the treatment of PD.

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Trinucleotide repeats: a structural perspective

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Trinucleotide repeat (TNR) expansions are present in a wide range of genes involved in several neurological disorders, being directly involved in the molecular mechanisms underlying pathogenesis through modulation of gene expression and/or the function of the RNA or protein it encodes. Structural and functional information on the role of TNR sequences in RNA and protein is crucial to understand the effect of TNR expansions in neurodegeneration. Therefore, this review intends to provide to the reader a structural and functional view of TNR and encoded homopeptide expansions, with a particular emphasis on polyQ expansions and its role at inducing the self-assembly, aggregation and functional alterations of the carrier protein, which culminates in neuronal toxicity and cell death. Detail will be given to the Machado-Joseph Disease-causative and polyQ-containing protein, ataxin-3, providing clues for the impact of polyQ expansion and its flanking regions in the modulation of ataxin-3 molecular interactions, function, and aggregation.

Keywords: amino acid-repeats, microsatellites, protein complexes, protein aggregation, amyloid, protein structure

TRINUCLEOTIDE REPEATS AND HUMAN DISEASE

Trinucleotide repeat (TNR) expansions and their association with neurological disorders have been known for the past 20 years (La Spada et al., 1991). Expansion of CAG, GCG, CTG, CGG, and GAA repeats located in coding or non-coding sequences of different genes (summarized in **Table 1**; **Figures 1** and **2**) are associated with a diverse range of human monogenic diseases such as Spinobulbar Muscular Atrophy (SBMA, a.k.a. Kennedy disease), Huntington Disease (HD), Spinocerebellar Ataxias (SCAs), Oculopharyngeal Muscular Dystrophy (OPMD), Myotonic Type 1 (DM1), Fragile X-Associated Tremor Ataxia Syndrome (FXTAS), and Friedreich Ataxia (FRDA) (for a review see Orr and Zoghbi, 2007), with longer repeats being correlated with earlier age at onset and increased disease severity. These TNR are highly unstable and the repeat tract length can change between affected individuals within the same family and can be different in different tissues (La Spada, 1997; Brouwer et al., 2009). More interestingly, in the brain of patients affected by CAG expansions, differences in repeat instability have been found between specific cell types (Pearson et al., 2005; Gonitel et al., 2008; Lopez Castel et al., 2010). GCG repeats are usually shorter and reveal a higher stability in different tissues and across generations than CAG repeats. The dynamic nature of these DNA repeat expansions is a consequence of their capability to form different secondary structures, which interfere with the cellular mechanisms of replication, repair, recombination and transcription (for a recent review see Lopez Castel et al., 2010). The molecular mechanisms underlying pathogenesis in those disorders, either associated with mental retardation, neuronal, or muscular degeneration, might result from alterations in the levels of gene expression and/or the function of the RNA or protein it encodes, mechanisms that likely act in concert to influence the pattern of selective cell toxicity. Some of those toxicity mechanisms will be briefly discussed below.

TRINUCLEOTIDE REPEATS AND RNA STRUCTURE

The formation of hairpin structures within the TNR RNA is related to the gain in RNA toxic function, the major pathogenic mechanism associated with CUG and CGG repeat expansions in non-coding regions of DM1 and FXTAS transcripts, which was also shown to contribute to pathogenesis in CAG repeat disorders such as HD and Machado-Joseph disease (MJD, a.k.a. SCA3) (reviewed in Krzyzosiak et al., 2012). These duplex structures, whose stability is positively correlated with the repeat size (Napierala and Krzyzosiak, 1997), sequester dsRNA binding proteins involved in mRNA splicing such as CUG-binding protein (CUGBP) and muscleblind protein 1 (MBNL1) (Miller et al., 2000), inducing aberrant splicing in affected cells, compromising multiple intracellular pathways, affecting cell-quality control regulation, and ultimately resulting in cell dysfunction (Li and Bonini, 2010). Structural studies on model trinucleotide CUG, CAG, and CGG repeats forming double-stranded chains revealed the features induced by periodic U-U, A-A, and G-G mismatches, and provided hints into the structural details of pathogenic RNAs that are recognized by RNA-binding proteins (Mooers et al., 2005; Kiliszek et al., 2010, 2011; Kumar et al., 2011; Parkesh et al., 2011). MBNL1 is composed of four zinc-containing RNA-binding domains arranged in two tandem segments, with the C-terminal zinc-finger pair displaying a GC-sequence recognition motif (Teplova and Patel, 2008) and interacting with the stem region of expanded CUG RNAs (Yuan et al., 2007). Electron microscopy analysis of MBNL1:CUG^{136} complexes showed that the pathogenic dsRNA forms a scaffold

Table 1 | Human diseases associated with nucleotide repeat expansions (adapted from Messaed and Rouleau, 2009; Lopez Castel et al., 2010; Matos et al., 2011).

Disease name	Repeat type	Repeat location	Gene	Protein (UniProt identifier, number of residues)	Biological process[a]	Normal repeat length	Disease repeat length	Protein structure determined?
Spinal and bulbar muscular atrophy (SBMA)	CAG	Protein coding region (polyQ)	*AR*	Androgen receptor (P10275, 919 residues)	Transcription, transcription regulation	9–36	38–62	Residues 20–30 and 671–919 (PDB code 1xow)
Huntington's disease (HD)	CAG	Protein coding region (polyQ)	*HTT*	Huntingtin (P42858, 3142 residues)	Apoptosis	6–34	36–121	Residues 5–18 (3lrh), Residues 1–17 (2ld0, 2ld2), Residues 1–64 (3io4, 3io6, 3ior, 3iot, 3iou, 3iov, 3iow)
Dentatorubral-pallidouysian atrophy (DRPLA)	CAG	Protein coding region (polyQ)	*ATN1*	atrophin 1 (P54259, 1190 residues)	Transcription, transcription regulation	7–34	49–88	No structural information
Spinocerebellar ataxia 1 (SCA1)	CAG	Protein coding region (polyQ)	*ATXN1*	ataxin 1 (P54253, 815 residues)	Transcription, transcription regulation	6–39	40–82	Residues 563–693 (1oa8)
Spinocerebellar ataxia 2 (SCA2)	CAG	Protein coding region (polyQ)	*ATXN2*	ataxin 2 (Q99700, 1313 residues)	No associated GO keywords for biological process	15–24	32–200	Residues 912–928 (3ktr)
Spinocerebellar ataxia 3 (SCA3)	CAG	Protein coding region (polyQ)	*ATXN3/MJD*	ataxin 3 (P54252, 364 residues)	Transcription, transcription regulation, Ubl conjugation pathway	10–51	55–87	Residues 1–182 (1yzb), Residues 222–263 (2klz)
Spinocerebellar ataxia 6 (SCA6)	CAG	Protein coding region (polyQ)	*CACNA1 A*	CACNA 1_A, P/Q-type α1A calcium channel subunit (O00555, 2505 residues)	Calcium transport, ion transport, transport	4–20	20–29	Residues 1955–1975 (3bxk)
Spinocerebellar ataxia 7 (SCA7)	CAG	Protein coding region (polyQ)	*ATXN7*	ataxin 7 (O15265, 892 residues)	Transcription, transcription regulation	4–35	37–306	Residues 330–401 (2kkr)
Spinocerebellar ataxia 17 (SCA17)	CAG	Protein coding region (polyQ)	*ATXN17*	TATA box binding protein (TBP) (P20226, 339 residues)	Transcription, transcription regulation, Host-virus interaction	25–42	47–63	Residues 159–337 (1cdw, 1c9b, 1jfi, 1nvp, 1tgh)
Multiple skeletal dysplasias (COMP)	GAC	Protein coding region (polyaspartate)	*COMP*	cartilage oligomeric matrix protein (a.k.a Thrombospondin-5) (P49747, 757 residues)	Apoptosis, cell adhesion	5	4, 6, 7	Residues 225–757 (3fby).
Synpolydactyly (HOXD13)	GCG	Protein coding region (polyA)	*HOXD13*	homeobox D13 (P35453, 343 residues)	Transcription, transcription regulation	15	22–29	No structural information

(Continued)

Table 1 | Continued

Disease name	Repeat type	Repeat location	Gene	Protein (UniProt identifier, number of residues)	Biological process[a]	Normal repeat length	Disease repeat length	Protein structure determined?
Oculopharyngeal Muscular Dystrophy (OPMD)	GCG	Protein coding region (polyA)	*PABPN1*	Polyadenylate-binding protein 2 (Q86U42, 306 residues)	mRNA processing	10	12–17	Residues 167–254 (3b4d, 3b4m, 3ucg)
Cleidocranial dysplasia (CBFA1)	GCG	Protein coding region (polyA)	*RUNX2*	Runt-related transcription factor 2 (Q13950, 521 residues)	Transcription; transcription regulation	17	27	No structural information
Holoprosencephaly (ZIC2)	GCG	Protein coding region (polyA)	*ZIC2*	Zinc-finger protein ZIC 2 (O95409, 532 residues)	Differentiation, neurogenesis, transcription, transcription regulation	15	25	No structural information
Hand-Foot-Genital Syndrome/HOXA13)	GCG	Protein coding region (polyA)	*HOXA13*	homeobox A13 (P31271, 388 residues)	Transcription, transcription regulation	18	24–26	No structural information
Blepharophimosis/ ptosis/epicanthus inversus syndrome type II (FOXL2)	GCG	Protein coding region (polyA)	*FOXL2*	Forkhead box like 2 (P58012, 376 residues)	Differentiation, transcription, transcription regulation	14	22–24	Residues 322–328 (2l7z)
Infantile spasm syndrome (ARX)	GCG	Protein coding region (polyA)	*ARX*	Aristaless-related homeobox (Q96QS3, 562 residues)	Differentiation, neurogenesis, transcription, transcription regulation	10–16	17–23	No structural information
Myotonic dystrophy type 1 (DM1)	CTG	3′ UTR	*DMPK*	Myotonic dystrophy protein kinase (DMPK) (Q09013, 639 residues)	No associated GO keywords for biological process	5–37	90–6500	Residues 11–420 (2vd5), Residues 460–537 (1wt6)
Friedreich ataxia (FRDA)	GAA	Intron	*FXN*	Frataxin (Q16595, 210 residues)	Heme biosynthesis, Ion transport, Iron storage, Iron, transport	6–32	>200	Residues 88–210 (1ekg), Residues 91–210 (1ly7), Residues 82–210 (3s4m, 3s5d, 3s5e, 3s5f, 3t3j, 3t3k, 3t3l, 3t3t, 3t3x)
Spinocerebellar ataxia 8 (SCA8)	CTG	3′ UTR	*ATXN8*	Ataxin-8 (a.k.a protein 1C2; (Present in SCA8-specific 1C2-positive intranuclear inclusions) (Q156A1, 80 residues)	Cell death	2–130	>110	Nostructural information

(Continued)

Table 1 | Continued

Disease name	Repeat type	Repeat location	Gene	Protein (UniProt identifier, number of residues)	Biological process[a]	Normal repeat length	Disease repeat length	Protein structure determined?
Spinocerebellar ataxia 12 (SCA12)	CAG	5′ UTR	*PPP2R2B*	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B β isoform (Q00005, 443 residues)	Apoptosis	7–45	55–78	No structural information
Huntington disease-like 2 (HDL2)	CAG	Alternative splice isoform 2 – polyA-expansion	*JPH3*	Junctophilin 3 (Q8WXH2, 748 residues)	No associated GO keywords for biological process	6–27	51–57	No structural information
FRAXA: fragile X syndrome	CGG	5′ UTR	*FMR1*	Fragile X mental retardation 1 protein (Q06787, 632 residues).	Transport; mRNA transport	6–52	230–2000	Residues 1–134 (2bkd), Residues 216–280 (2fmr), Residues 216–425 (2qnd), Residues 527–541 (2la5)
FXTAS: fragile X tremor/ataxia syndrome	CGG	5′ UTR	*FMR1*	Fragile X mental retardation 1 protein (Q06787, 632 residues).	Transport; mRNA transport	6–52	59–230	Residues 1–134 (2bkd), Residues 216–280 (2fmr), Residues 216–425 (2qnd), Residues 527–541 (2la5)
FRAXE: fragile X syndrome	CGG	5′ UTR	*FMR2*	Fragile X mental retardation 2 protein (P51816, 1311 residues)	mRNA processing, mRNA splicing	4–39	200–900	No structural information

UTR, untranslated region.

[a] *Biological Function based on Gene Ontology as annotated in UniProt.*

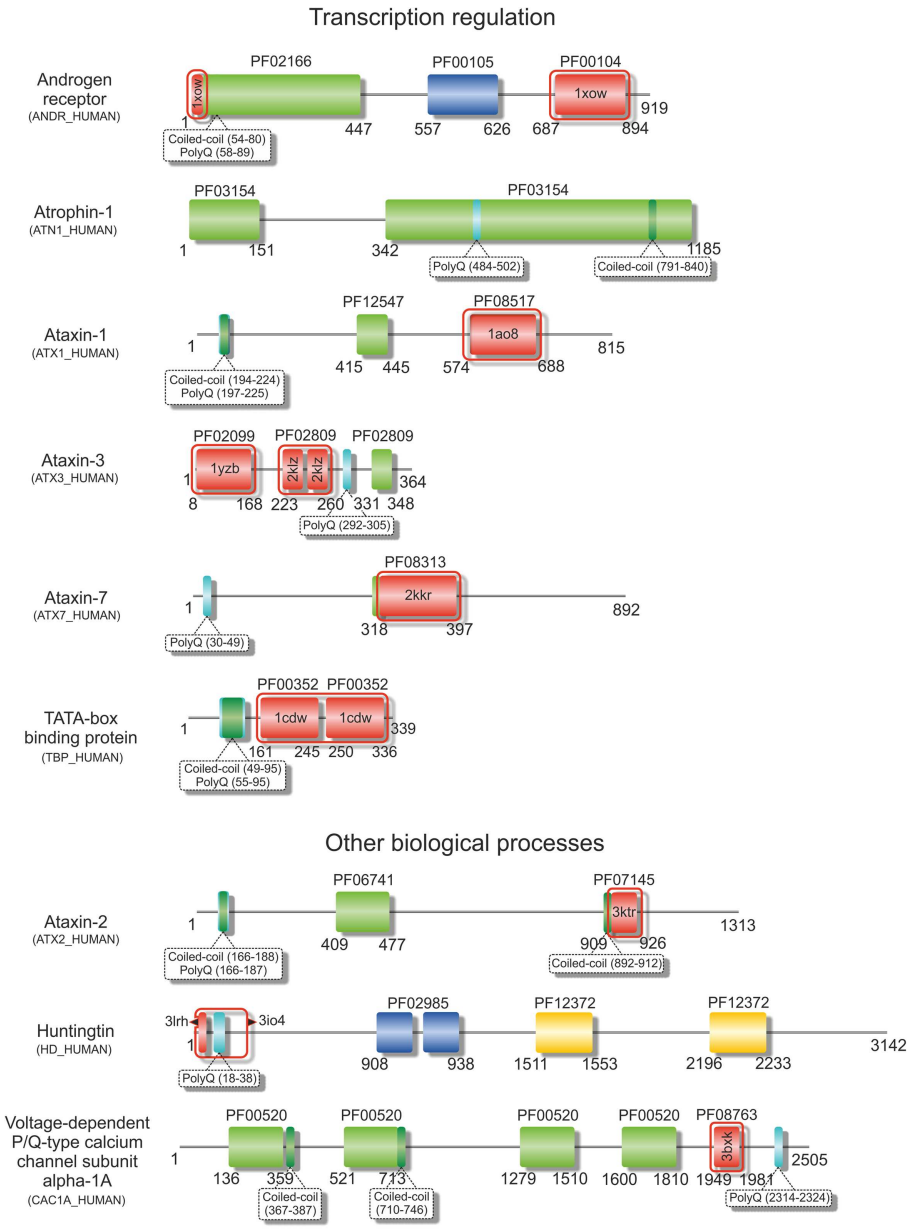

FIGURE 1 | Structural variability of proteins encoded by TNR-containing genes. Illustrative domain graphics of the multi-domain structure of proteins associated with polyQ-expansion diseases. All proteins shown are referenced by their name as annotated in UniProt. The protein domains for which information is annotated in the Pfam database are shown as colored boxes with Pfam family accession code referenced above the domain box. Complete names of domains can be assessed by searching the specific Pfam accession code at http://pfam.sanger.ac.uk/. Numbers below the domain schemes represent amino acid residue numbers. Regions containing the amino acid repeats and with a prediction for formation of coiled-coils (as annotated in UniProt) are shown as well as regions with known 3D structure (boxed in red, with PDB accession codes shown). Notice the predominant location of the repeat regions within the N-terminal regions of the proteins.

with tandem spaced MBNL1 binding sites were MBNL1 oligomers with a ring-like structure can assemble, possibly leading to the formation of the ribonuclear foci identified in cell models of these TNR diseases (Yuan et al., 2007; de Mezer et al., 2011). The structure and stability of the TNR hairpin structures formed depends on the presence of interruptions as well as on the nature of the flanking regions. This might be related with the ability of individual repeats to participate in the RNA toxicity mechanisms (Krzyzosiak et al., 2012).

In FRDA and FXTAS, pathogenesis results predominantly from decreased expression of the associated genes (*FXN* and *FMR1/FMR2*) caused by the expansion of GAA and CGG repeats, respectively, which results in loss of function of key proteins involved in iron-sulfur cluster biogenesis and mRNA translation

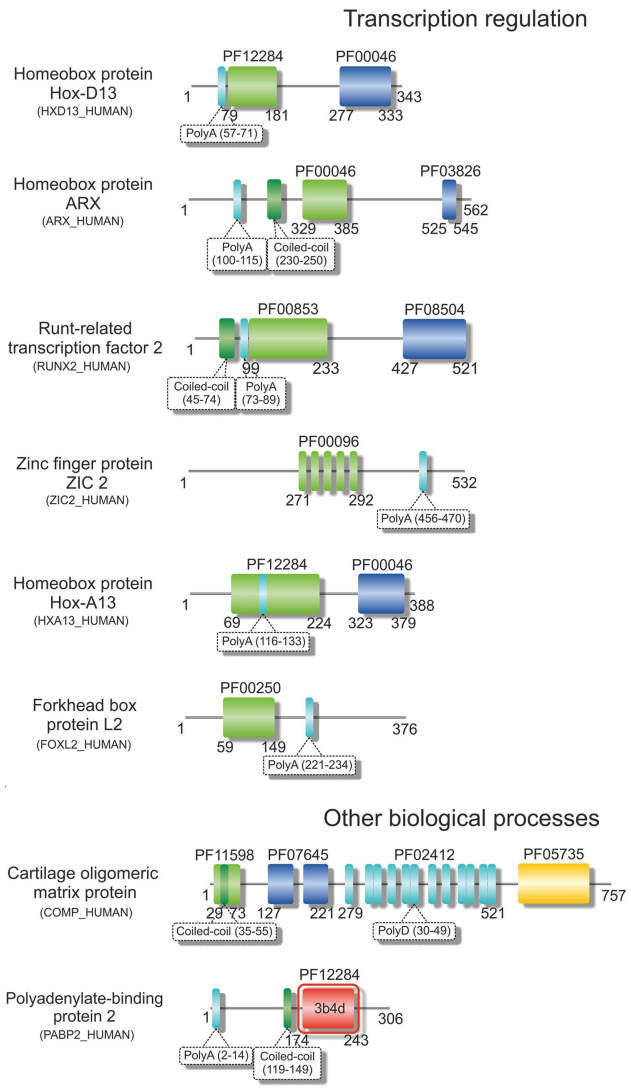

FIGURE 2 | Structural variability of proteins encoded by TNR-containing genes. Illustrative domain graphics of the multi-domain structure of proteins associated with polyD- and polyA-expansion diseases. All proteins shown are referenced by their name as annotated in UniProt. The protein domains for which information is annotated in the Pfam database are shown as colored boxes with Pfam family accession code referenced above the domain box. Complete names of domains can be assessed by searching the specific Pfam accession code at http://pfam.sanger.ac.uk/. Numbers below the domain schemes represent amino acid residue numbers. Regions containing the amino acid repeats and with a prediction for formation of coiled-coils (as annotated in UniProt) are shown as well as regions with known 3D structure (boxed in red, with PDB accession codes shown). Notice the predominant location of the repeat regions within the N-terminal regions of the proteins.

at synapses. Nevertheless, in FXTAS RNA toxicity is also proposed to play a role in pathogenesis (Li and Bonini, 2010). The recently discovered mechanisms of pathogenesis in spinocerebellar ataxia type 8 (SCA8) uncovered the extreme complexity of TNR disorders. In fact, SCA8 is caused by expansion of CTG/CAG repeats in the affected gene, which are transcribed bi-directionally leading to the generation of expanded CUG and CAG-containing transcripts further translated into homopolymeric proteins, so that pathogenesis can be mediated by both RNA and protein toxicity (Merienne and Trottier, 2009). Curiously, recent data have highlighted the possibility of non-ATG translation across expanded TNR in all possible reading frames, which might further contribute to the generation of novel toxic proteins and RNAs adding to the multi-parametric character of the pathogenic mechanisms associated with TNR diseases (Li and Bonini, 2010; Pearson, 2011; Sicot et al., 2011).

TRINUCLEOTIDE REPEATS WITHIN PROTEIN CODING REGIONS

Over 20 years ago, the finding that the expansion of CAG repeats within the coding sequence of the androgen receptor gene was the genetic basis of SBMA (La Spada et al., 1991) represented a hallmark in the discovery of these novel dynamic mutations and their association with human disease. Some years later, the identification of intracellular inclusions containing the expanded proteins (Paulson et al., 1997) provided a clue to pathogenesis, directing research in the field into an extensive search for the mechanisms of polyQ-induced protein aggregation. The moderate expansion of GCG and CAG repeats, which are translated into polyA and polyQ tracts in the affected proteins (**Figures 1** and **2**), results in protein misfolding and aggregation, in accordance with a general, although not always unique, toxic gain of function mechanism of pathogenesis (Williams and Paulson, 2008). The appearance of insoluble cytoplasmic or nuclear inclusions enriched in the expanded polyA- or polyQ-containing protein constitutes a characteristic fingerprint of these diseases (Messaed and Rouleau, 2009; Orr, 2012a), regardless of their controversial role in pathogenesis. While the proteins containing polyA repeats are predominantly transcription factors with a role in development (see **Table 1** and Amiel et al., 2004; Messaed and Rouleau, 2009), most of the proteins linked to polyQ-expansion diseases are involved in DNA-dependent regulation of transcription or neurogenesis and often contain multiple intermolecular partners (Butland et al., 2007). Despite the overall lack of sequence or structural homology, both polyQ- and polyA-repeat expansions are associated with formation of ß-rich amyloid-like protein inclusions, and with the wider group of protein misfolding disorders. These inclusions are enriched in ubiquitin, proteasome subunits, and chaperones, and often recruit macromolecules that are part of the macromolecular interaction networks associated with the proteins' native functions (Williams and Paulson, 2008). As an example, the poly(A)-binding protein PABNP1 forms insoluble inclusions upon alanine expansion, co-aggregating together with poly(A)-mRNA, proteasome subunits, ubiquitin, heat-shock proteins, and SKIP, a transcription factor associated with muscle-specific gene expression (Brais, 2003; Tavanez et al., 2009; Winter et al., 2013).

The simplistic view of the predominant role of the inclusions in polyQ-induced pathogenesis was later challenged by the failure of this mechanism to explain the cell-specific vulnerability characteristic for each disease and by the identification of numerous examples of neuronal toxicity in the absence of visible intracellular inclusions (Arrasate et al., 2004). Indeed, the inclusions were shown to be fibrillar and display amyloid-like properties both *in vivo* and *in vitro* (Huang et al., 1998; Bevivino and Loll, 2001;

Sathasivam et al., 2010) and, in a mechanistic parallel with the pathogenic mechanisms proposed for "classical" amyloids, many studies suggested that the insoluble inclusions played a protective role, sequestering toxic, and misfolded protein conformers (Arrasate et al., 2004; Rub et al., 2006; Miller et al., 2010). Indeed, soluble intermediates in the aggregation pathway such as misfolded β-sheet rich polyQ protein monomers and oligomers have latter been identified and proposed to represent the major toxic species (Kayed et al., 2003; Gales et al., 2005; Nagai et al., 2007; Miller et al., 2011). Also, in OPMD, the primary toxic species are proposed to be the soluble variants of the expanded polyA-repeat protein PABPN1 (Messaed et al., 2007). It is currently accepted that in polyQ disorders the expanded region plays a role in inducing the self-assembly of the carrier protein, which engages in pathogenic interactions and leads to the formation of toxic monomers or oligomers (Takahashi et al., 2008; Weiss et al., 2008) latter converted to insoluble intracellular amyloid-like oligomers where both expanded and "normal" protein are sequestered along with other macromolecular partners (reviewed in Williams and Paulson, 2008; Matos et al., 2011; Costa and Paulson, 2012). As more biochemical data is gathered, more is understood about the role of amino acid expansions in modulating the interaction with macromolecular partners. As an example, expansion of the polyA tract in PABPN1 results in increased association with Hsp70 chaperones and type I arginine methyl transferases (Tavanez et al., 2009). This indicates that the distinct neuropathological features arising from this amino acid-repeat expansion might at least partially result from alterations on the native biological functions and macromolecular interactions of the carrier protein, which might vary in different intracellular environments.

Recent data have shown that expansion of polyA repeats is frequently associated with loss of normal function altering a multitude of cellular pathways with consequences in cell functionality (Amiel et al., 2004; Messaed and Rouleau, 2009), although protein aggregation might also play a dominant role in some of the polyA-associated disorders (Messaed and Rouleau, 2009; Winter et al., 2013). Studies with polyQ proteins have shown that pathogenesis might result from a subtle imbalance in the association of the mutant protein with multiple cellular partners and that toxicity and neuronal death could result from a combination of protein self-assembly and functional alterations (Friedman et al., 2007; Li et al., 2007b; Lim et al., 2008; Kratter and Finkbeiner, 2010; Orr, 2012b; Pastore and Temussi, 2012). In fact, neuronal death as a result of polyQ-expansion seems to resemble that of linker cell in *C. elegans* (Pilar and Landmesser, 1976; Chu-Wang and Oppenheim, 1978; Blum et al., 2012, 2013) which involves the polyQ protein pqn-4, pointing for a common mechanism for linker cell death, and neuronal death in polyQ diseases (Blum et al., 2013).

Polyglutamine diseases constitute a representative and largely studied group of neurodegenerative disorders where considerable amounts of data have been collected on the role of expanded polyQ for disease pathogenesis. However, given the proposed function of polyQ regions in mediating protein–protein interactions, which might be modulated by polyQ-expansion (Schaefer et al., 2012), the information on the role of these regions for native protein function, structure, and dynamics is still limited. Structural and functional information on the role of these repeat sequences in protein function is crucial to better understand how expansion affects selected neuronal subpopulations. Below, we briefly discuss the current knowledge on the function and structure of polyQ repeats and their role on macromolecular interactions, and finally focus on the known structural and functional information on ataxin-3, the protein whose mutation causes MJD.

FUNCTION OF PolyQ ON PROTEIN–PROTEIN INTERACTIONS AND EVOLUTION

Until recently, the function of many amino acid-repeat-containing proteins and the role of homopeptide regions were somewhat obscure. However, several global analysis studies on single amino acid-repeat-containing proteins shed light onto their function and onto the biological significance of the repeated region, in particular of polyQ, the most prevalent amino acid repetition in humans (Alba and Guigo, 2004). It is now accepted that TNR, particularly those located within protein-coding regions, are considered important mutators providing the genetic variability required for driving evolution (King, 1994; Kashi et al., 1997; Kashi and King, 2006; Nithianantharajah and Hannan, 2007). In fact, simple or low-complexity amino acid-repeats are rare within prokaryotic but extremely abundant within eukaryotic proteins, particularly over-represented in *Plasmodium* (49–90% of the total proteome), *D. discoideum* (52%), *D. melanogaster* (20%), *C. elegans* (9%), and *H. sapiens* (14%) (Haerty and Golding, 2010). Among all homopolymeric repeats, the most common on eukaryotic proteins are glutamine, asparagine, alanine, and glutamate repeats (Faux et al., 2005). This seems to indicate that there has been a strong negative selection against the appearance of hydrophobic amino acid-repeats with high tendency to aggregate, such as polyisoleucine, polyleucine, polyphenylalanine, and polyvaline (Oma et al., 2005, 2007).

The homopeptide regions seem to be particularly relevant for brain development and function, since these repeated regions can be found in various neurodevelopmental genes (Nithianantharajah and Hannan, 2007). Indeed, the sexual behavior of prairie voles (Hammock and Young, 2005), as well as human pair-bonding (Walum et al., 2008), seems to be dependent on the repeat length in the vasopressin 1A receptor gene. A wide study of the distribution and function of homopeptide-containing proteins could also demonstrate a clear trend in humans, *D. melanogater*, and *C. elegans*, with the majority of homopeptide-containing proteins performing roles in transcription/translation and signaling processes and to a less extend in transport and adhesion processes (Faux et al., 2005). A similar profile was also found in a comparative analysis of proteins with amino acid-repeats in human and rodents (Alba and Guigo, 2004) and also on a comparative genomic study in domestic dogs, which unveiled an association between morphological variations and the length of the repeated region in the transcription factor-encoded genes *ALX4* and *RUNX2* (Fondon and Garner, 2004). Analysis of the human genome also revealed the existence of 64 CAG repeat-containing genes involved in biological processes such as regulation of transcription, binding of transcriptional co-activators and transcription factors, and in neurogenesis in general (Butland et al., 2007). Additionally, a detailed analysis of the human polyQ database (http://pxgrid.med.monash.edu.au/polyq/) (Robertson et al., 2011) also indicated that the

majority of polyQ-containing proteins display domains involved in development (Homeobox domain-containing proteins, Fibroblast growth factor receptor), chromatin remodeling (Bromodomain and PHD-containing proteins), and signal transduction (PDZ domain-containing proteins), all biological processes that are highly dependent on protein–protein interactions and associated with the formation of multicomponent protein complexes. As for humans, analysis of bovine polyQ proteins revealed an enrichment for large multi-domain transcriptional regulators (Whan et al., 2010).

It is currently accepted that the majority of repeat-containing proteins perform roles in processes that require the assembly of large multiprotein or protein/nucleic acid complexes (Faux et al., 2005; Hancock and Simon, 2005; Whan et al., 2010). Supporting this notion is the fact that homopolymeric amino acid-repeats are considered to be unstructured (Gojobori and Ueda, 2011) and that intrinsically unstructured regions are suggested to constitute macromolecular docking sites, which become structured only when bound to cognate ligand partners (Huntley and Golding, 2002; Simon and Hancock, 2009). In fact, "hub proteins" contain significantly longer and more frequent repeats or disordered regions, which facilitate binding to multiple partners (Dosztanyi et al., 2006). Recently, Fiumara et al. (2010) found an overrepresentation of coiled-coils domains in polyQ-containing proteins and in their interaction partners, which are able to form α-helical supersecondary structures, often inducing protein oligomerization (Parry et al., 2008). Thus, polyQ tracts due to their intrinsic structural flexibility, which is largely influenced by the flanking residues (see PolyQ: A Simple Sequence Repeat with a Polymorphic Structure below), may act as stabilizers of intra- and intermolecular protein interactions, possibly by extending a neighboring coiled-coil region to promote its interaction with a coiled-coil region in an interacting protein partner (Schaefer et al., 2012). A detailed analysis revealed heptad repeats typical of coiled-coils in regions flanking or overlapping polyQ stretches, whose disruption is sufficient to impair CHIP-huntingtin interaction, indicating that coiled-coils are crucial for polyQ-mediated protein contacts. Importantly, coiled-coils also seem to be important for the regulation of aggregation and insolubility of polyQ-containing proteins (see below and Fiumara et al., 2010) as recently proposed by Petrakis et al. (2012), which discovered a recurrent presence of coiled-coil domains in ataxin-1 misfolding enhancers, while such domains were not present in suppressors.

Based on the several observations on the function of polyQ-containing proteins it is suggested that a general function of polyQ, as for the majority of repeat sequences, is to aid in the assembly of macromolecular complexes, either through tethered distant domains or through interactions with the polyQ itself (Gerber et al., 1994; Korschen et al., 1999; Faux et al., 2005). By affecting protein interactions, and being present in particular functional classes such as transcription factors, polyQ is considered central to the evolution of this type of proteins and consequently crucial to the evolution of cellular signaling pathways (Hancock and Simon, 2005).

A structural analysis of polyQ repeats and its flanking domains as well as its role in protein aggregation will be discussed in greater detail in the next sections.

STRUCTURAL STUDIES ON PolyQ REPEATS

Since the discovery that polyQ repeats are associated with human neurodegenerative diseases that a huge effort has been made to determine the structure of polyQ and to understand how expansion of the repeat affects the structure of the carrier protein and/or the normal interaction with molecular partners. The first evidence from the aggregation-prone character of polyQ-rich proteins came from studies with glutamine-rich cereal storage proteins and synthetic glutamine polypeptides (Beckwith et al., 1965; Krull et al., 1965). After the discovery that a number of neurological disorders were triggered by expansion of a polyQ tract in different and unrelated proteins (La Spada et al., 1994), and before intracellular inclusions enriched in the polyQ-expanded protein were identified as a major fingerprint in these diseases (Davies et al., 1997; Paulson et al., 1997), Perutz (1994) anticipated that the expanded polyQ tract could mediate protein–protein interactions causing protein aggregation in neurons and recruiting other polyQ-rich proteins such as transcription factors leading to cellular dysfunction. Below, the structural features and self-assembly properties of polyQ sequences are briefly discussed (for a detailed review on the biophysical and structural features of polyQ, see Wetzel, 2012).

PolyQ: A SIMPLE SEQUENCE REPEAT WITH A POLYMORPHIC STRUCTURE

In order to elucidate the structure of the glutamine repeat and to uncover the structural changes induced by polyQ expansion, several strategies have been put forward including (a) the structural analysis of polyQ-containing peptides of different lengths, (b) the characterization of proteins of well-known structure after insertion of an exogenous polyQ repeat, and structural determination of (c) polyQ-antibody complexes, or (d) natural polyQ-rich proteins.

Using synthetic peptides containing 15 glutamine repeats, Perutz and coworkers proposed that polyQ stretches could self-associate forming hydrogen bonds between their side-chain amide groups and the main chain of a neighboring β-strand, to form cross-β structures (polar zippers) (Perutz, 1994). This study was followed by many reports where synthetic polyQ peptides were used as models of the biophysical properties of polyQ-rich proteins, which established that polyQ-containing peptides have a tendency toward self-assembly into amyloid-like structures (Chen et al., 2002a). Moreover, the results obtained *in vitro* reflected disease features observed *in vivo* such as the correlation between larger polyQ size, increased protein aggregation, and earlier disease onset (Chen et al., 2002b; Kar et al., 2011). Circular dichroism studies of polyQ peptides in solution have shown that their monomeric forms lack regular secondary structure (Altschuler et al., 1997; Klein et al., 2007) and additional biophysical experiments proposed that these peptides can adopt collapsed (Crick et al., 2006; Dougan et al., 2009; Peters-Libeu et al., 2012) or extended (Singh and Lapidus, 2008) coils in solution whose compactness was strongly correlated with the polyQ size (Walters and Murphy, 2009). The determination of the structure of monomeric polyQ peptides with atomic detail is however still lacking as a result of their intrinsic conformational flexibility and tendency to aggregate into heterogeneously sized β-rich oligomers. From the combination of experimental and theoretical methods a picture for polyQ

structure and aggregation is emerging, where the monomeric polyQ adopt an ensemble of conformations lacking regular secondary structures that assemble into β-structures in a polyQ-length dependent fashion (Vitalis et al., 2009; Walters and Murphy, 2009, 2011; Williamson et al., 2010; Kar et al., 2011). Divergent results proposing the existence of predominantly extended or collapsed conformations or the minimum size for polyQ aggregation are likely due to the differences in the introduction of variable flanking residues (Kar et al., 2011). They might result from the insertion of different polyQ tract interrupting residues (Walters and Murphy, 2011), or be a consequence of the protocols used for the preparation and disaggregation of the peptides used for the biophysical studies (Jayaraman et al., 2011). Most results obtained with these peptides do not generally take into account the possible effects of the protein context on the structural properties of the polyQ stretches, a particularly relevant feature considering that the role of non-polyQ domains in protein aggregation has been reported for ataxin-1 (de Chiara et al., 2005), ataxin-3 (Gales et al., 2005), and huntingtin (Tam et al., 2009; Thakur et al., 2009; Liebman and Meredith, 2010).

In a pioneer work, Stott et al. (1995) inserted a G-Q_{10}-G peptide into the inhibitory loop of chymotrypsin inhibitor 2 (CI2), a soluble small protein from barley seeds, showing that this CI2-polyQ chimera has an increased tendency for self-assembly. Even though a CI2 variant with four glutamines crystallized, the structure of the CI2-Q_4 dimer showed that the polyQ region was disordered and that oligomerization was mediated by domain swapping (**Figure 3A**) and not by direct polyQ association (Chen et al., 1999). A structure resembling the proposed polar zipper was later observed between two asparagines in the hinge loop of the major domain swapped dimer of bovine pancreatic ribonuclease A (Liu et al., 2001) (**Figure 3B**). Insertion of a 10 glutamine repeat within this hinge loop of ribonuclease A, resulted in domain swapping, oligomerization, and amyloid-like fiber formation, but strikingly the enzyme within the fibers was catalytically active, retaining its native fold (Sambashivan et al., 2005). However, although the structure of the domain swapped dimer was solved by X-ray crystallography, the repeat region was not visible in the electron density maps.

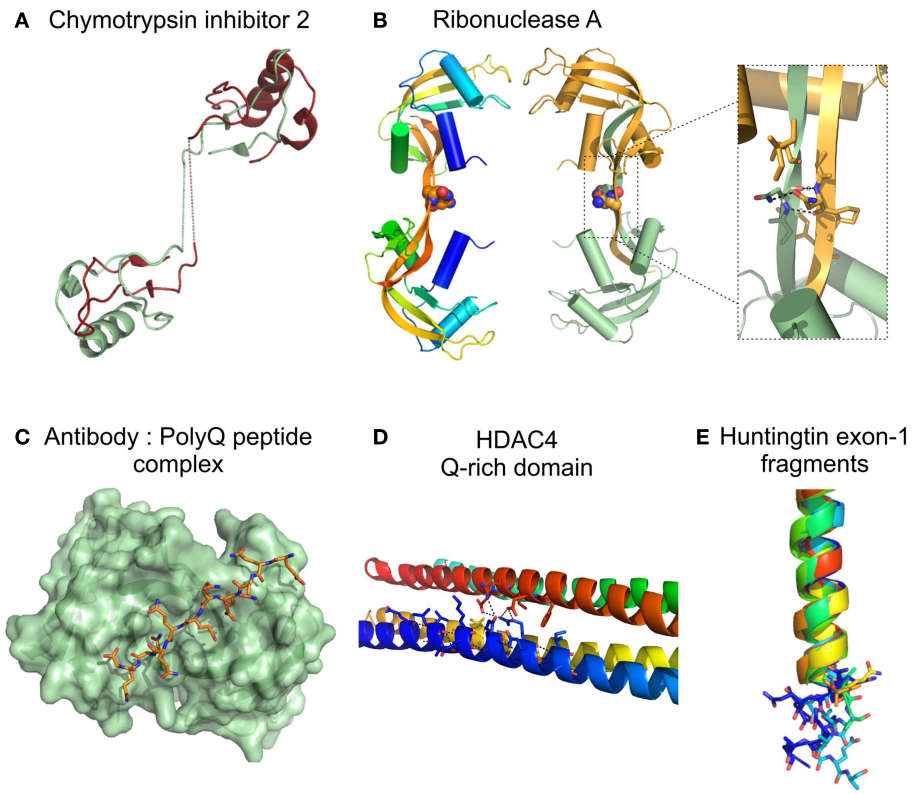

FIGURE 3 | Structure of proteins/protein domains containing polyQ regions. **(A)** Cartoon representation of the domain swapped dimer of chymotrypsin inhibitor 2 with a 4 glutamine insertion [(Chen et al., 1999); PDB accession code 1cq4], dotted lines represent the polyQ linker not visible in the X-ray crystal structure. **(B)** Cartoon representation of domain swapped major dimers of ribonuclease A. Inset shows a short segment resembling the polar zipper formed by asparagine residues in the linker region [(Liu et al., 2001); PDB accession code 1f0v]. **(C)** Surface representation Fv fragment of a monoclonal antibody in complex with a polyQ peptide shown as sticks [(Li et al., 2007a), PDB accession code 2otu]. **(D)** Cartoon representation of the glutamine-rich domain from HDAC4 showing details of the polar interactions (dotted lines) at the oligomer interfaces involving glutamine residues [(Guo et al., 2007), PDB accession code 2o94]. **(E)** Cartoon representation of the crystal structures of huntingtin exon-1 fragments observed in different crystal forms, highlighting the different orientations of the C-terminal polyQ residues shown as sticks. The 17 glutamine stretch adopts variable conformations in the structures: α helix, random coil, and extended loop. [(Kim et al., 2009), PDB accession codes 3io4, 3iow, 3iov, 3iou, 3iot, 3ior, 3io6].

A first overview of a short polyQ stretch at atomic resolution resulted from the structure of a $polyQ_{10}$ peptide ($GQ_{10}G$) (**Figure 3C**) bound to MW1, an antibody against polyQ. This structure reveals that polyQ adopts an extended, coil-like structure in which contacts are made between side chains and/or main chain atoms of all 10 glutamines and the antibody-combining site (Li et al., 2007a). The peculiar structural features of these repeat-containing regions were also revealed by the crystallographic structure of a glutamine-rich domain of human histone deacetylase (HDAC4), that folds into a tetramer-forming straight α-helix (**Figure 3D**). The protein interfaces consist of multiple hydrophobic patches separated by polar interaction networks, in which clusters of glutamines engage in extensive intra- and interhelical interactions (Guo et al., 2007). Further details on the structure of polyQ were unveiled by the high-resolution crystal structures of huntingtin (HD) exon 1, containing 17 glutamines (Htt17Q) (Kim et al., 2009). Htt17Q in fusion with maltose-binding protein (MBP) folds into an amino-terminal α-helix followed by a $polyQ_{17}$ region that adopts multiple conformations in the different crystal forms, including α-helix, random coil, and extended loop, and a polyproline helix formed by the $polyP_{11}$ and mixed P/Q regions (**Figure 3E**). The authors suggested that the shallow equilibrium between α-helical, random coil, and extended conformations can be subtly altered by the size of polyQ sequence, the neighboring protein context, protein interactions, or by changes in cellular environment, and that this polymorphic behavior is a common characteristic of many amyloidogenic proteins (Kim et al., 2009).

SELF-ASSEMBLY AND AGGREGATION OF PolyQ REPEATS

The first approaches to characterize polyQ-induced protein aggregation and pathogenesis in the context of a full-length protein included the insertion of the polyQ peptides into well-known non-pathogenic protein carriers such as hypoxanthinephosphoribosyl transferase (HPRT), which resulted in a neurological phenotype mimicking that observed in mice expressing the mutant HD truncated protein (Ordway et al., 1997). *In vitro* studies aiming at better characterizing the structure and function of polyQ repeats in the context of full-length soluble proteins, included the insertion of ectopic polyQ stretches into well-characterized and soluble proteins such as CI2 (Stott et al., 1995; Chen et al., 1999), myoglobin (Mb) (Tanaka et al., 2001; Tobelmann and Murphy, 2011), glutathione S transferase (GST) (Masino et al., 2002; Bulone et al., 2006) and the B domain from *Staphylococcus aureus* Protein A (SpA) (Saunders et al., 2011). Fusion of the polyQ sequences with stable and soluble proteins moderates the intrinsic polyQ peptide aggregation propensity, but induces the self-assembly of carrier proteins into fibrillar amyloid-like structures, a nucleation-dependent process whose kinetics is directly proportional to the size of the inserted polyQ repeat. Likewise, polyQ peptides are able to seed the aggregation of intracellular soluble polyQ-containing proteins when added to cell cultures, conferring a heritable phenotype of self-sustaining seeding, resembling a prion-like mechanism (Ren et al., 2009), reviewed in Cushman et al. (2010).

The impact of the polyQ tract and its expansion on the perturbation of the structure of flanking sequences and domains is critically dependent on the location of the amino acid-repeats, revealing impressive location-dependent changes in structural stability, and fibril morphology of the host proteins (Robertson et al., 2008; Saunders et al., 2011; Tobelmann and Murphy, 2011). Curiously, the studies with these model proteins showed that stability and structure of the carrier protein remained unaltered by polyQ expansion when the repeat was inserted at the N- or C-terminus of the structured domain (Robertson et al., 2008), mimicking the location of polyQ tracts in most disease-related proteins (**Figure 1**).

The role of the flanking regions in modulating protein fibril formation in polyQ disease proteins is well supported by experimental data (de Chiara et al., 2005; Gales et al., 2005; Bhattacharyya et al., 2006; Saunders and Bottomley, 2009; Tam et al., 2009; Thakur et al., 2009; Liebman and Meredith, 2010), in agreement with the knowledge that different polyQ-containing proteins have a diverse threshold for aggregation. For example, addition of a polyproline extension after the polyQ repeat slows down aggregation (Bhattacharyya et al., 2006), while protein domains outside the polyQ tract [e.g., Josephin domain (JD) of ataxin-3 and AHX domain of ataxin-1] have been shown to contribute to protein aggregation (Masino et al., 2004; de Chiara et al., 2005; Gales et al., 2005; Ellisdon et al., 2006, 2007). The multitude of data on the polyQ-induced aggregation of disease and non-disease-proteins highlights the complex interplay between the polyQ region and the adjacent protein domains. In light of the polymorphic nature of the polyQ and the modulation of its structural features by the protein context, two general mechanisms have been proposed for polyQ-mediated toxicity (Kim et al., 2009): (a) the expanded polyQ stretch adopts a novel conformation that mediates toxicity or is the precursor to toxic species; (b) intra- or intermolecular protein interactions mediated by expanded polyQ in the random coil conformation are sufficient to result in pathological effects. In both cases the affinity of the interactions involving the expanded polyQ region could be higher with selected target proteins, leading to a preference of the disease proteins for some of the protein partners, a fact that is in agreement with the hypothesis raised by Zuchner and Brundin (2008), which postulate that resistance to NMDA receptor-mediated excitotoxicity occurring in some mouse models for HD is a consequence of a differential binding of partner proteins, in a polyQ tract size dependent manner, to the proline-rich domain of huntingtin. In this context, differences in molecular interactions occurring in a cell- and tissue-specific manner would result in different toxicities according to particular cellular environments.

Given the above mentioned studies, it is nowadays clear that the polyQ region influences aggregation of proteins, but this process is highly dependent on the surrounding protein context. Therefore, even though the structural information on peptides and proteins with polyQ expansions is a useful guideline for the investigation of the pathogenic effects of polyQ expansion, each of the proteins involved in polyQ diseases shows distinctive characteristics, cellular roles, and structural properties causing difficulties in the formulation of structural hypothesis that could explain how different monomeric conformations of polyQ leads to various aggregated species and how they contribute to neurotoxicity.

PolyQ REPEATS IN ATAXIN-3 FUNCTION AND DYSFUNCTION

Machado-Joseph disease is an inherited neurodegenerative disorder of adult onset originally described in people of Portuguese Azorean descent but later shown to be the most common autosomal dominant spinocerebellar ataxia worldwide. Clinically, it is characterized by ataxia, ophthalmoplegia, and pyramidal signs, associated in variable degree with dystonia, spasticity, peripheral neuropathy, and amyotrophy (Coutinho and Andrade, 1978). Pathologically, the disorder is associated with degeneration of the deep nuclei of the cerebellum, pontine nuclei, subthalamic nuclei, substantia nigra, and spinocerebellar nuclei (Coutinho et al., 1982; Rosenberg, 1992; Margolis and Ross, 2001). It is caused by an expansion of a repetitive CAG tract within the *ATXN3* gene (Kawaguchi et al., 1994). While in the healthy population the number of CAG repeats ranges between 10 and 51, in MJD patients the length of ataxin-3 polyQ tract exceeds 55 consecutive residues. Ataxin-3 is a modular protein, located both in the nucleus and the cytoplasm (Perez et al., 1999; Antony et al., 2009; Macedo-Ribeiro et al., 2009), encompassing an N-terminal globular JD, with structural similarity to cysteine proteases (Scheel et al., 2003; Albrecht et al., 2004), followed by an extended tail composed of two ubiquitin interaction motifs (UIMs), the expandable polyQ tract, and a C-terminal region (Matos et al., 2011). The C-terminal region of ataxin-3 may contain a third UIM, depending on the splice variant (Goto et al., 1997), with the 3UIM isoform of ataxin-3 being predominantly found in the brain (Harris et al., 2010). Currently, the physiological function of ataxin-3, as well as the molecular mechanism by which expanded polyQ sequences causes selective neurodegeneration remain mostly unknown. However, since it is ubiquitously expressed and cell death is region specific, neurodegeneration is currently viewed as depending on sequence and structural features outside the ataxin-3 polyQ tract [reviewed in Matos et al. (2011) and references therein].

ATAXIN-3 BIOLOGICAL ROLES

ATXN3 orthologs have been identified in eukaryotic organisms including protozoans, plants, fungi, and animals (Albrecht et al., 2004; Costa et al., 2004; Rodrigues et al., 2007). Several functions have been ascertained to ataxin-3 based on studies with orthologs. Specifically, a role in cell structure and/or motility was proposed for mouse ataxin-3 as it is highly abundant in all types of muscle and in ciliated epithelial cells (Costa et al., 2004). In fact, ataxin-3 is able to interact with tubulin through its JD domain (**Figure 4**), with nM affinity (Mazzucchelli et al., 2009), which supports a role in cell structure. Interestingly, data on ataxin-3 *C. elegans* ortholog not only reinforces a function in structure/motility and signal transduction (Rodrigues et al., 2007), but also indicate a function in development as absence of *ATXN3* strongly modifies expression of several development-related genes. *ATXN3* knock-out animals showed no obvious deleterious phenotype, probably due to a putative redundant function between ataxin-3 and other JD-encoding proteins, such as ataxin-3-like protein, Josephin 1 and Josephin 2, all containing a typical cysteine protease catalytic triad. However the studies with *ATXN3* knock-out animals revealed an overall increase in the levels of ubiquitinated proteins (Schmitt et al., 2007) and signs of altered expression of core sets of genes associated with the ubiquitin-proteasome and signal transduction pathways (Rodrigues et al., 2007), pointing to a dual function of ataxin-3 in the ubiquitin-proteasome system and transcriptional regulation (Matos et al., 2011; Orr, 2012a).

Ataxin-3 function as transcriptional regulator

The putative role of ataxin-3 in transcriptional regulation is proposed to entail the modulation of histone acetylation and deacetylation at selected promoters. Ataxin-3 interacts with the major histone acetyltransferases cAMP-response-element binding protein (CREB)-binding protein (CBP), p300, and p300/CREB-binding protein-associated factor (KAT2B/PCAF, **Figures 4** and **5**), and is proposed to inhibit transcription in specific promoters (e.g., MMP-2 promoter) either by blocking access to histone acetylation sites or through recruitment of histone deacetylase 3 (HDAC3) and nuclear receptor co-repressor (NCOR1; **Figures 4** and **5**) (Li et al., 2002; Evert et al., 2006). Although, the interaction sites have not been mapped in detail for all these proteins, co-immunoprecipitation experiments showed that KAT2B/PCAF, p300, and CBP bind exclusively to the polyQ-containing C-terminal region of ataxin-3 (**Figure 4**), apparently in a polyQ-size dependent manner (Li et al., 2002). Experimental evidence also indicates that ataxin-3 forms part of a CREB-containing complex, although no direct interaction has been observed between the two proteins (Li et al., 2002). In contrast, the N-terminal region of ataxin-3 directly binds histones H3 and H4 (**Table 2**; **Figure 4**) (Li et al., 2002). Of note, p300 and CBP, as well as NCOR1, also encompass amino acid repetitions in its sequence. Interestingly, in huntingtin and in ataxin-1, polyQ interferes with CBP-activated gene transcription via interaction of their glutamine-rich domains (Shimohata et al., 2000; Nucifora et al., 2001) and mutant huntingtin targets specific components of the core transcriptional machinery, in a glutamine-tract length-sensitive manner (Zhai et al., 2005), pinpointing once again the role of the amino acid-repeat region in the establishment of protein–protein interactions.

Ataxin-3 molecular function: ubiquitin hydrolase

A role for ataxin-3 in ubiquitin-dependent pathways was proposed by bioinformatic analysis (Scheel et al., 2003; Albrecht et al., 2004), and its ability to bind and cleave poly-ubiquitin chains and polyubiquitinated proteins was later demonstrated experimentally (Burnett et al., 2003; Chai et al., 2004). Importantly, inhibition of ataxin-3 catalytic activity results in the increase of polyubiquitinated proteins, resembling the effects of proteasome inhibition (Berke et al., 2005), indicating that ataxin-3 is involved with proteins targeted for proteasomal degradation. The function of ataxin-3 in the ubiquitin-proteasome system was further supported by the identification of its association with the ubiquitin-like domain of the human homologs of the yeast DNA repair protein Rad23, HHR23A, and HHR23B (Wang et al., 2000; Doss-Pepe et al., 2003; Nicastro et al., 2005, 2009), with valosin-containing protein (VCP)/p97 (Hirabayashi et al., 2001; Doss-Pepe et al., 2003; Boeddrich et al., 2006; Zhong and Pittman, 2006), and with the ubiquitin ligase E4B (Matsumoto et al., 2004) (**Figures 4** and **5**). Strikingly, the weak direct association between ataxin-3 and E4B is strongly reinforced by the addition of VCP/p97, indicating that these proteins form part of a higher order macromolecular

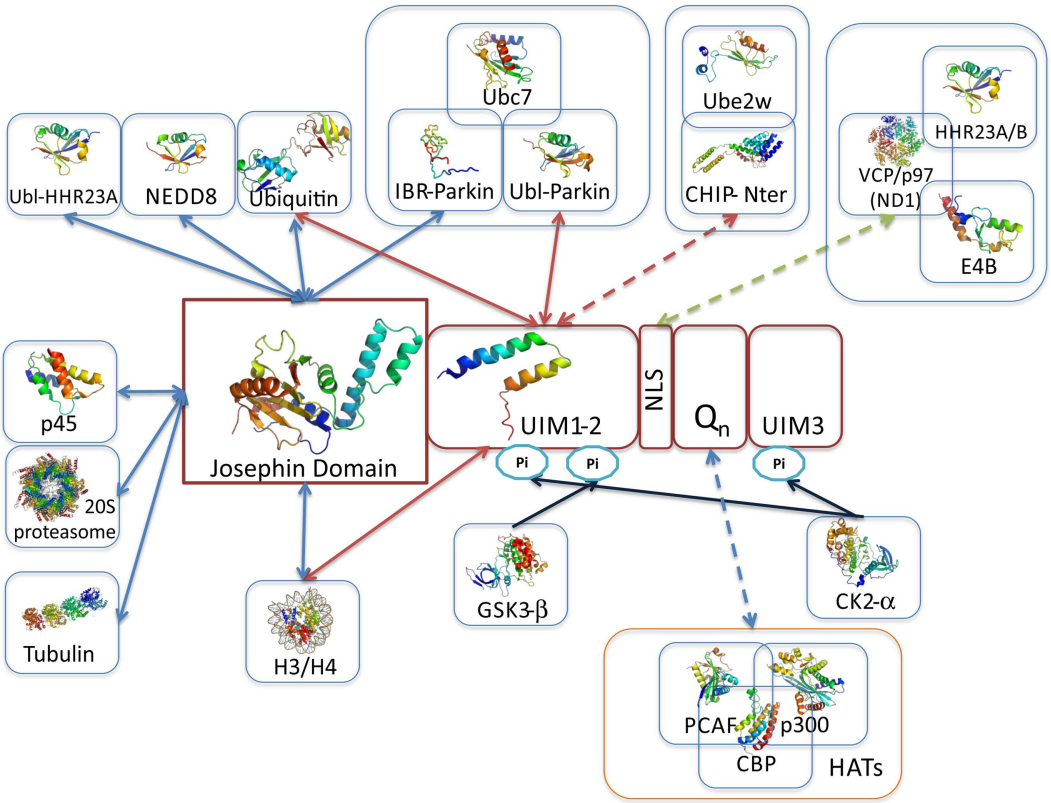

FIGURE 4 | Overview of ataxin-3 structural information. Schematic illustration of ataxin-3 (isoform 2; a.k.a. 3UIM isoform) domain structure highlighting the regions involved in protein–protein interactions. The solution structures of the Josephin domain (PDB accession code 1yzb) and UIMs1-2 (PDB accession code 2klz) are shown colored from N-(blue) to C- terminus (red). JD-, UIM-, NLS-, and polyQ-mediated interactions are represented by blue, red, green, and purple arrows, respectively; blue arrows indicate the location of post-translational modification sites, resulting from the interaction and phosphorylation by CK2 and GSK3. Representative multi-subunit complexes where ataxin-3 participates are boxed (Li et al., 2002; Matsumoto et al., 2004; Scaglione et al., 2011; Durcan et al., 2012). One of the main questions in the quest for ataxin-3 interacting proteins is whether polyQ-expansion of the disease-protein modulates the binding affinities. Current data indicates that polyQ-expansion increments the ataxin-3 affinity for CHIP (Scaglione et al., 2011), VCP/p97 (Matsumoto et al., 2004; Boeddrich et al., 2006; Zhong and Pittman, 2006), and the transcription regulators p300, CBP, and PCAF (Li et al., 2002) (interactions represented by broken lines). Strikingly, all these interactions are mediated by ataxin-3 flexible tail, which includes the polyQ tract. Moreover the transcriptional regulators p300, CBP, and NCOR all contain amino acid repeats.

complex to regulate the degradation of misfolded ER proteins (Matsumoto et al., 2004; Zhong and Pittman, 2006) (**Figure 5**).

Biochemical studies showed that ataxin-3 displays a strong preference for chains containing four or more ubiquitins (Chai et al., 2004) and that full-length ataxin-3 and its JD both display proteolytic activity toward either linear substrates containing a single ubiquitin molecule (Burnett et al., 2003; Chow et al., 2004b; Weeks et al., 2011) or K48/K63-linked poly-ubiquitin chains (Winborn et al., 2008; Todi et al., 2009), displaying also the capacity to bind the ubiquitin-like protein NEED8 in a substrate-like fashion (Ferro et al., 2007). Moreover, ataxin-3-like protein, Josephin 1 and Josephin 2, also display ubiquitin protease activity (Tzvetkov and Breuer, 2007; Weeks et al., 2011), although the relative activities are highly variable in spite of their high sequence similarity. Characterization of ataxin-3 ubiquitin hydrolase activity has also revealed that the full-length protein preferentially cleaves Lys-63-linked and mixed-linkage chains with more than four ubiquitins (Burnett et al., 2003; Winborn et al., 2008). This specificity is dictated by the UIMs, as the isolated JD shows a preference toward the disassembly of Lys-48-linked chains (Nicastro et al., 2009, 2010). Altogether, this indicates that ataxin-3 ubiquitin hydrolase activity is likely to be associated with delivery of target substrates to the proteasome rather than with their rescue from degradation, as it happens with most of the other deubiquitinases (Ventii and Wilkinson, 2008; Matos et al., 2011; Scaglione et al., 2011). Interestingly, ubiquitin hydrolase activity of ataxin-3 is not affected by polyQ expansion and both normal and expanded ataxin-3 are able to increase the cellular levels of a short-lived GFP normally degraded by the ubiquitin-proteasome pathway (Burnett et al., 2003).

The 3D structures for JD alone or in the presence of ubiquitin as well as that of the tandem UIM1-UIM2 have already been determined (Mao et al., 2005; Nicastro et al., 2005, 2009; Song et al., 2010), giving a structural perspective on the ubiquitin hydrolase function of ataxin-3. The JD contains two ubiquitin binding sites, both of hydrophobic nature, with site 1 being negatively charged to

Table 2 | Human ataxin-3 associated proteins.

Ataxin-3 interacting protein (UniProt accession code)	Protein name	Direct interaction?	Interaction domains		Reference
			Ataxin-3	Partner protein	
CELL-QUALITY CONTROL (PROTEIN HOMEOSTASIS)					
HHR23A/B (P54725/P54727)	UV excision repair protein RAD23 homolog A/B	Yes, kD (JD:Ubl) = 12 μM	JD	Ubiquitin-like (Ubl) N-terminal domain	Wang et al. (2000), Doss-Pepe et al. (2003), Nicastro et al. (2005, 2009)
Poly-ubiquitin (P0CG48/P0CG47)	Polyubiquitin-C/Polyubiquitin-B	Yes, kD (atxn3:K48-tetraUb) = 0.2 μM, kD (atxn3:Ub) = 50 μM	UIMs, JD	K48- and K63-linked Ub (≥4 Ub), K48-linked diUb	Burnett et al. (2003), Doss-Pepe et al. (2003), Chai et al. (2004), Nicastro et al. (2009, 2010)
Ubiquilin-1 (Q9UMX0)	Protein linking IAP with cytoskeleton 1	n.d.	n.d.	n.d.	Heir et al. (2006)
NEDD8 (Q15843)	Ubiquitin-like protein Nedd8	Yes	JD	NEDD8	Ferro et al. (2007)
Parkin (O60260)	E3 ubiquitin-protein ligase parkin	Yes	JD, UIMs	IBR domain, Ubiquitin-like (Ubl) domain	Durcan et al. (2011, 2012)
Ubc7 (P62253)	Ubiquitin-conjugating enzyme E2 G1	Yes (transient interaction detected using cross-linking reagents)	n.d.	n.d.	Durcan et al. (2012)
p45 (P62195)	26S proteasome regulatory subunit 8	Yes	N-terminal atxn3 region (residues 1–133)	n.d.	Wang et al. (2007)
20S Proteasome (P25786, P25787, P25788, P25789, P28066, P60900, O14818, P20618, P49721, P49720, P28070, P28074, P28072, Q99436)	Proteasome subunits α types 1-7 and β types 1-7	n.d.	N-terminal atxn3 region (residues 1–150)	n.d.	Doss-Pepe et al. (2003)
CHIP (Q9UNE7)	E3 ubiquitin-protein ligase CHIP	Yes, kD (atxn3:CHIP) = 2.2 μM, kD (atxn3:Ub-CHIP) = 0.1 μM	Atxn3 C-terminus (residues 133–357)	CHIP N-terminus	Jana et al. (2005), Scaglione et al. (2011)
VCP/p97 (P55072)	Transitional endoplasmic reticulum ATPase	Yes	Residues 277–281 (includes arginine/lysine-rich NLS)	N domain, residues 1-199	Hirabayashi et al. (2001), Doss-Pepe et al. (2003), Matsumoto et al. (2004,?) Boeddrich et al. (2006), and Zhong and Pittman (2006)
E4B (O95155)	Ubiquitin conjugation factor E4 B	Yes (with 79Q-ataxin-3)	n.d.	n.d.	Matsumoto et al. (2004)

(Continued)

Table 2 | Continued

Ataxin-3 interacting protein (UniProt accession code)	Protein name	Direct interaction?	Interaction domains		Reference
			Ataxin-3	Partner protein	
OTUB2 (Q96DC9)	Ubiquitin thioesterase OTUB2	n.d.	n.d.	n.d.	Sowa et al. (2009)
USP13 (Q92995)	Ubiquitin carboxyl-terminal hydrolase 13	n.d.	n.d.	n.d.	Sowa et al. (2009)
KCTD10 (Q9H3F6)	BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3	n.d.	n.d.	n.d.	Sowa et al. (2009)
Tubulin dimer (Q71U36/P68363)	Tubulin α-1A, Tubulin β-2B	Yes, kD (atxn3:tubulin) = 50–70 nM	JD	n.d.	Mazzucchelli et al. (2009)
Dynein (Q9Y6G9)	Cytoplasmic dynein 1 light intermediate chain 1	n.d.	n.d	n.d.	Burnett and Pittman (2005)
HDAC6 (Q9UBN7)	Histone deacetylase 6	n.d.	n.d.	n.d.	Burnett and Pittman (2005)
TRANSCRIPTIONAL REGULATION					
p300 (Q09472)	Histone acetyltransferase p300	Yes	PolyQ-containing C terminus of atxn3 (residues 288–354)	n.d.	Li et al. (2002)
CBP (Q92793)	cAMP-response-element binding protein (CREB)-binding protein	Yes	PolyQ-containing C terminus of atxn3 (residues 288–354)	n.d.	Li et al. (2002)
PCAF (Q92831)	p300/CREB-binding protein-associated factor: histone acetyltransferase KAT2B	Yes	PolyQ-containing C terminus of atxn3 (residues 288–354)	n.d.	Li et al. (2002)
Histone H3/H4 (P68431/P62805)	Histone	Yes	JD + UIM1 and 2 (residues 1–288)	n.d.	Li et al. (2002)
HDAC3 (O15379)	histone deacetylase 3	Yes	n.d.	n.d.	Evert et al. (2006)
NCOR1 (O75376)	Nuclear receptor corepressor 1	n.d.	n.d.	n.d.	Evert et al. (2006)
MAML3 (Q96JK9)	Mastermind-like protein 3	n.d.	n.d.	n.d.	Ravasi et al. (2010)
EWSR1 (Q01844)	RNA-binding protein EWS	n.d.	n.d.		Vinayagam et al. (2011)

(Continued)

Table 2 | Continued

Ataxin-3 interacting protein (UniProt accession code)	Protein name	Direct interaction?	Interaction domains		Reference
			Ataxin-3	Partner protein	
SIGNAL TRANSDUCTION					
CK2 (P19784)	Casein kinase II subunit α	Yes	n.d.	n.d.	Tao et al. (2008), Mueller et al. (2009)
GSK3B (P49841)	Glycogen synthase kinase-3 β	Yes	n.d	n.d	Fei et al. (2007), Vinayagam et al. (2011)
DNM2 (P50570)	Dynamin-2	n.d.	n.d.	n.d.	Vinayagam et al. (2011)
CDKN1A (P38936)	Cyclin-dependent kinase inhibitor 1	n.d.	n.d.	n.d.	Vinayagam et al. (2011)
ANXA7 (P20073)	Annexin A7	n.d.	n.d.	n.d.	Vinayagam et al. (2011)
RPS6AK1 (Q15418)	Ribosomal protein S6 kinase α-1	n.d.	n.d.	n.d.	Vinayagam et al. (2011)
TK1 (P04183)	Thymidine kinase, cytosolic	n.d.	n.d.	n.d.	Vinayagam et al. (2011)
MKNK1 (Q9BUB5)	MAP kinase-interacting serine/threonine-protein kinase 1	n.d.	n.d.	n.d.	Vinayagam et al. (2011)
ATAXIOME					
TEX11 (Q8IYF3)	Testis-expressed sequence 11 protein	n.d.	n.d.	n.d.	Lim et al. (2006)
C16orf70 (Q9BSU1)	UPF0183 protein C16orf70	n.d.	n.d.	n.d.	Lim et al. (2006)
ARHGAP19 (Q14CB8)	Rho GTPase-activating protein 19	n.d.	n.d.	n.d.	Lim et al. (2006)
PICK1 (Q9NRD5)	PRKCA-binding protein	n.d.	n.d.	n.d.	Lim et al. (2006)

Boxes shaded in gray represent associations identified in high-throughput interactome screenings.

Atxn3, ataxin-3; IBR, In Between Ring fingers; JD, Josephin domain; n. d., not determined; NLS, nuclear localization sequence; Ub, ubiquitin; UBA, ubiquitin associated domain; Ubl, ubiquitin-like domain; UIM, ubiquitin-interacting motifs.

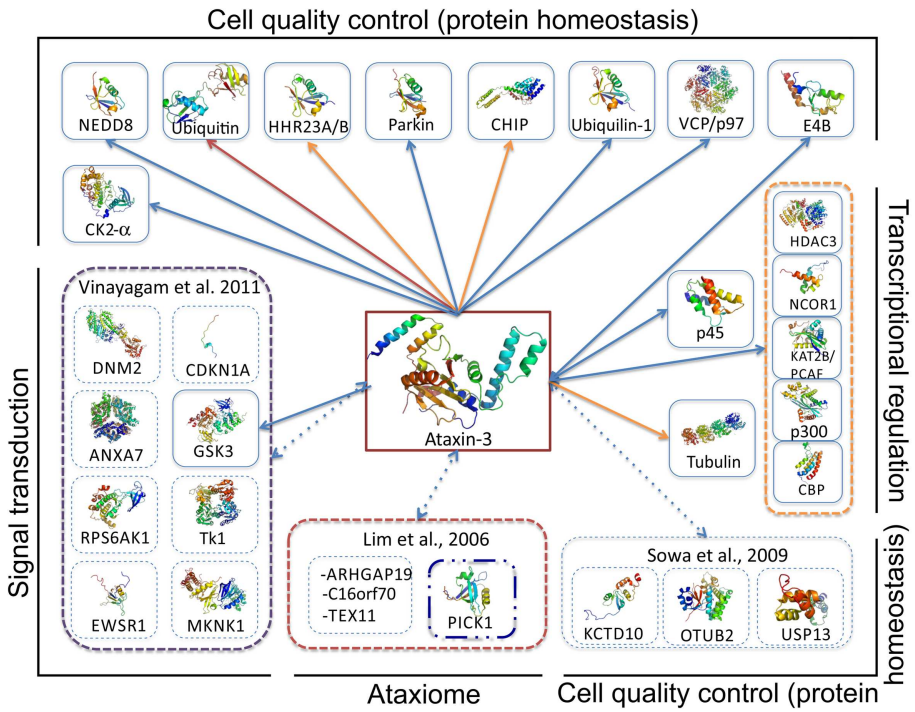

FIGURE 5 | Overview of ataxin-3 protein interaction network. Data on the ataxin-3 interactors was obtained by analysis of Interactome3D (Mosca et al., 2012), MINT (Ceol et al., 2010), and Dr. PIAS (Sugaya and Furuya, 2011) protein interaction databases, and completed with data compiled from current literature on ataxin-3 protein associations obtained with a diverse set of experimental approaches (see complete information on **Table 2**). Red arrows indicate interactions for which structural data has been obtained, while orange arrows indicate that biophysical data on interaction affinity *in vitro* is known (**Table 2**). Broken arrows represent interactions that result from high-throughput interactome analysis that still require detailed biochemical and functional analysis. Proteins are grouped according to their biological role.

facilitate docking of the positively charged ubiquitin C-terminus close to the catalytic site. Binding of ubiquitin to site 1 is of crucial importance for both JD and full-length ataxin-3 activity as ubiquitin hydrolase (Nicastro et al., 2010). Site 2 confers ubiquitin-chain linkage preference to ataxin-3 and it overlaps with the surface for interaction of the ubiquitin-like domain in HHR23B (Nicastro et al., 2005, 2010). Solution structure for the two UIMs (UIM1 and UIM2), which are separated by a short 2 amino acid spacer, revealed that they fold into two α-helices separated by a flexible linker (Song et al., 2010). Upon ubiquitin binding, this structure adopts a typical helix-loop-helix folding pattern, where hydrophobic interactions dominate the complex formation (Song et al., 2010). When in tandem, UIM1 and UIM2 show higher binding affinity for mono- or poly-ubiquitin than individual UIMs (Song et al., 2010), suggesting a cooperative binding mechanism (Song et al., 2010). The effect of the presence of UIM3 in ataxin-3 binding affinity for ubiquitin has not been shown, but its role in ubiquitin chain binding and recognition is unlikely to be of relevance to ataxin-3 activity, since no differences in proteolytic activity were identified when the 2UIM and 3UIM isoforms were compared. In the model proposed for ataxin-3 ubiquitin chain proteolysis, the UIMs (UIM1-UIM2) select and recruit poly-ubiquitin substrates, presenting them to the catalytic JD for cleavage (Mao et al., 2005).

Even though ataxin-3 functions as ubiquitin hydrolase, its proteolytic activity is rather low, indicating that either ataxin-3/JD requires additional factors (post-translational modifications, cofactors, intracellular interactions) to exhibit significant proteolytic activity or the substrates used *in vitro* so far are not optimal. Interestingly, only three amino acid mutations are sufficient to significantly increase the proteolytic activity of ataxin-3, to a value close to that of ataxin-3-like protein (Weeks et al., 2011). Under physiological conditions, one candidate for an activating signal is mono-ubiquitination at K117, which has been shown to increase the enzyme's rate of cleavage of Lys-63 linked substrates (Todi et al., 2009). However, the molecular mechanism by which ubiquitination increases enzyme activity is not still clear, nor is it known whether other cellular signals (e.g., phosphorylation by CK2 or GSK3b; Fei et al., 2007; Tao et al., 2008) may also modulate the activity of ataxin-3. Interestingly the JD-containing protein, Josephin 1 was also demonstrated to cleave ubiquitin chains only after it is mono-ubiquitinated (Seki et al., 2013). The regulation of ataxin-3 activity through ubiquitination might depend on the interaction of ataxin-3 with several E3 ubiquitin ligases (Durcan and Fon, 2013), such as the C-terminus of 70 kDa heat-shock protein (Hsp70)-interacting protein (CHIP), parkin, and E4B (**Figure 5**), since all were shown to promote ataxin-3 ubiquitination and regulate its degradation by the proteasome (Matsumoto et al., 2004; Jana et al., 2005; Miller et al., 2005). Association of ataxin-3 with CHIP is a multistep process regulated by mono-ubiquitination of the N-terminal region of

CHIP by the E2-conjugating enzyme Ube2w, and occurs through the region encompassing polyQ and UIM1 and 2 (Jana et al., 2005) (**Figure 4**). As observed for other interactions involving the C-terminal region of ataxin-3, the ataxin-3-CHIP complex is affected by polyQ expansion and the polyQ-expanded protein displays a sixfold increase in binding affinity (Scaglione et al., 2011). The presence of ataxin-3 in multicomponent E3-ligase complexes is also supported by the identification of a direct interaction with parkin, an association that stabilizes the interaction between parkin and the E2-conjugating enzyme Ubc7 (Durcan et al., 2011). In contrast with what is observed in the ataxin-3:CHIP complex, ataxin-3 association with parkin remains unaltered by polyQ expansion (Durcan et al., 2012) (**Figure 4**). However, we still do not understand the mechanisms that regulate shuttling of ataxin-3 between these functional complexes or how its distribution is modulated by polyQ expansion. Further biochemical studies are required to establish the correlation between these macromolecular interactions and their relevance for ataxin-3 aggregation and neurodegeneration in MJD patients

ATAXIN-3 AGGREGATION: A MULTISTEP PATHWAY MODULATED BY THE PROTEIN CONTEXT

A characteristic hallmark of MJD and other polyQ-expansion diseases is the appearance of intracellular inclusions enriched in the disease protein and containing components from the cell-quality control machinery (e.g., ubiquitin, proteasome subunits, and chaperones), indicating that these diseases form part of the larger family of protein misfolding disorders (Williams and Paulson, 2008). Early *in vitro* studies showed that expansion of the polyQ tract within the pathological range induced formation of insoluble β-rich fibrils with the capacity to bind amyloid-specific dyes (Bevivino and Loll, 2001). Later it was demonstrated that non-pathological ataxin-3 could also form insoluble fibrillar aggregates upon destabilization of its structure by temperature, pressure or denaturing agents (Marchal et al., 2003; Chow et al., 2004c). Unexpectedly, under partially destabilizing conditions the JD was also able to form insoluble fibrils, indicating that even though polyQ repeats are themselves toxic, the structure of ataxin-3, besides polyQ, has an important role in aggregation and fibril formation (Masino et al., 2004). Structural studies have shown that in ataxin-3, the addition of a polyQ tract destabilized the protein but polyQ expansion within the pathogenic range did not further induce significant structural changes (Chow et al., 2004a). In fact, alterations in ataxin-3 stability were later shown not to be a requirement for amyloid fibril formation since both non-expanded ataxin-3 and the isolated JD were shown to self-assemble and form fibrils under near-physiological conditions (Gales et al., 2005). Since kinetics of aggregation is slower for non-expanded ataxin-3 (Ellisdon et al., 2007), early events in the oligomerization pathway could be identified, with the formation of JD-mediated dimers proposed to be the first step in the ataxin-3 self-assembly pathway (Gales et al., 2005; Masino et al., 2011; Scarff et al., 2012). In a striking parallel with the classical amyloids the kinetics of fibril formation follows a nucleation-dependent polymerization mechanism, where these intermediate species might represent the most toxic species (Kayed et al., 2003; Laganowsky et al., 2012).

The current model for polyQ-expanded ataxin-3 aggregation consists of two steps. A JD-dependent step that leads to the formation of SDS-soluble protofibrils, followed by the formation of detergent-resistant fibrils similar to those found in MJD, where the polyQ-expanded tracts play a key role (Ellisdon et al., 2006, 2007). On the contrary, non-expanded polyQ ataxin-3 undergoes a single step aggregation event resulting in SDS-soluble aggregates, which does not involve the polyQ tract (Ellisdon et al., 2006) but directly depends on conformational changes outside the polyQ repeat. In the initial stages of aggregation, JD retains a native-like secondary structure, but is deployed of catalytic activity pointing to a subtle conformational change before fibril assembly (Masino et al., 2011). Although the JD plays a central role in this aggregation process, recent studies show that the ataxin-3 flexible region (Masino et al., 2003; Scarff et al., 2012), encompassing UIM1, and UIM2 also contributes to aggregation of the full-length protein (Santambrogio et al., 2012). This multistep aggregation modulated by the polyQ protein context seems to be common in other polyQ disorders such as HD (Thakur et al., 2009) and SCA1 (de Chiara et al., 2005). The presence of an expanded polyQ tract leads subsequently to rapid inter-fibril association and formation of large, highly stable amyloid-like fibrils. This indicates that the non-polyQ domains predispose ataxin-3 for aggregation and that the presence of a pathological polyQ tract introduces an additional step resulting in the formation of a highly stable amyloid-like aggregate.

This complex aggregation mechanism, involving domains outside the amino acid-repeat region, is also found in PABPN1(Tavanez et al., 2005; Winter et al., 2013), a multi-domain polyA-containing protein consisting of an N-terminal segment containing the alanine repeat, a coiled-coil domain, a RNA recognition motif (RRM), and a C-terminal domain. As observed for ataxin-3, the propensity of PABPN1 to aggregate and form amyloid fibrils (Scheuermann et al., 2003) is not exclusive of the expanded protein and can also be observed in the non-disease protein (Tavanez et al., 2005; Rohrberg et al., 2008). This indicates that polyA-flanking regions/domains influence the aggregation process, and that this is likely a broader mechanism common in homopeptide repeat-containing proteins. In fact, analysis of PABPN1 sequence indicates higher scores for aggregation propensity within the RRM domain (Tavanez et al., 2009), with mutations in the RRM being sufficient to prevent aggregate formation (Tavanez et al., 2005).

Aggregation of homopeptide repeat-containing proteins is therefore a multiparametric process that culminates in cell-specific degeneration, whose toxicity might be explained by the context-dependent molecular interactions and post-translational modifications. The relation between protein interactions, function, and aggregation will be discussed below, with a particular focus on the polyQ-containing protein, ataxin-3.

ATAXIN-3 FUNCTION VS. AGGREGATION

Several studies have been focused on the search for specific ataxin-3 interactors, some of which have identified direct physical association between the protein partners and provided clues into ataxin-3 biological role (**Table 2** and references herein; **Figure 5**). Recently, different high throughput interactome screens focused on the search for protein complexes associated with ubiquitin hydrolases

(Sowa et al., 2009), ataxia-related proteins (Lim et al., 2006), signal transduction pathways (Vinayagam et al., 2011), and transcriptional regulation (Ravasi et al., 2010), contributing with data on novel putative ataxin-3 binding proteins (**Table 2**; **Figures 4** and **5**).

An overview of the current data on the ataxin-3 interactome shows that a large number of interactions map to the catalytic JD. The intrinsic tendency of JD to self-associate involves the hydrophobic patches on its surface, which overlap with the functionally relevant ubiquitin binding sites 1 and 2 (Matos et al., 2011; Pastore and Temussi, 2012), providing a direct link between protein function and aggregation and exposing a role for intracellular interactors, such as ubiquitin, in protecting against ataxin-3 self-assembly (Gales et al., 2005; Masino et al., 2011; Matos et al., 2011; Pastore and Temussi, 2012). Likewise, in the polyA-containing protein PABPN1 the RRM domain responsible for the interaction with the mRNA (Banerjee et al., 2013) and with polyadenylate-specific RNA polymerase is also involved in the aggregation process (Winter et al., 2013). In addition, the fact that heat-shock proteins including Hsp70, and type I arginine methyl transferases (PRMT1 and PRMT3) associate preferentially with expanded PABPN1 raises the question whereas proteotoxicity of expanded PABPN1 might also be caused by altered protein networking. Destabilization of the ataxin-3 JD by specific mutations revealed that any conformational change in this region is directly linked with aggregation of the full-length protein (Saunders et al., 2011), emphasizing the putative therapeutical potential associated with the identification of macro-molecules with the ability to stabilize this N-terminal region. In fact, interaction of JD with protein partners (Masino et al., 2011) or chaperones (Robertson et al., 2010) is sufficient to induce stabilization of JD leading to a reduction in ataxin-3 self-assembling properties (Matos et al., 2011; Pastore and Temussi, 2012).

Concerning the C-terminal flexible tail of ataxin-3, the interaction with VCP/p97 is probably the one for which more experimental data is available (Hirabayashi et al., 2001; Doss-Pepe et al., 2003; Matsumoto et al., 2004; Boeddrich et al., 2006; Zhong and Pittman, 2006). This association is dependent on the arginine/lysine-rich motifs close to the polyQ tract of ataxin-3 (Boeddrich et al., 2006), and several reports point to a stronger interaction with the disease-protein containing longer polyQ stretches (Wang et al., 2000; Matsumoto et al., 2004; Boeddrich et al., 2006; Zhong and Pittman, 2006). Functional interaction with VCP/p97 is able to modulate the fibrillogenesis of a C-terminal fragment of expanded ataxin-3 (71Q) in a concentration-dependent manner, with equimolar concentrations of VCP/p97 stimulating fibrillogenesis, while a four-fold excess of VCP prevented aggregation (Boeddrich et al., 2006). This study provides clues toward the role of interactors targeting the C-terminal region of ataxin-3 as modulators of its oligomerization properties, indicating that this mostly unstructured region (Masino et al., 2003; Scarff et al., 2012) may also represent a bridge between physiological interactions, function, and aggregation. In the field of polyQ disorders, the search for protein interactors is an active area of research, uncovering novel macromolecular partners often acting as disease modifiers (Goehler et al., 2004; Kaltenbach et al., 2007; McGurk and Bonini, 2012). A recent screen for modifiers of ataxin-1 aggregation and toxicity in mammalian cells showed that toxicity enhancers often contained coiled-coil domains. Importantly, coiled-coil formation by ataxin-3 polyQ stretch and its flanking domains were also predicted (Fiumara et al., 2010), however neither the consequences of its expansion nor its functional properties were experimentally assessed. Since coiled-coil structures are known to be involved in protein–protein interactions (Parry et al., 2008; Fiumara et al., 2010), it would be interesting to access the role of protein interactions mediated by the polyQ region of ataxin-3 in regulating its aggregation behavior (**Figure 4**). In fact, some of the interactors associated with ataxin-3 nuclear functions, rich in polyQ- (p300, NCOR, CBP), and polyA-repeats (NCOR), target this region and are predicted to bind better to the expanded protein (**Figures 4** and **5**). Therefore it is tempting to speculate that these anomalously stronger interactions with the expanded protein in the nucleus might be associated with increased aggregation and toxicity observed when a strong nuclear localization sequence (NLS) is fused to ataxin-3 (Perez et al., 1998; Bichelmeier et al., 2007; Macedo-Ribeiro et al., 2009) or when the protein shuttles to the nucleus upon increased cellular stress (Reina et al., 2010). Heat-shock induces phosphorylation of a serine residue located on ataxin-3 JD and drives the protein to the nucleus leading to its dissociation from the cytosolic VCP/HHR23A complex (Reina et al., 2010), highlighting the dynamic nature of ataxin-3 partition between macromolecular assemblies and making it tempting to speculate that polyQ expansion might affect this distribution.

CONCLUDING REMARKS

Trinucleotide repeats are keen for driving evolution by providing genetic variability, with homopeptide-encoded regions being crucial for the establishment of protein interactions. However, as unstable regions, expansion of the homopeptide regions might occur, being responsible for several neurodegenerative and muscular diseases. Homopeptide repeats such as polyQ and polyA seem to influence and to drive the repeat-containing protein toward self-assembly and aggregation. On the other hand, structural studies were able to reveal that aggregation of homopeptide-containing proteins also depends on the homopeptide protein context with additional protein domains playing a role in a multi-domain self-assembly mechanism. Differential expression of protein isoforms generated by alternative splicing, post-translational modifications and, additionally, differences in macromolecular interactions are currently advanced as hypotheses that, by their ability to modulate protein function and aggregation, could explain the cell-specific toxicity of the homopeptide-expanded proteins.

Ataxin-3 is an excellent example of a repeat-containing protein that, upon polyQ-expansion, does not undergo drastic structural and functional changes, but achieves an increased tendency toward self-assembly and aggregation. The protein has deubiquitinase activity and plays a role in the cell-quality control system, and in transcriptional regulation. As a result of its modular multi-domain structure, ataxin-3 engages in multiple macromolecular interactions and several evidences show that it associates with several functional multiprotein complexes, in some cases in a polyQ-dependent manner. The structural and mechanistic details regulating ataxin-3 redistribution between different cellular machineries are still unclear, although post-translational modifications of the protein subunits assembled in

these complexes are likely to play a role. Different cellular events such as proteotoxic stress or aging might unbalance the association of expanded ataxin-3 with its molecular partners and contribute to the alteration of ataxin-3 normal cellular functions. Since ataxin-3 self-assembly is a complex process that involves several protein domains, including JD, the relocalization of the protein to different complexes might induce the exposure of aggregation-prone regions and lead to the appearance of the characteristic intracellular protein inclusions.

Since macromolecular interactions seem to be either protective or exacerbate aggregation of the homopeptide-containing proteins, they might be targeted therapeutically. However, an in-depth understanding about the effect of homopeptide-expansion in the function of the containing-protein and on the interactions with molecular partners is required, in order to understand how they contribute to neurodegeneration. The combination of biochemical and computational approaches to the identification of disease-protein interaction networks is critical for defining their normal function, identifying new markers for disease prognosis and also for the development of tools to selectively target those interactions with potentially reduced side effects.

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Multiple aspects of gene dysregulation in Huntington's disease

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Huntington's Disease (HD) is a genetic neurodegenerative disease caused by a CAG expansion in the gene encoding Huntingtin (*Htt*). It is characterized by chorea, cognitive, and psychiatric disorders. The most affected brain region is the striatum, and the clinical symptoms are directly correlated to the rate of striatal degeneration. The wild-type Htt is a ubiquitous protein and its deletion is lethal. Mutated (expanded) Htt produces excitotoxicity, mitochondrial dysfunctions, axonal transport deficit, altered proteasome activity, and gene dysregulation. Transcriptional dysregulation occurs at early neuropathological stages in HD patients. Multiple genes are dysregulated, with overlaps of altered transcripts between mouse models of HD and patient brains. Nuclear localization of Exp-Htt interferes with transcription factors, co-activators, and proteins of the transcriptional machinery. Another key mechanism described so far, is an alteration of cytoplasmic retention of the transcriptional repressor REST, which is normally associated with wild-type Htt. As such, Exp-Htt causes alteration of transcription of multiple genes involved in neuronal survival, plasticity, signaling, and mitochondrial biogenesis and respiration. Besides these transcriptional dysregulations, Exp-Htt affects the chromatin structure through altered post-translational modifications (PTM) of histones and methylation of DNA. Multiple alterations of histone PTM are described, including acetylation, methylation, ubiquitylation, polyamination, and phosphorylation. Exp-Htt also affects the expression and regulation of non-coding microRNAs (miRNAs). First multiple neural miRNAs are controlled by REST, and dysregulated in HD, with concomitant de-repression of downstream mRNA targets. Second, Exp-Htt protein or RNA may also play a major role in the processing of miRNAs and hence pathogenesis. These pleiotropic effects of Exp-Htt on gene expression may represent seminal deleterious effects in the pathogenesis of HD.

Keywords: transcription, epigenetics, chromatin remodeling, histone modifications, REST, miRNAs

INTRODUCTION

Huntington's disease (HD) is a dominantly inherited genetic disorder induced by an abnormal expansion of a CAG trinucleotide repeat at the 5′ terminal part of the Huntingtin (Htt) gene leading to a polyglutamine expansion in the Htt protein (1). It is the most frequent genetic disease induced by a polyglutamine expansion with a prevalence of three to seven for 100,000 persons. Individuals with 39 CAG repeats or more will develop the clinical symptoms and signs of HD including neuropsychiatric, motor, and cognitive abnormalities that cause a progressive loss of functional capacity and shorten life span (2, 3). Intermediate alleles repetitions (between 36 and 39 repeats) are usually associated with late onset disease and may express a variable penetrance as the patient may die before disease onset (4–6). HD has a well-defined neuropathology, and informative pre-manifest predictive genetic testing. Brain weight may be reduced by as much as 25–30% in advanced HD cases. Gross pathology in HD is mainly observed in the brain, with atrophy predominating in the caudate-putamen, and to a lesser extent, the cerebral cortex. Furthermore, despite the early expression of mutated Htt (Exp-Htt) in all neuronal cells the first symptoms and neuropathological hallmarks appear at adulthood, around 40–45 years old. The age of onset of the disease is conversely proportional to the number of CAG repeats in the affected allele. Once the first symptoms have appeared, the disease progresses and leads progressively to death. As neuro-degeneration progresses in the striatum, the severity of symptoms increases (2). Magnetic resonance imaging (MRI) studies indicate that striatal atrophy begins up to 15 years before predicted onset and continues through the period of manifest illness (7). Therefore, the pre-symptomatic phase in HD provides a unique window for therapeutic intervention and neuro-protection.

The clinical features of HD can be divided into three groups: movement disorders, cognitive impairment, and psychiatric manifestations [see Ref. (8) for review]. Chorea is the most characteristic movement disorder of HD and is characterized by brief, involuntary, abnormal movements, which appear unpredictably in all the parts of the body. Cognitive impairment can precede motor symptoms or occur during the course of the disease, and usually leads, in turn, to dementia. Neurobehavioral symptoms include irritability, agitation, apathy, anxiety, social

withdrawal, impulsiveness, alcohol abuse, obsessive-compulsive disorder. Mood disorders are very frequent, including depression and HD patients have a risk of suicide that is 10 times higher than in the general population.

There is no cure for HD, although medication can be given to help control the emotional and movement problems associated with HD. While medicines may help keep clinical symptoms under control, they are unable to stop or reverse the course of the disease.

Basic research has provided new insights into the complex cellular and molecular alterations involved in the pathogenesis of HD. The wild-type Htt is an ubiquitous protein, expressed in most cells and within all cellular compartments (9). It is required for normal embryonic development, and *Htt* knock-out mice show early lethality (E8,5) (10, 11). Furthermore, selective knock-down of the Htt protein in neurons and testis produces apoptosis in these tissues (12). Whether neuronal degeneration in HD is due to loss of normal function of wild-type Htt, or gain of toxic functions of Exp-Htt is still a debate. Expansion of polyglutamine in Htt leads to protein aggregation (9), a mechanism thought to be primarily involved in several neurological disorders caused by CAG repeats. It still remains to be established whether the mutant Htt aggregates are incidental, pathogenic, or neuroprotective. Expansion of polyglutamine in Htt produces by itself multiple cellular dysfunctions, including excitotoxicity, altered mitochondrial functions, axonal transport deficit, altered proteasome activity, and gene dysregulation, that were extensively described in other reviews (8, 13). Among these alterations, transcriptional dysregulation occurs at early neuropathological stages in HD and seems to be seminal in the neuropathological process.

TRANSCRIPTIONAL DYSREGULATION IN HD

Dysregulation of transcription was first described in HD brain tissues at early neuropathological stages and then found in pre-symptomatic HD transgenic mice. Expression of enkephalin, substance P, dopamine D1 and D2 receptor mRNAs were shown to be altered in the caudate-putamen of HD patients in *post mortem* tissue in the early grade using *in situ* hybridization (14). Subsequently, cDNA microarray performed on genetically engineered HD mouse models allowed thousands of genes to be monitored, and provided a global genomic view of striatal dysfunctions in HD. From these analysis, neurotransmitter receptors, enzymes, and proteins involved in neuron structure, stress response, and axonal transport were found to be dysregulated (15–20). These changes were reproducibly observed in various HD mouse models and in the human HD caudate-putamen (19). Altogether these observations strongly supported that changes in transcription underlie neuro-degeneration rather than unspecific degradation of all RNAs in affected neurons.

Importantly, more than 81% of striatal-enriched genes (genes with higher relative expression in the striatum when compared to other brain regions) are decreased in a HD mouse model and in the caudate of HD patients (21). Down-regulation of novel striatal-enriched genes involved in vesicle transport and trafficking, tryptophan metabolism and neuroinflammation have also been identified in both HD mouse striatum and caudate from HD patients (22). Transcriptional dysregulation occurs in large genomic regions, in a coordinated fashion and is associated with disease progression. Hence genome-wide expression profiling of the blood from HD patients revealed significant differences in symptomatic patients (23) but not moderate-stage patients (20). Thus, these biomarkers need to be further validated before their widespread use in clinical trials.

PATHOGENIC INTERACTION OF Exp-HTT WITH NUCLEAR PROTEINS

Huntingtin has multiple interacting partners, among which are transcription factors or co-activators of the transcriptional machinery, some of them exhibiting enhanced binding with Exp-Htt, while a handful prefers binding with wild-type Htt (24, 25). Due to its polyglutamine expansion, Exp-Htt abnormally interacts with several proteins involved in transcription regulation. These include the global transcriptional regulator TATA-binding protein/TFIID (26), TAFII130, a co-activator involved in cAMP-responsive element binding protein (CREB)-dependent transcription (27). An abnormal interaction of Exp-Htt has also been shown with specificity protein 1 (Sp1) (28), p53, CREB binding protein (CBP) (29, 30), and nuclear receptor co-repressor (NCoR) (31). The global consequence of these pathogenic interactions is a widespread transcriptional dysregulation. Thus, overexpression of Sp1 and TAFII130 in cultured striatal cells reverses the transcriptional inhibition of the dopamine D2 receptor gene caused by Exp-Htt, and protects neurons from Exp-Htt-induced cellular toxicity (28). Exp-Htt induces upregulation of p53 and its downstream targets, Bax and Puma, both *in vitro* and in postmortem brains of HD patients (32, 33). This results in mitochondrial membrane depolarization and decreased complex IV activity. p53 inhibition or its genetic deletion ameliorates mitochondrial defects in HD cell cultures (33).

CRE-regulated genes have been well described for their role in neuronal survival (34) and impairment of CRE-dependent transcription can account for the neurodegenerative process in HD. One of the CRE-regulated genes that has been directly associated with striatal neuro-degeneration is the peroxisome proliferator-activated receptor co-activator-1α (PGC-1α), a transcriptional co-activator that controls the expression of genes involved in mitochondrial biogenesis, respiration and glucose/fatty acid metabolism (35). Exp-Htt is known to cause energy dysfunction that is mainly related to mitochondrial abnormalities (36–38). Expression of PGC1-α is down-regulated in HD patients and HD mice (39). This down-regulation is explained by an interference of Exp-Htt with the CREB/TAF4-dependent transcriptional pathway. Cross-breeding of *Pgc-1α* knock-out mice with HD knock-in mice leads to increased degeneration of striatal neurons and motor abnormalities in the HD mice, whereas lentiviral-mediated overexpression induces neuro-protection. Decreased expression of PGC1-α accounts for abnormal myelination in HD, since Exp-Htt-induced down-regulation of PGC1-α in oligodendrocytes leads to inhibition of genes involved in myelination (40). PGC1-α can also control extrasynaptic NMDAR activity in neurons, which contributes to excitotoxicity in HD (41). Suppression of PGC1-α contributes to Exp-Htt-induced increase in extrasynaptic NMDAR activity and vulnerability. Others key regulators of PGC-1α, are Mitogen and Stressed-activated protein Kinase-1 (MSK-1), and SIRT3. MSK-1 is a striatum-enriched nuclear protein

kinase, targeted by the pro-survival Extracellular-signal Regulated Kinase (ERK) signaling pathway. By regulating CREB phosphorylation, along with histone H3 phosphorylation, MSK-1 is directly involved in the expression levels of PGC-1α, and as such protects against Exp-Htt-induced striatal death *in vitro* and *in vivo* (42, 43) (see below). SIRT3 is one the seven mammalian homologs of the sirtuin gene family. This mitochondrial deacetylase, initially described in brown adipocytes, regulates mitochondrial functions and thermogenesis (44). In response to exercise, SIRT3 controls CREB phosphorylation and PGC-1α expression, via AMP-activated protein kinase (AMPK) (45). Exp-Htt induces decreased deacetylase activity of SIRT3 and further leads to reduction in cellular NAD(+) levels and mitochondrial biogenesis in cells. Viniferin, a natural compound that activates AMPK and enhances mitochondrial biogenesis, is neuroprotective in HD cellular models, an effect that tightly depends on SIRT3 activity (46). Strikingly, the sirtuin family members seem to be intimately linked to pathogenesis in HD, since the NAD+-dependent deacetylase activity of SIRT1 is also involved in the regulation of transcription in HD. SIRT1 is a nuclear protein that normally controls CREB phosphorylation levels via TORC1 (Regulated transcription co-activator 1 (TORC1) activity (47, 48). By interacting with SIRT1, Exp-Htt inhibits its deacetylase activity, and causes hyperacetylation of TORC1. This results in a decrease of CREB-regulated genes, including BDNF, and probably PGC1-α.

Altogether, these data strongly support that transcriptional dysregulation in HD plays a major role in mitochondrial dysfunctions and energy metabolism deficit, two important hallmarks of the pathology.

IMPAIRMENT OF CYTOSOLIC SEQUESTRATION OF REST

Wild-type Htt sequesters R element-1 silencing transcription factor (REST), a transcriptional repressor of neuronal survival factors, including brain-derived neurotrophic factor (BDNF). This neurotrophic factor is expressed by cortical neurons, which project to the striatum, and is critical for striatal survival. Interestingly, both transcriptional regulation and axonal transport of BDNF (49, 50) are altered in HD. Htt interacts with REST in the cytoplasm, and this interaction is impaired by Exp-Htt. Thus, increased nuclear translocation of REST is observed in the presence of Exp-Htt. Locally, REST exerts a potent inhibitory role on *Bdnf* transcription and other neuronal genes (49, 51, 52). In this context, the consequence of the loss of function of Htt is directly correlated with HD pathogenesis. Expression level of BDNF is decreased in the striatum of HD patients and in the cortex of HD mouse models (49, 53, 54). Down-regulation of BDNF in the striatum specifically worsens the HD phenotype, whereas elevating BDNF expression in the forebrain alleviates it (54–58). The role of REST in HD may not be restricted to the regulation of *Bdnf* transcription since several REST targets are known to be dysregulated in HD (52, 58). REST seems to have a widespread role on gene dysregulation in HD, since it also controls non-coding RNAs (see below). *In vivo* delivery of a dominant negative form of REST in the motor cortex restores the expression of BDNF mRNA and protein along with other REST-regulated genes in this region (59). Surprisingly, despite this important effect on gene regulation, no therapeutic effects were found in motor function in HD mouse models. These data raised the question as to whether a more widespread rescue of REST-regulated genes in the brain may be necessary.

CHROMATIN REMODELING IN HUNTINGTON'S DISEASE

Chromatin remodeling is an "above the genome" molecular mechanism, that gates DNA access, and hence transcription. It is critically controlled by post-translational modifications (PTM) of histones (H2A and H2B, H3 and H4), a group of highly basic proteins tightly linked to DNA. By modifying the electrostatic interactions between the N-terminal domain of histones and DNA, PTM of histones contribute to the chromatin structure, and access of the transcriptional machinery to the DNA (60). In particular, the methylation or acetylation state of histones is closely linked to regions of transcriptional activity, by regulating transcription factor access to promoter regions in the DNA.

The enzymes that catalyze histone acetylation are histone acetyltransferases (HATs) whereas Histone Deacetylases (HDACs), catalyze the reverse deacetylation reaction (60–62). By interacting with CBP and p300/CBP-associated factor (P/CAF), Exp-Htt blocks their intrinsic HAT activity (29, 30, 63, 64). This results in a global reduction of histones H3 and H4 acetylation levels, along with CBP-regulated gene transcription. Overexpression of CBP reduces Exp-Htt-induced toxicity (30).

Determining experiments were performed to demonstrate that HDAC inhibitors (HDACis), including SAHA, sodium butyrate, or phenylbutyrate improved behavioral performance and increased neuronal survival in several HD models (64–67). These data lead to the general concept that HDACi could be a new therapeutic avenue in HD. This concept is however weakened by the toxicity of the aforementioned HDACi compounds at therapeutic doses. Furthermore, it must be emphasized that the levels of acetylated histones are not decreased globally in HD mouse models, but rather selectively in the promoters of genes that are specifically down-regulated in HD (68).

So far, HDACis act broadly on the HDAC family, which comprises 11 members divided into four classes: I (HDAC1, 2, 3 and 8), IIa (HDAC4, 5, 7 and 9), IIb (HDAC6 and 10), and IV (HDAC11) (69). Their relative toxicity can be due to either inhibition of a pro-survival HDAC isoform, or low substrate specificity, a single enzyme being capable of deacetylating multiple sites within histones (60). Thus, it was postulated that inhibitors targeting one specific HDAC might produce a better benefit to side effect ratio.

To unravel this issue, genetic invalidation of each single HDAC was investigated in the R6/2 mouse model. These studies revealed that reduction of Hdac3, 5, 6, 7, and 9 expression had no effect on HD-related phenotype (70–72), whereas reduction of Hdac4 expression showed a significant beneficial effect (73). This raises the interesting question as to whether specific HDAC4 inhibitors may be more adapted for HD treatment, an issue that is now under investigation (73).

Methylation of histones affects lysine and arginine residues and is associated to either activation or repression of transcription, depending on the modified residues. One of the proteins involved in methyltransferase activity at histone H3 (K9) is ERG-associated protein with SET domain (ESET). ESET expression is increased in HD patients and R6/2 HD mice (74). Sp1 acts as a transcriptional activator of the ESET promoter at guanosine-cytosine

(GC)-rich DNA binding sites (75). Inhibiting Sp1 binding to these sites using mitramycin (a clinically approved antitumor antibiotic) suppressed basal ESET promoter activity in a dose dependent manner and lead to extended survival, enhanced motor performance and improved brain histopathology in R6/2 mice (74). Interestingly, the reduction of H3K9 hypermethylation induced by mithramycin or chromomycin, is associated with an increased acetylation of the same residue (76). On the other hand, the beneficial effect of the HDACi phenylbutyrate in HD mice is accompanied by an increase in H3 and H4 acetylation and a concomitant decrease in H3 methylation (67). These data illustrate that a crosstalk between acetylation and methylation participates to the nucleosomal dynamics, and that disequilibrium between these two epigenetic marks can be corrected by the inhibition of either deacetylation or methylation.

Monoubiquitylation of histones has also been implicated in HD-related transcriptional dysregulation. This modification, which involves E3-ubiquitin ligase complexes, affects lysine residues of histone H2A (uH2A) and H2B (uH2B) and is associated to either activation or repression of transcription depending on the modified residues. Exp-Htt expression alters the activity of specific E3-ubiquitin ligases, and modifies uH2A and uH2B. Knocking down the H2A E3-ubiquitin ligase reduces uH2A and rescues transcriptional repression in Exp-Htt knock-in cells. In contrast, knocking down the H2B E3-ubiquitin ligase induces transcriptional repression in wild-type Htt knock-in cells (77).

Core histones can be post-translationally modified by transglutaminases (TG), which catalyze transamidation of glutamine residues. All four mammalian core histones, H2A, H2B, H3, and H4, were shown to be glutaminyl substrates of TG2, a nuclear TG, and their crosslinking contributes to chromatin condensation *in vitro* (78–80). Total TGs activity is elevated in brain extracts from HD patients (81) and treatment of R6/2 mice with a TG competitive inhibitor, cystamine, extends survival, reduces tremor and abnormal movements and ameliorates weight loss in these mice (82). Therefore, TGs were suggested to participate to chromatin remodeling and gene expression dysregulation in HD. McConoughey and colleagues showed that TG2 polyaminates H3 N-terminal tail which increases its positive charge and therefore its propensity to more tightly interacts with DNA (83). TG2 occupies the promoter/enhancer regions of two genes essential for energy production, PGC1-α and cytochrome *c*, and a selective inhibition of TG2 in a HD striatal cell line corrects gene dysregulation. Therefore TG2 inhibition has emerged as a HDAC-independent epigenetic therapeutic strategy for HD.

Histone phosphorylation is mainly described as an activating chromatin mark of gene activation. This PTM affects serine, threonine and tyrosine residues. Histone phosphorylation is controlled by the interplay between kinases and phosphatases that respectively add and remove phosphate onto each of these residues. Histone kinases phosphorylate the hydroxyl group of the targeted amino-acid side chain therefore leading to a change of the global charge of histones, a reduced interaction between histones and DNA and a relaxation of chromatin (60). Phosphorylation of H3S10 involves MSK-1 that was shown to be down-regulated in HD cells, mice and patients (42). Restoration of MSK-1 expression in striatal neurons *in vitro* and in the lentiviral-based rat model of HD protects against neuronal dysfunctions induced by Exp-HTT (42, 43). In contrast, MSK-1 knock-out mice exhibit spontaneous striatal atrophy when they age, and a higher sensitivity to the 3-nitropropionic acid (3NP), a mitochondrial neurotoxin that induces selective degeneration of striatal neurons and HD-like symptoms in humans, monkeys, and rodents (43). In addition to its H3S10-kinase activity, MSK-1 phosphorylates, and activates CREB, leading to the regulation of PGC-1 α, both *in vitro* and *in vivo* (**Figure 1**).

In addition to histone PTMs, chromatin remodeling is controlled by DNA methylation. A recent study showed that Exp-HTT induces an extensive alteration of DNA methylation on a large proportion of genes that change in expression in HD (84). The authors of this study identified more specifically two transcriptional regulators, AP-1 and Sox2, associated with DNA methylation changes. Since these epigenetic changes are more stable, this could explain the long-term modifications of gene expression in HD.

HD AND miRNAs

About 98% of human transcribed genome is dedicated to non-protein-coding RNAs (NcRNAs) genes, with regulatory properties on gene expression. Among ncRNAs, microRNAs (miRNAs) are 21–23 nucleotide RNA molecules that regulate gene expression by promoting either degradation or translational-inhibition of target mRNAs (85). The miRNA pathway starts in the nucleus with the RNA polymerase II-mediated transcription of primary (pri-miRNAs) hairpins, which are cleaved into precursor of miRNAs (pre-miRNAs) by the nuclear proteins Drosha and DiGeorge syndrome critical region 8 (DGRC8) (86, 87). Pri-miRNAs are then transported to the cytoplasm (88, 89) and processed into 22nt duplex mature miRNAs by the RNAseIII Dicer (90), which is then assembled into the RNA-induced silencing complex (RISC) with the protein Argonaute (Ago) (91). MiRNAs suppress post-transcriptional expression of genes by guiding RISC interaction with their specific sequence motifs within the 3′untranslated region (3′UTR). This results in either degradation or translational-inhibition of the target mRNAs (92–95).

Many miRNAs are selectively and abundantly expressed in the CNS where they play key roles in the elaboration of the neuronal transcriptome (96) and seem to be important mediators of plasticity (97). MiRNA dysregulation has been associated with several human disorders of the CNS. The first evidence came from studies showing that Dicer or DGCR8 ablation impairs neuronal differentiation, produces synaptic dysfunctions, disturbs axonal path-findings, and induces neuro-degeneration, suggesting that miRNAs play important roles in neurological disorders (98, 99). Evidence of miRNA dysregulation in HD exists. Two different and complementary aspects of this dysregulation arise from the recent literature. First, there is now increasing evidence that multiple neural miRNAs are decreased in HD neurons, with concomitant de-repression of downstream target mRNAs (58, 100–102). Second, several elegant studies demonstrate that Exp-HTT protein (103) or RNA (104) may play a major role in the processing of miRNAs and hence pathogenesis.

Using an *in silico* approach, the group of Cataneo identified 17 miRNA genes as likely targets of REST (58) (**Figure 2**). The regulation of these miRNAs by REST was evaluated in embryonic

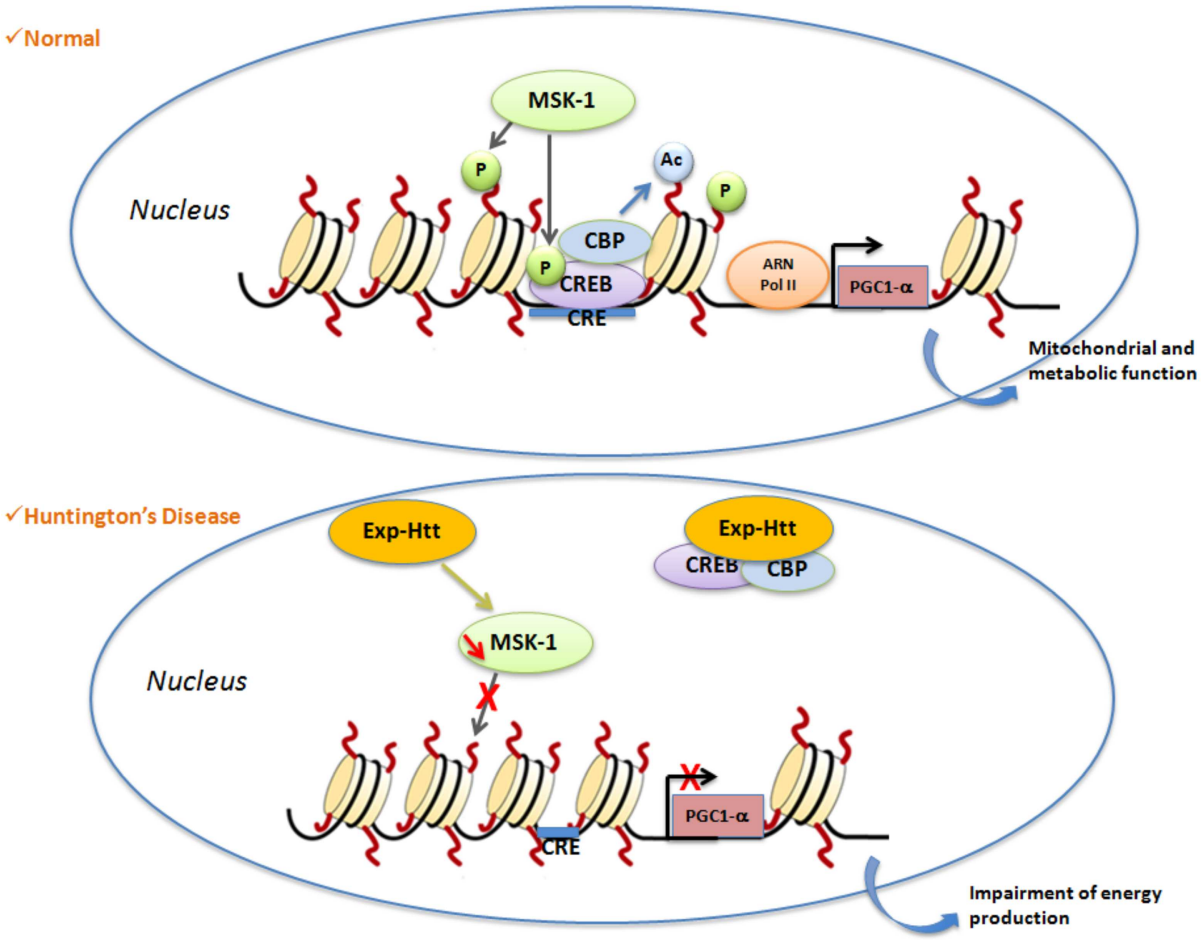

FIGURE 1 | Illustration of a signaling pathway involved in histone PTMs in HD. Under normal conditions, activation of nuclear MSK-1 induces phosphorylation of (1) histone H3 and (2) the transcriptional factor CREB. Phosphorylated CREB recruits CBP, and activates its histone acetyl transferase (HAT) properties. Post-translational modifications (PTM) of histones (phosphorylation, acetylation) lead to modification of chromatin structure from heterochromatin to euchromatin, which allows gene transcription to occur. One of the key genes regulated by CREB is *PGC1α*, a crucial gene involved for the regulation of mitochondrial and metabolic functions. In Huntington's disease, Exp-Htt expression results on (1) down-regulation of MSK-1 expression and (2) sequestration of CREB and CBP within Exp-Htt aggregates. Therefore, the heterochromatin structure is favored, a structure that impairs transcription of CREB target genes.

striatal cell lines, and mir-29a, mir-124a, mir-132, and mir-135b were shown to be significantly upregulated upon loss of REST function and in the cortex of 12-week-old R6/2 mice. In humans, mir-132 expression level is significantly lower in HD samples compared to control. In contrast, mir-29a and mir-330 expression is significantly higher in HD samples. Packer et al. (102) used a screen of predicted REST-regulated miRNAs from HD patient brain samples, and found significant decreases of miR-9, miR-9*, and miR-29b as well as a significant increase of miR-132 at late stages. They also found that the bi-functional brain enriched miR-9/miR-9* targets two components of the REST complex: miR-9 targets REST and miR-9* targets CoREST. A characterization of miRNAs profiling and sequence modification was performed by Illumina sequencing in the frontal cortex and the striatum. It showed a strong deregulation of miRNA and IsomiRs (miRNAs containing length and sequence heterogeneity) in HD, most being common to both frontal cortex and striatum (105). Of interest, the co-regulated miRNAs contained regulatory sequences for REST and p53, suggesting a key role of these genes in down-regulation of gene expression in HD. Profiling of miRNAs expression was also performed in the YAC128 and R6/2 mice, showing that nine miRNAs (miR-22, miR-29c, miR-128, miR-132, miR-138, miR-218, miR-222, miR-344, and miR-674*) are commonly down-regulated in 12-month-old YAC128 mice and 10-week-old R6/2 mice (100). Concomitantly, the expression of Dicer is decreased at the late stages in these two mouse lines, indicating that miRNA biogenesis is altered in HD. More recently, Soldati and collegues, found the same results in HD cell lines (101). Rescuing miR-22 expression in *in vitro* HD models, protected against Exp-Htt-induced neurotoxicity (106). Very recently, miR-196a was shown to reduce the expression of Exp-HTT *in vitro*, and to improve molecular, pathological, and behavioral phenotypes in a HD transgenic

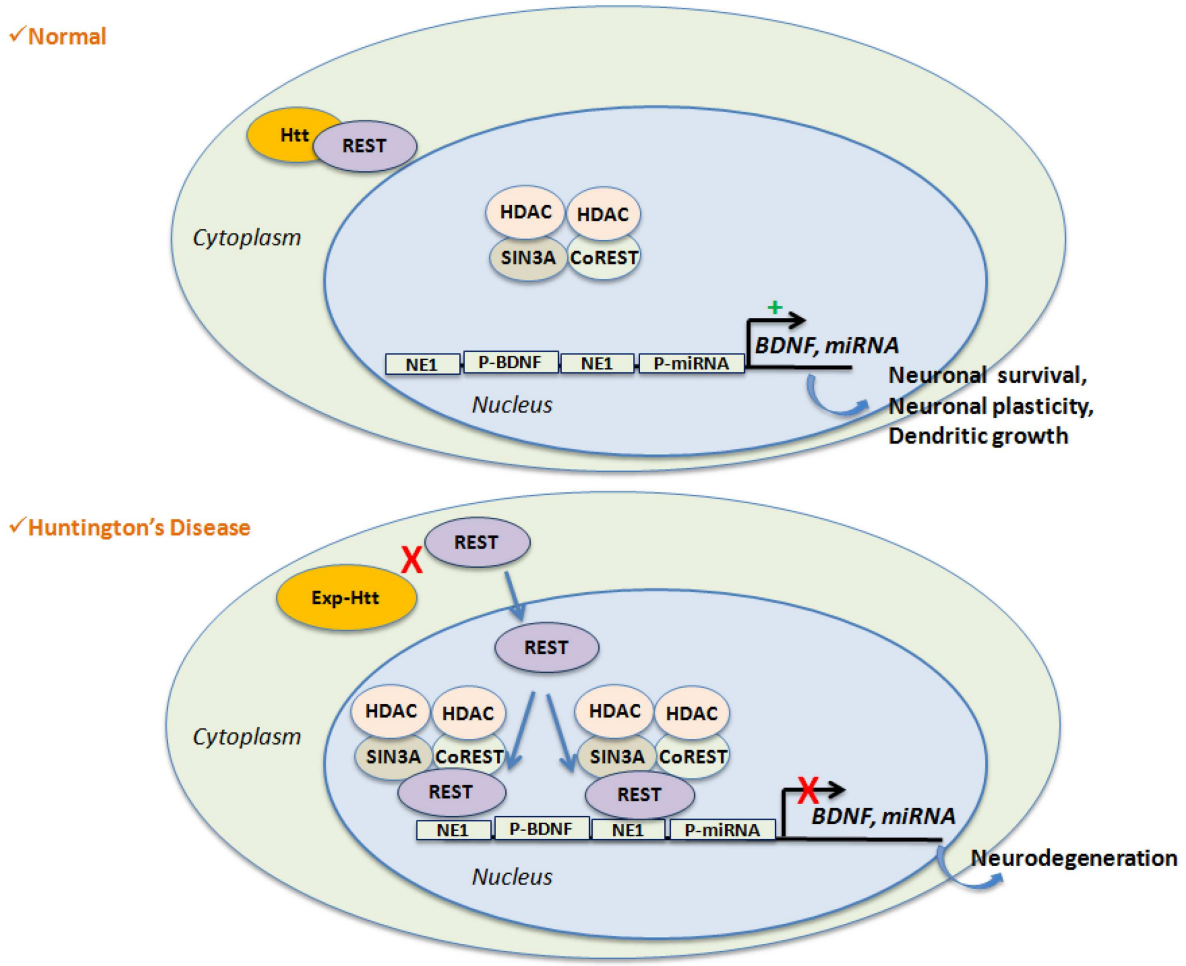

FIGURE 2 | REST-mediated gene repression is favored in HD. Wild-Type Htt interacts with REST, a transcriptional repressor, within the cytoplasm. This leads to de-repression of REST target genes like *BDNF* or non-coding miRNAs, which are essential for neuronal survival, neuronal plasticity or dendrites growth. Expansion of Htt disrupts its binding with REST and facilitates nuclear entry of REST along with the formation of the repressor complex on the RE1 site. Activation of RE1 site results on target gene silencing and participates to neuro-degeneration.

mouse model (107). Of importance, miR-196a ameliorated the formation of aggregates in iPSC (inducible Pluripotent Stem Cells) from HD patients, when differentiated in the neuronal stage. The down-regulation of Exp-HTT by miR-196a is of prime importance, since it forms the bases of new strategies for allele-specific silencing in HD. miR-196a did not regulate Exp-Htt levels directly, but rather indirectly, probably through the regulation of the ubiquitin-proteasome system, gliosis, and the CREB pathway.

Additionally, HTT has a more direct role in post-transcriptional gene silencing by miRNAs. An elegant study from Naoko Tanese's group (103) has shown that HTT itself contributes to RNA-mediated gene silencing through its association with Ago in Processing bodies (P-bodies). Mouse striatal cells expressing Exp-Htt showed fewer P-bodies and reduced reporter gene silencing activity compared to wild-type. More recently, a pathogenic role of the Exp-*HTT* RNA was provided (104). The authors showed that Exp-*HTT* mRNA generates small CAG-repeated RNAs (sCAGs) having a neurotoxic activity. This toxic effect was dependent on Dicer and Ago proteins, as they were inhibited by their knock-down. They thus provide the first demonstration that these sCAGs generated by Exp-HTT may contribute significantly to the neuro-degeneration pattern observed in HD.

CONCLUSION

Most of the cellular dysfunctions in HD are due to alterations of gene expression: from mitochondrial dysfunctions and metabolism energy deficit, to excitotoxicity. Furthermore, dysregulation of transcription is a widespread, reproducible, and early event in the pathogenic process of HD. Therefore, new therapeutic approaches targeting transcription factors, chromatin remodeling, or miRNAs can be proposed. Obviously targeting signaling pathways that control expression levels of the trophic factor BDNF or the mitochondrial gene PGC1-α will provide interesting perspective. Targeting the REST transcriptional repressor, CREB, or Sirtuins remain interesting strategies. Although therapeutic trials, including safety

and tolerability studies, with the global HDACi, phenylbutyrate, have been conducted in patients, these compounds remain highly unspecific, since they act on multiple classes of HDACs, hence on numerous non-selected genes and sometimes non-nuclear targets. Alternative approaches could be to design compounds that target more specifically one type of HDAC, for example HDAC4 – an issue that is under investigation – or to target other PTMs of histones (including phosphorylation, methylation, ubiquitylation, or polyamination), each PTM targeted alone, or in combination.

It was recently discovered that non-coding RNAs are dysregulated in HD. Because one miRNA can target multiple pathways, this suggests that miRNAs could have pleiotropic, widespread effects on HD pathogenesis. An elegant demonstration of this assumption was recently made both *in vitro* and *in vivo*, using miR-196a, including in IPSC from HD patients. One important finding in this regard was that Exp-HTT itself was down-regulated by miR-196a. Therefore, a new and fascinating therapeutic avenue is now offered with miRNAs in HD.

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A tale of two maladies? Pathogenesis of depression with and without the Huntington's disease gene mutation

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Huntington's disease (HD) is an autosomal dominant disorder caused by a tandem repeat expansion encoding an expanded tract of glutamines in the huntingtin protein. HD is progressive and manifests as psychiatric symptoms (including depression), cognitive deficits (culminating in dementia), and motor abnormalities (including chorea). Having reached the twentieth anniversary of the discovery of the "genetic stutter" which causes HD, we still lack sophisticated insight into why so many HD patients exhibit affective disorders such as depression at very early stages, prior to overt appearance of motor deficits. In this review, we will focus on depression as the major psychiatric manifestation of HD, discuss potential mechanisms of pathogenesis identified from animal models, and compare depression in HD patients with that of the wider gene-negative population. The discovery of depressive-like behaviors as well as cellular and molecular correlates of depression in transgenic HD mice has added strong support to the hypothesis that the HD mutation adds significantly to the genetic load for depression. A key question is whether HD-associated depression differs from that in the general population. Whilst preclinical studies, clinical data, and treatment responses suggest striking similarities, there are also some apparent differences. We discuss various molecular and cellular mechanisms which may contribute to depression in HD, and whether they may generalize to other depressive disorders. The autosomal dominant nature of HD and the existence of models with excellent construct validity provide a unique opportunity to understand the pathogenesis of depression and associated gene-environment interactions. Thus, understanding the pathogenesis of depression in HD may not only facilitate tailored therapeutic approaches for HD sufferers, but may also translate to the clinical depression which devastates the lives of so many people.

Keywords: Huntington's disease, neurodegeneration, depression, psychiatric disorders, serotonin, BDNF, stress, polyglutamine disease

INTRODUCTION

Huntington's disease (HD) is a progressive neurodegenerative disorder that affects mood, cognition, and movement. HD provides a unique opportunity to study pathogenesis from its earliest stages, as the genetic cause of this autosomal dominant disease, with its array of divergent symptoms, is known. HD is caused by an abnormal expansion mutation of a tract of CAG trinucleotide tandem repeats close to the 5′ end of the *huntingtin* gene on chromosome 4 (The Huntington's Disease Collaborative Research Group, 1993). HD was first described by the physician George Huntington in 1872, and our understanding of the mechanisms underlying HD has increased exponentially in the 20 years since the tandem repeat expansion in *huntingtin* was found to be the causative gene mutation. Despite this, a cure for this disease remains elusive. In light of this, much attention has been focused on symptom management. As a late onset disease, alleviation of especially early symptoms can greatly lengthen and improve the largely normal and productive periods of patients' lives. HD presents with a classic triad of relentlessly progressive symptoms. Diagnosis is based on the appearance of motor symptoms, which most commonly appear in patients in the fourth or fifth decade of life. Some rare cases of juvenile onset are seen, with motor symptoms manifesting during or prior to teenage years; however juvenile onset HD, which is caused by exceptionally long CAG repeat expansions, accounts for only about 5% of all HD cases (Nance and Myers, 2001). Completing the triad are cognitive and psychiatric symptoms, which can arise years, often decades, prior to the inception of motor symptoms. There are also a plethora of non-CNS, peripheral symptoms, although it is not yet clear whether specific symptoms originate from cellular dysfunctions in central and/or peripheral tissues (van der Burg et al., 2009).

Psychiatric symptoms abound in HD (Leroi et al., 2002), including psychosis (Lovestone et al., 1996), irritability, anxiety, apathy (van Duijn et al., 2007), and obsessive and compulsive symptoms (Beglinger et al., 2008; van Duijn et al., 2008). However, depression is among the most prevalent of psychiatric symptoms, with a lifetime prevalence of major depression reported to be up to and over 50% in patients; moreover, depression is often diagnosed years if not decades prior motor symptom onset (Shiwach, 1994; Naarding et al., 2001; Paulsen et al., 2001, 2005; Duff et al., 2007;

van Duijn et al., 2008; Gargiulo et al., 2009). This occurrence is hugely disproportionate when compared to lifetime prevalence of major depression in the general population, which has been found to be around 15% (Hasin et al., 2005; Kessler et al., 2005).

More than merely prevalent, depression has been rated by patients as one of the most debilitating symptoms of HD, affecting perceived quality of life more so than motor or even cognitive aspects of the disease (Ho et al., 2009). Depression increases the risk of suicide (Jensen et al., 1993; Harris and Barraclough, 1998; Cavanagh et al., 2003). In HD, almost 30% of patients attempt suicide at least once and death due to suicide among HD patients is four times that of the normal populace – a similar rate of death by suicide to that of those suffering from affective disorders (Farrer, 1986; Inskip et al., 1998). The presence of depression also leads to negative collateral effects such as the hastening of cognitive decline (Nehl et al., 2001; Smith et al., 2012). Whilst there is currently no cure available for HD, depression is largely seen as treatable. However, understanding of the etiology and pathophysiology of this early-onset symptom is required to allow early detection of depression and to apply the most suitable treatments.

Huntington's disease is the most notorious of a group of neurodegenerative diseases caused by an expansion of a CAG trinucleotide repeat encoding poly-glutamine (poly-Q). Other diseases in this group include dentatorubropallidoluysian atrophy (DRPLA), spinocerebellar ataxia type 3 (SCA-3), spinal bulbar muscular atrophy (SBMA), and spinocerebellar ataxia types 1, 2, 6, 7, and 17 (David et al., 1997; Kakizuka, 1997; Koshy and Zoghbi, 1997; Robitaille et al., 1997; Ross, 1997; Nakamura, 2001; Nakamura et al., 2001). SCA patients show depression prevalence of between 15 and 17% in a large study with 526 genetically confirmed and clinically affected patients (117 SCA1, 163 SCA2, 139 SCA3, and 107 SCA6) (Schmitz-Hübsch et al., 2011), a rate similar to the prevalence in the general population (∼15%) and certainly less than that seen in HD. Other members of this group are very rare and studies have not been done specifically examining rates of depression. The overrepresentation of depression in HD may be due to the neurological importance of the huntingtin protein, its ubiquitousness and the subsequent consequences of the mutation.

Whilst the link between the length of CAG expansion and speed of deterioration has been established, evidence from the literature does not support the idea that depression in HD is affected by the length of CAG repeats. In a study with a cohort of 79 HD patients, no correlations were observed between CAG repeat length and personality change, psychosis, depression, or non-specific alterations (Weigell-Weber et al., 1996). No relationship was discerned even after correcting for predicted age of neurological onset in neurologically asymptomatic patients (Berrios et al., 2001). A more recent study examining 72 HD patients found that whilst the number of CAG repeats associated negatively with the age of onset of psychiatric disorders, neither the probability of developing psychiatric disorders nor the severity of psychiatric symptoms was correlated with the number of CAG repeats (Vassos et al., 2008). It therefore seems that although higher repeat number is associated with faster disease progression as a whole, it is insufficient to induce depression.

Although the ultimate cause of depression in HD may be different from that of depression in the HD gene-negative population (which we will refer to forthwith as "clinical depression"), recent research has shown that there are certainly perceptible similarities in the symptoms and even physiological anomalies. These similarities are worth examining as they may hold clues to the etiology of depression in HD and may be informative in establishing how best to treat this debilitating affective disorder. Various aspects of HD pathogenesis, including disrupted transcription, trafficking, signaling, homeostasis, synaptic and neuronal function, have been recently reviewed (Milnerwood and Raymond, 2010; Raymond et al., 2011; Ross and Tabrizi, 2011; Nithianantharajah and Hannan, 2012). Here, we discuss potential molecular and cellular mechanisms specifically involved in the etiology of depression in HD. This includes evidence implicating the dysregulation of serotonergic signaling, alteration of hypothalamic-pituitary-adrenal (HPA)-axis activity, and disruption of BDNF expression and trafficking as key pathogenic processes.

DIAGNOSING DEPRESSION IN HD

Notwithstanding evidence of overwhelming prevalence of psychiatric symptoms, treatment of psychopathologies in HD has been scantly examined. Studies of the efficacy of various drugs in treating these symptoms have been few and far between, mostly with small cohorts, often case reports of a single patient with no controls (Naarding et al., 2001). While conventional antidepressants have been applied in the treatment of depression in HD, the effectiveness of different types of antidepressants, doses, treatment durations, and other factors have not been thoroughly examined. Having a different origin of disease, i.e., the mutation in the *huntingtin* gene, it is possible that disparities exist in HD which may translate to more efficacious treatment strategies. For instance, a study has found that passivity is a risk factor to earlier disease onset versus patients who were more active (Trembath et al., 2010). This reflects findings in mouse models where transgenic mice raised in enriched cages which provided enhanced sensory stimulations were rescued from depressive-like behavioral phenotypes seen in mice raised in standard cages (Pang et al., 2009; Du et al., 2012; Renoir et al., 2013). This suggests enhanced engagement and participation may have benefits in the treatment of depression. Little has been done with regards to behavioral therapy in HD but a study has shown that remotivational therapy improving quality of life of HD patients (Sullivan et al., 2001). A recent study on the use of antidepressants in pre-onset HD gene carriers found that about 22% of 787 prodromal patients are already on antidepressants, much more than in the control group, with around 13% on antidepressants (Rowe et al., 2012). However, a consensus on the prevalence of depression in HD has yet to be reached. The inconsistency in rates of depression reported between different studies is due to a variety of factors such as small sample sizes, differences in methodologies utilized, lack of control groups, and differences in the disease stages of participants (van Duijn et al., 2007). In particular, the usage of different assessment tools [Unified Huntington's Disease Rating Scale (UHDR), Diagnostics and Statistics Manual of Mental Disorders (DSM), Neuropsychiatric Inventory (NPI), Beck Depression Inventory-II (BDI-II), Hamilton Rating Scale for Depression (Ham-D), Hospital Anxiety and Depression Scale (HADS), Depression Intensity Scale Circles (DISCs)] have resulted

in very disparate estimations of the prevalence of depression in HD, ranging from 33 to 69%.

This large variation highlights the endogenous differences between how depression manifests with and without the HD gene mutation, causing the use of tools not tailored for depression in HD to produce inconsistent results. For example, standard rating scales for clinical depression contain items relating to symptoms of HD that are present regardless of the presence or absence of depression and therefore may skew the diagnosis. Weight loss and sleep disturbances are items included in many diagnostic tools as symptomatic of depression even though they are common symptoms in HD patients without depression. Therefore these symptoms may well be nothing but red herrings if included for diagnosis of depression in HD. Scales that contain less "somatic" items, such as the HADS and the DISCs were better able to accurately identify depression in HD (De Souza et al., 2010). Comparison between the BDI-II and the Ham-D found that the items best able to discriminate depression in HD patients, other than depressed mood, which had high correlations [correlation coefficient (r) = 0.834 for the BDI and 0.917 for the Ham-D], are those items measuring thoughts and attitudes around mood such as "discouraged about the future" ($r = 0.653$) and "satisfaction in life" ($r = 0.64$). Weight loss ($r = 0.193$ for BDI and 0.128 for Ham-D), loss of appetite ($r = 0.319$ for BDI, 0.297 for Ham-D), and other vegetative symptoms associated with clinical depression were, on the other hand, poor indicators of depression in HD (Rickards et al., 2011). Depression is notoriously heterogeneous in its presentation and these findings suggest possible dominance of a different cluster of symptoms in HD compared to most clinical depression patients. Accurate discriminators of depression in HD are required for better screening, diagnosis, and the gaging of treatment efficacy; areas that have not so far been systematically studied. Whether or not the differences reflected in the screening tools point to underlying pathophysiological divergence between depression in HD and clinical depression require further investigations. Besides the obvious consequences of depression itself, another important reason for the accurate and early detection of depression in HD is that depression has the negative effects of hastening cognitive decline and correlates with poorer cognitive performance in prodromal patients (Nehl et al., 2001; Smith et al., 2012), suggesting that earlier intervention can prolong the functional years of a patient's life.

It is tempting to speculate, and indeed has been a predominant view, that depression in HD is a natural consequence to the reactive stress induced by the knowledge of being at risk of inheriting the disease and/or of positive genetic or clinical diagnosis of HD (Shiwach and Norbury, 1994). Whilst psychosocial stress undoubtedly adds to the psychological burden inducing depression (Codori et al., 2004; Larsson et al., 2006), it alone is now regarded as insufficient to fully account for the psychiatric co-morbidity. Prior to clinical onset, HD gene-positive carriers presented a higher current prevalence of major depression despite being ignorant of their gene status at the time of psychiatric assessment (Horowitz et al., 2001; Julien et al., 2007). That overrepresentation suggests that depression in HD patients is a behavioral manifestation of early neuropathology. Interestingly, the greater susceptibility of females to developing depression in the general population is also apparent within the HD population (Zielonka et al., 2012). These clinical aspects have been recapitulated by studies of the R6/1 transgenic and knock-in HdhQ111 mouse models (that are by definition unaware of their gene status) which report a female-specific depression-like behavioral phenotype (Pang et al., 2009; Pouladi et al., 2009; Du et al., 2012; Orvoen et al., 2012; Renoir et al., 2012), circumventing the caveat of psychosocial stress. Whilst sexual dimorphism has been reported in the CAG(n51) transgenic HD rats, the nature of the affective dysfunction in this model is less clear (Bode et al., 2008; Faure et al., 2011). Depressive-like behaviors have also been identified in the YAC transgenic HD mice (Pouladi et al., 2009), suggesting that it is a fundamental aspect of HD phenotypes. The study of other rodent models of HD might be relevant for the understanding of other specific psychiatric aspects of HD such as anxiety (Orvoen et al., 2012; Abada et al., 2013).

Although the pathophysiology leading to depression in HD arises from a different source than those of clinical depression, the successes of antidepressant treatments in ameliorating depression in HD, albeit mostly from case studies, suggest similarities in the underlying pathophysiology. Therefore it is important to take advantage of the extensive knowledge garnered in the study of clinical depression and to compare the two diseases in order to apply the most effective and tailored treatment for depression in HD.

THE SEROTONERGIC SYSTEM

Symptomatic treatment of depression in HD and the efficacy of the range of antidepressant drugs often prescribed to HD patients have not been thoroughly examined. Case studies report benefits of selective serotonin reuptake inhibitors (SSRIs), selective noradrenergic reuptake inhibitors (SNRIs) (venlafaxine), atypical antipsychotics (olanzepine), monoamine oxidase inhibitors (MAOI), tetracyclic, and tricyclic antidepressants on small numbers of patients (Patel et al., 1996; Squitieri et al., 2001; Bonelli et al., 2003; Ciammola et al., 2009). A study of 26 HD patients with diagnoses of major depression treated with venlafaxine for 4 weeks showed significant improvement albeit a high rate of side effects such as irritability (Holl et al., 2010). In a more recent study by Rowe et al. (2012) of 787 prodromal HD participants, it was reported that 20% of prodromal patients were prescribed antidepressants with the vast majority using SSRIs (e.g., paroxetine, fluoxetine, and sertraline). However, the effectiveness SSRIs versus other types of antidepressants in diminishing the depression symptoms has yet to be specifically documented. Indeed the overall effectiveness of SSRI interventions in treating depression in HD has not been studied despite its frequent use.

Dysregulation of the serotonin (5-HT) signaling system has long been scrutinized in the field of clinical depression as an etiological cause (Castro et al., 1998; Yohrling et al., 2002). Compromising the 5-HT system affects its downstream elements; 5-HT upregulates the expression of cyclic adenosine monophosphate (cAMP) (Vaidya and Duman, 2001), which ultimately results in the activation of cAMP response element-binding protein (CREB). A loss of CREB signaling impairs adult hippocampal neurogenesis and disrupts normal hippocampal function (Jacobs et al., 2000; Urani et al., 2005), both of which have been proposed to be key pathologies of depression (Petersen et al., 2008; Lucassen et al., 2010). Based on findings from animal models, CREB signaling in

HD is also disrupted, due to sequestration of its binding partner, CREB binding protein (CBP). Thus, this is a common molecular pathology that the disease shares with depression, and one which, along with the target gene BDNF as described below, is likely to mediate the deficits in hippocampal neurogenesis which is the cellular consistently observed in animal models of HD. Chronic treatment of different mouse models of HD with a variety of SSRIs have been found to rescue the neurogenesis deficits but there has yet to be a study demonstrating a possible rescue of neurogenesis through modulation of CREB signaling. That would be an interesting avenue of investigation for the future since compounds that increase phosphorylation of CREB and cAMP levels are reportedly associated with a rescue of cognitive deficits in the R6/1 transgenic mouse model (Giralt et al., 2013).

Serotonin reuptake inhibitors work in part by desensitizing 5-HT1A autoreceptors (Dawson et al., 2002; Rossi et al., 2008), which act to inhibit 5-HT production. Desensitization of these autoreceptors therefore increases 5-HT neurotransmission (Blier et al., 1998). It was also found that a single nucleotide polymorphism (SNP) on the promoter of 5-HT1A receptor which leads to the over-expression of 5-HT1A autoreceptors resulted in increased susceptibility to developing major depression in those with the mutation (Albert and Francois, 2010). As levels of 5-HT in the human brain can only be measured post-mortem, markers are used in patients to reflect 5-HT levels. One of these is measuring the primary metabolite of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid (CSF), which is deemed to reflect brain 5-HIAA levels (Wester et al., 1990). It has been found that 5-HIAA is reduced in the CSF of a sizeable proportion of patients with major depression, a finding replicated by *post-mortem* studies (Asberg, 1976; Asberg et al., 1976; van Praag and Plutchik, 1984; Roy et al., 1989). Reduced 5-HIAA levels have also been reported in HD patients, which is a clear indicator that there is a dysregulation of serotonin metabolism in HD (Caraceni et al., 1977; Jongen et al., 1980). Closely related, monoamine oxidase A, an enzyme that metabolizes 5-HT thereby decreasing the amount of available 5-HT, was elevated significantly in many brain regions of depressed patients (Meyer et al., 2006). This abnormal increase in metabolism of 5-HT has similarly been documented in the putamen and substantia nigra pars compacta of the basal ganglia, and in the pons of HD brains through quantitative enzyme radioautography (Richards et al., 2011). Interestingly, platelet monoamine oxidase activity was reported to be unchanged in HD patients with overt symptoms (Markianos et al., 2004) which suggests a dissociation between central and peripheral pathology in HD. Tryptophan, an essential amino acid from which serotonin is synthesized, has also been found to be reduced in a large proportion of patients with depression (Moller et al., 1983; Quintana, 1992). Experimentally induced tryptophan depletion via a special diet resulted in rapid relapse in remitted depression patients hours after ingestion of diet (Delgado et al., 1990). Plasma total and protein-bound tryptophan levels were found to be reduced in HD patients (Belendiuk et al., 1980) and while oral tryptophan administration increases blood 5-HT levels in healthy individuals, this response not observed in HD patients which is a further demonstration of a disease-associated impairment of 5-HT metabolism (Christofides et al., 2006). A recent study looking at the raphe nucleus, where 5-HT synthesis occurs, found a significant correlation between the level of depression in HD patients and stem raphe echogenicity using transcranial sonography (Krogias et al., 2011). This is similar to what is seen in clinical depression patients (Walter et al., 2007), further strengthening the evidence that an abnormality in 5-HT production may underlie both diseases. Recent work on compounds which influence tryptophan metabolism rescue neurodegeneration in the R6/2 mouse model of HD (Zwilling et al., 2011) but the effectiveness of such compounds to treat depressive behaviors have yet to be determined.

Besides changes to 5-HT levels, dysregulation of the receptors of 5-HT has also been implicated in depression. Major depression has been implicated with increased 5-HT1A autoreceptor density in the dorsal raphe nucleus (Stockmeier et al., 1998). Reduced post-synaptic 5-HT1A/2 receptor function has also been linked with depression with patients who had suffered major depression exhibiting global reduction in 5-HT1A and 5-HT2A receptor binding (Drevets et al., 1999, 2007; Messa et al., 2003; Moses-Kolko et al., 2008). This is reflected in animal models where genetic 5-HT1A receptor knock-out mice showed phenotypes of anxiety (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998) whereas over-expression of 5-HT1A receptor reduced anxiety-related behaviors (Kusserow et al., 2004). In *post-mortem* HD brains, reduced 5-HT1 receptor binding has been reported in the putamen, hippocampus (Cross et al., 1986), and in the basal ganglia and substantia nigra (Waeber and Palacios, 1989). Similar reductions in 5-HT1A receptor binding have been reported in the R6/2 transgenic mouse model, as well as a marked decrease in enzymatic activity of tryptophan hydroxylase (TPH) activity (the rate limiting enzyme for the biosynthesis of 5-HT) (Yohrling et al., 2002). The decrease in receptor binding is likely attributable to down-regulation of mRNA levels of various 5-HT receptors which is evident in R6/1 HD mice (Pang et al., 2009). All these culminate in abnormal physiological responses to the administration of 5-HT1A receptor agonist 8-OH-DPAT by R6/1 HD mice (Renoir et al., 2011a, 2012), thereby demonstrating how molecular neuropathology manifests as disease pathophysiology. Interestingly, chronic application of the SSRI sertraline was able to correct the hypersensitivity of the 5-HT1A autoreceptor in female R6/1 mice as well as depression-like phenotype (Renoir et al., 2012), suggesting an etiological link between disturbances to the 5-HT system with the HD-associated depression-like phenotype.

THE HPA-AXIS AND STRESS RESPONSES

The HPA-axis is the major endocrine system responsible for stress adaptation. Stress activates the axis, resulting in the production of the stress hormone cortisol (corticosterone in rodents) (Papadimitriou and Priftis, 2009). Abnormally elevated HPA-axis activity is one of the most replicated biological findings in major depression (Pariante and Lightman, 2008; Stetler and Miller, 2011). Cortisol levels are usually tightly regulated because prolonged exposure to cortisol is damaging to the brain: reducing neurogenesis (Cameron and Gould, 1994; Wong and Herbert, 2006; Brummelte and Galea, 2010) and increasing apoptosis in the hippocampus (Sapolsky, 1986; Crochemore et al., 2005; Andrés et al., 2006; Liu et al., 2011) resulting in atrophy in the hippocampus of rats (Woolley et al.,

1990) and monkeys (Sapolsky et al., 1990) as well as neuronal atrophy (Cerqueira et al., 2005a) and volume reduction in the prefrontal cortex (Cerqueira et al., 2005b). Dysregulation of the 5-HT neurotransmission has been linked with the HPA-axis abnormalities (Hery et al., 2000; Froger et al., 2004). The serotonin system is able to modulate anxiety and depression (Meaney et al., 1994; Ramboz et al., 1998; Harada et al., 2008; Brummett et al., 2012), possibly by regulating the HPA-axis through influencing glucocorticoid receptor (GR), whose activation is important in regulating the 5-HT system both *in vivo* and *in vitro* (Lanfumey et al., 2000; Erdeljan et al., 2001; Wang et al., 2009; Falkenberg and Rajeevan, 2010; Belay et al., 2011). For example, it has been found that serotonin neurotransmission is modulated by HPA-axis activity, with GR activation increasing 5-HT1A signaling (Hesen and Joels, 1996). Adrenalectomy reduced dentate gyrus neuronal morphology and this effect was reversed by using the 5-HT1A agonist ipsapirone (Huang et al., 1997). But sustained elevation of corticosteroid significantly reduced 5-HT1A receptor mRNA level as well as 5-HT1A binding density in the hippocampus and this is reversed by chronic administration of antidepressants (Lopez et al., 1998).

Studies have also described HPA-axis hyperactivity in HD patients. Earlier studies with small sample sizes found higher basal cortisol levels in moderate stage HD patients compared to controls at both morning (Leblhuber et al., 1995) and evening (Heuser et al., 1991). More recently, hyperactivity of the HPA-axis has been found in a small sample of clinically diagnosed, early stage HD patients compared to controls, with higher 24 h cortisol production, particularly in the morning and early afternoon periods (Aziz et al., 2009). Examining salivary cortisol in pre-symptomatic HD patients in comparison to controls, subtle but altered cortisol awakening responses were found, with higher cortisol concentration in patients at early morning periods, just after awakening (van Duijn et al., 2010). This indicates that HPA-axis dysregulation is an early pathophysiology in HD. However, another study with a comparatively large sample of patients found increased urine cortisol levels but only in moderate to late clinically diagnosed patients whereas pre-symptomatic and early stage patients did not display any differences in urine cortisol compared to 68 healthy controls (Björkqvist et al., 2006). But it is worthy to note that in this study, samples were collected in a narrow time window (14:00–17:00), when cortisol level is ebbing, leaving the possibility that the difference identified in the previous studies was masked in the early stage patients examined. Furthermore, analysis of patients was confounded by limitations to recruitment such as imbalance of gender (or indeed disregarding gender as a factor), differences in stages of disease progression as well as environmental confounders such as smoking, alcoholism, depression, and use of psychotropic medications.

Increased baseline corticosterone was initially found in R6/2 mice from an early age (Björkqvist et al., 2006). However, because of their rapid motor onset and deterioration, the R6/2 model is not an optimal one for examining pre-motor onset symptoms and pathophysiologies. Recently, it was found that at a pre-motor onset age, female R6/1 mice exhibit increased corticosterone release after physiological and pharmacological stresses whilst baseline levels did not differ between WT and R6/1 females (Du et al., 2012). This is more akin to what is seen in early, pre-motor symptomatic HD patients with little change in baseline cortisol levels, suggesting that in prodromal patients, differences may only become apparent when the HPA-axis is induced by stressors. Interestingly, the sex-dimorphic display of this physiological abnormality correlates with prior findings of depression-like behavioral phenotypes in pre-motor onset female R6/1 mice that were also absent in the males of the same age (Pang et al., 2009; Renoir et al., 2011b). Curiously, using pharmacological means as well as *in vitro* analysis, it was found that the source of the hyperactivity in the female R6/1 mice is the adrenal gland (Du et al., 2012). Studies examining the adrenal glands in HD have so far been scant. However, this finding raises the possibility of an early peripheral change which may influence central brain function. More research is required to examine the HPA-axis of HD patients in terms of its regulation and response to stress. Whilst adrenal hyperplasia has been found in victims of suicide (Szigethy et al., 1994) and a few studies reporting increased adrenal volume in depressed patients compared to controls, the lack of numbers and heterogeneity between studies makes it hard to draw useful conclusions about the role adrenal-specific pathology may play in the development of depression (Kessing et al., 2011).

BDNF AND NEUROTROPHIN SIGNALING

Depression is a complex affective disorder and is thought to result at least partially from an inability of the brain to make appropriate adaptations to environmental stressors, and may involve impaired neural plasticity (Duman et al., 2000; Duman, 2002; Manji et al., 2003; Czeh and Simon, 2005). This is evidenced by studies showing altered brain structures in depression subjects such as reduced cell number, cell density, and body size as well as reduced glial density in frontal cortical and hippocampal brain regions (Ongur et al., 1998; Cotter et al., 2001; Beasley et al., 2002; Rajkowska, 2002). These observations led to the hypothesis that a loss of neurotrophic factors, which are vital for the survival, development, and maintenance of neurons (Lewin and Barde, 1996; McAllister, 2001) as well as regulating synaptic and morphological plasticity (McAllister et al., 1999; Thoenen, 2000), is directly involved in the pathophysiology of depression and that its restitution may lie at the heart of successful treatment (Altar, 1999; Duman, 2004). Indeed, recently it was found that HD patients exhibited a significant decrease in peripheral BDNF gene expression (Krzyszton-Russjan et al., 2012). HD patients (Ciammola et al., 2007) and rodent models of HD (Strand et al., 2007) also show reduced levels of BDNF protein. Huntingtin has important roles in regulating both the transcription (Zuccato et al., 2001) and trafficking (DiFiglia et al., 1995; Gauthier et al., 2004) of BDNF, actions that are affected by the mutation in HD.

BDNF helps support the survival and integrity of existing neurons and encourage growth, migration, and differentiation of new neurons and synapses in the central as well as peripheral nervous systems (Cowansage et al., 2010). It also enhances neurogenesis in the hippocampus (Zigova et al., 1998; Benraiss et al., 2001; Pencea et al., 2001). The expression of BDNF is regulated via neuronal activity through calcium mediated mechanisms (Tabuchi, 2008) whilst its receptor tyrosine kinase-coupled receptor (TrkB), is also regulated in an activity-dependent manner (Nagappan

and Lu, 2005). As compromised synaptic and structural plasticity is significantly associated with depression, the importance of BDNF in depression has therefore garnered considerable attention. Lower levels of BDNF in depression patients suggest a role of BDNF in the pathogenesis of depression (Yoshimura et al., 2010; Yoshida et al., 2012). Serum BDNF was found to be significantly reduced in antidepressant-naïve depression patients compared to those who were treated with antidepressants and there was a significant negative correlation between BDNF levels and the Hamilton Rating Scale for Depression (Shimizu et al., 2003). Hippocampal BDNF levels in post-mortem brains of depression patients were found to be higher in those treated with antidepressants at time of death (Chen et al., 2001; Karege et al., 2005). This suggests that antidepressants increase BDNF levels and may account, at least in part, for their potency. In animal models, a similar result was seen. It was found that early life stress lowers BDNF and alters stress sensitivity later in life (Cirulli et al., 2009), whereas increases in BDNF, either via social enrichment or direct infusion into the brain, was found to reduce susceptibility to depression-like behavior (Siuciak et al., 1997; Cirulli et al., 2010).

However, studies also found that increases in BDNF through communal nesting induced an anxiety phenotype by reducing latency to immobility and increasing immobility time in the forced-swim test (FST) (Branchi et al., 2006). Other studies examining the role of BDNF in depression have also found that heterozygous knock-out of BDNF did not alter anxiety profile on the elevated-plus maze (Montkowski and Holsboer, 1997; MacQueen et al., 2001; Gorski et al., 2003). BDNF heterozygotes or mice expressing the dominant negative TrkB receptor TrkB.T1, did not show any differences from WT mice in their performance on the FST, therefore showing that reduced BDNF signaling does not cause depression *per se* (Saarelainen et al., 2003). However, the fact that a lack of BDNF signaling negated the effects of antidepressant treatment argues that whilst it is not predictive of depression, it is integral for the potency of antidepressant action.

BDNF seems to mediate the positive effect of several classes of antidepressants including SSRIs, tricyclics, SNRI, and MAOI, which have been found to increase BDNF in serum, cortical astrocytes, prefrontal cortex, and hippocampus (Duman and Monteggia, 2006; Allaman et al., 2011; Molendijk et al., 2011). It is also upregulated by exercise and environmental enrichment, contributing to beneficial effects such as induction of cell survival, proliferation, and dendritic development, leading to augmented cognitive outcomes (Acheson et al., 1995; Cotman and Berchtold, 2002; Choi et al., 2009; Sun et al., 2010; Kazlauckas et al., 2011). Environmental enrichment and exercise (voluntary wheel running) have been found to rescue BDNF deficits in R6/1 HD mice as well as ameliorating affective, cognitive, and motor abnormalities (van Dellen et al., 2000; Spires et al., 2004; Pang et al., 2006, 2009; Nithianantharajah et al., 2008; Zajac et al., 2010; Renoir et al., 2013). A diet rich in omega-3 fatty acids, which has been shown to have positive effects on depression (Freeman, 2009), also increases BDNF levels in the hippocampus (Venna et al., 2009).

In BDNF-deficient mice, the behavioral effects of antidepressants were abolished (Saarelainen et al., 2003; Monteggia et al., 2004). TrkB T1 over-expressing transgenic mice, which show reduced TrkB activation in the brain, are resistant to the effects of antidepressants (Saarelainen et al., 2003), whereas overexpression of TrkB led to resistance to depression-like behavior, with SSRI administration unable to further increase this resistance (Koponen et al., 2005) indicating that TrkB signaling is required for the behavioral benefits of antidepressants. These findings suggest that BDNF may be a key molecule involved in various antidepressant treatment strategies. A possible mechanism mediating the antidepressant-induced increase of BDNF is the upregulated expression of CREB, a transcription factor that upregulates BDNF and TrkB (Nibuya et al., 1996). Interestingly, CREB-mediated transcription regulation requires the aid of CBP, whose expression is downregulated by mutant huntingtin (Kazantsev et al., 1999; Nucifora et al., 2001).

Antidepressants are widely used in the treatment of HD patients (Sackley et al., 2011). Recent studies suggest that chronic treatment with the SSRIs fluoxetine or sertraline increased hippocampal neurogenesis, ameliorated cognitive deficits, and depression-like behavioral symptoms in R6/1 mice (Grote et al., 2005; Renoir et al., 2012) and increased BDNF levels and neurogenesis in R6/2 mice (Peng et al., 2008). Chronic antidepressant treatment in depressed patients resulted in upregulation of CREB protein expression (Nibuya et al., 1996), CREB phosphorylation (Saarelainen et al., 2003), BDNF (Chen et al., 2001), and TrkB (Bayer et al., 2000) in the hippocampus. BDNF has been proposed to be a mediator of the effects of antidepressants (Koponen et al., 2005), by augmenting the survival and differentiation of adult-born neurons in the dentate gyrus (Groves, 2007). These results led to the hypothesis that depression in HD coincides with decreased activity in the serotonin-CREB-BDNF-TrkB pathway, resulting in cellular dysfunction and reduced neurogenesis in the hippocampus.

The link between HPA-axis, depression, and BDNF has been explored in rodent models of depression and given much attention. Social stress has been widely used as a useful model of depression (Henn and Vollmayr, 2005). Stressors such as forced immobilization (Smith et al., 1995) and social defeat (Pizarro et al., 2004) were found to decrease BDNF expression in the hippocampus and cortical and subcortical regions of rodent models. Induced elevation of corticosterone, mimicking the effect of stress, has also been associated with reduced levels of BDNF mRNA and protein in the hippocampus and frontal cortex of rodent models (Schaaf et al., 1997, 1998; Chao et al., 1998; Dwivedi et al., 2006). Adrenalectomy surgery caused an increase of BDNF in the hippocampus (Chao et al., 1998), whilst chronic GR activation reduces both CREB phosphorylation and BDNF expression (Focking et al., 2003). This suggests regulatory ability of glucocorticoids on BDNF expression. GR was also found to interact with the BDNF receptor TrkB and corticosterone reduces TrkB-GR interaction, causing reduced BDNF-triggered glutamate release and BDNF-stimulated PLC-γ (Numakawa et al., 2009). Thus, taken together, increased HPA-axis activity may initiate a chain reaction, leading to altered 5-HT signaling, reduced CREB-mediated transcription of BDNF and damage to the hippocampus and other brain regions, which in turn, reduces negative feedback on the HPA-axis in a negative cycle (**Figure 1**). The

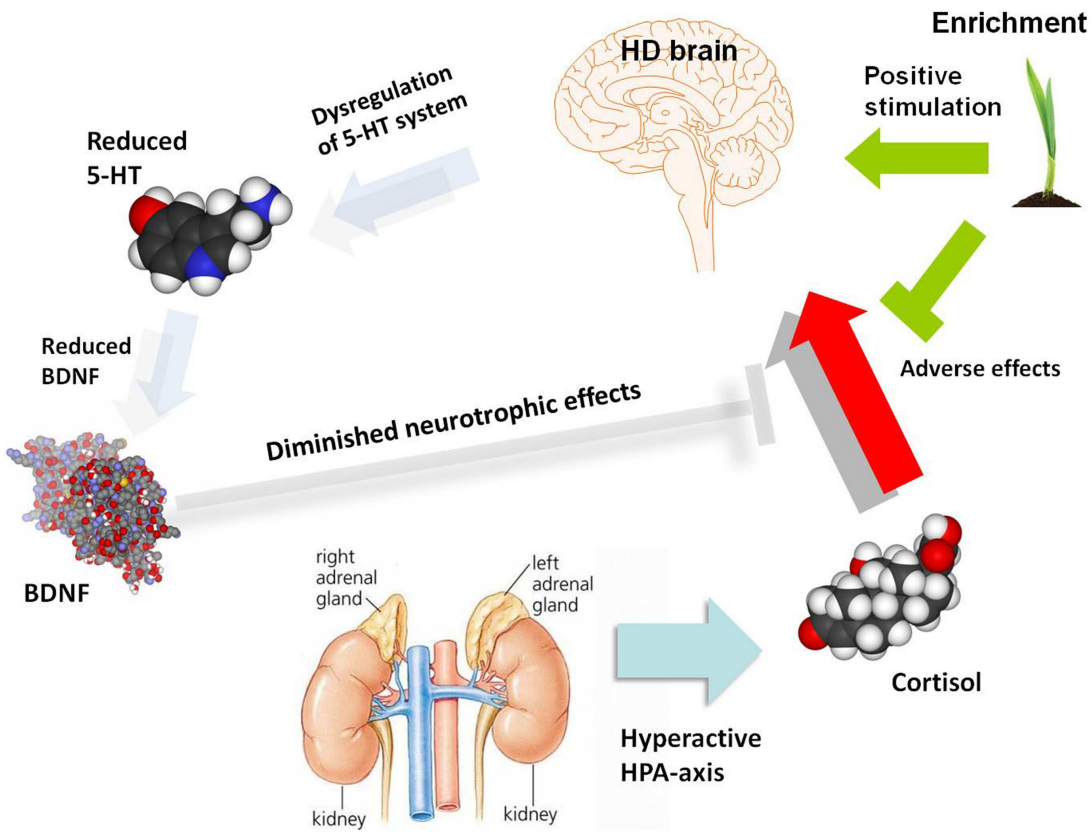

FIGURE 1 | A schematic diagram illustrating potential molecular and cellular mediators of HD pathogenesis, with a focus on serotonergic and neurotrophin signaling as well as the HPA-dysfunction which may give rise to depression and associated symptoms. The role of environmental enrichment in ameliorating specific aspects of molecular, cellular, and systems dysfunction is also illustrated. There are clearly many other molecular and cellular candidates implicated in HD pathogenesis which are not shown in this diagram, which outlines a specific hypothesis regarding the etiology of depression in HD. 5-HT, serotonin; BDNF, brain-derived neurotrophic factor; HD, Huntington's disease; HPA-axis, hypothalamic-pituitary-adrenal axis.

molecular and cellular processes may be impacted by environmental modulators, such as the cognitive stimulation and physical exercise induced by environmental enrichment. Complex gene–gene interactions and associated gene-environment interactions are presumably responsible for the variable incidence of depression both within HD patients (where each tandem repeat expansion mutation is embedded in a genome possessing a range of genetic modifiers) and the general population. Elucidation of this complexity at molecular, cellular, and systems levels will require a new generation of sophisticated animal models and clinical investigations.

SUMMARY

As a cure for HD remains elusive, effective strategies targeting symptom management are of great interest. This is especially pertinent with early onset psychiatric and cognitive symptoms, which tend to occur in the most productive years. Depression, being both early onset and having devastating consequences, is a priority target.

Collectively, HD shares certain hallmarks with a large portion of clinical depression patients: namely, disturbances of the 5-HT system, BDNF expression, and HPA-axis dysregulation. This is not perhaps surprising as the use of conventional antidepressants have shown to be equally efficacious in treating depression in HD. However, due to the relentless deterioration of the brain seen in HD, it is paramount to diagnose depression both early and accurately in the hope of stemming the decline with appropriate interventions. The inconsistent diagnostic results reflect the need for a tailored diagnostic tool for HD, taking into account many somatic symptoms such as weight loss which often seem to have little to do with depression. Furthermore, besides antidepressants such as SSRIs, other methods may be examined in detail as potential therapeutics. Strategies such as exercise interventions, occupational therapy, and other cognitive/behavioral treatments are under-utilized in HD and worthy of systematic investigation, both on their own and as adjuncts to pharmacological treatments.

Ultimately, the treatment of HD may require a sophisticated understanding of the pathogenesis of psychiatric, cognitive and motor symptoms, and the source of their heterogeneity in clinical populations. For example, it would be expected that genetic polymorphisms/mutations which predispose to depression in the general population might have additive effects on predisposition when combined with the HD

mutation. Similarly, the kind of stressors which can trigger depression in genetically vulnerable individuals might be equally toxic for those who are HD gene-positive. A natural consequence of such detailed insights into pathogenesis could be pharmacogenomics, polypharmacy, and other tailored therapeutic approaches to improve the lives of those suffering from HD and depression.

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Ataxin-3 and its E3 partners: implications for Machado–Joseph disease

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Machado–Joseph disease (MJD) is the most common dominant inherited ataxia worldwide, caused by an unstable CAG trinucleotide expansion mutation within the *SCA3* gene resulting in an expanded polyglutamine tract within the ataxin-3 protein. Ataxin-3 functions as a deubiquitinating enzyme (DUB), within the Ub system and whilst many DUBs are known to partner with and deubiquitinate specific E3-Ub ligases, ataxin-3 had no identified E3 partner until recent studies implicated parkin and CHIP, two neuroprotective E3 ligases. MJD often presents with symptoms of Parkinson disease (PD), which led to identification of parkin as a novel E3-Ub ligase whose activity was regulated by ataxin-3-mediated deubiquitination. Findings from these studies also revealed an unexpected convergence upon the E2-Ub-conjugating enzyme in the regulation of an E3/DUBenzyme pair. Moreover, mutant but not wild-type ataxin-3 promotes the clearance of parkin via the autophagy pathway, raising the intriguing possibility that increased turnover of parkin may contribute to the pathogenesis of MJD and help explain some of the Parkinsonian features in MJD. In addition to parkin, the *U*-box E3 ligase CHIP, a neuroprotective E3 implicated in protein quality control, was identified as a second E3 partner of ataxin-3, with ataxin-3 regulating the ability of CHIP to ubiquitinate itself. Indeed, ataxin-3 not only deubiquitinated CHIP, but also trimmed Ub conjugates on CHIP substrates, thereby regulating the length of Ub chains. Interestingly, when expanded ataxin-3 was present, CHIP levels were also reduced in the brains of MJD transgenic mice, raising the possibility that loss of one or both E3 partners may be a contributing factor in the pathogenesis of SCA3. In this review we discuss the implications from these studies and describe the importance of these findings in helping us understand the molecular processes involved in SCA3 and other neurodegenerative disorders.

Keywords: parkin, ataxin-3, CHIP, Machado–Joseph disease, Parkinson's disease, polyglutamine expansion

ATAXIN-3: A DEUBIQUITINATING ENZYME

Machado–Joseph disease (MJD) is one of nine polyQ disorders caused by a CAG expansion mutation within the *SCA3/MJD1* gene that encodes the *ataxin-3* protein (Kawaguchi et al., 1994). Expansion of its polyglutamine (polyQ) tract is believed to lead to a toxic gain of function, with calpain-dependent proteolysis of the mutant ataxin-3 generating expanded polyQ fragments and ultimately insoluble aggregates (Paulson et al., 1997; Fujigasaki et al., 2000; Chai et al., 2001; Koch et al., 2011). However, despite the shared mechanisms between these disorders, differences exist at both the clinical and neuropathological level, which cannot be accounted for by the polyQ expansion alone. Indeed, understanding the normal function of ataxin-3 can help explain why expansion of the polyQ tract in ataxin-3 is causing some of the specific features associated with MJD.

A well-characterized function of ataxin-3 is its role in the ubiquitin (Ub)-proteasome (UPS) system (Burnett et al., 2003; Doss-Pepe et al., 2003; Scheel et al., 2003) as a deubiquitinating enzyme (DUB). As a DUB, ataxin-3 possesses two distinct features for it to function in the UPS. The first is its N-terminal Josephin domain, that confers ataxin-3 with a cysteine protease activity for hydrolyzing Ub linkages (Scheel et al., 2003; Nicastro et al., 2005). The second critical feature are its three Ub-interacting motifs (UIMs), through which ataxin-3 binds Ub conjugates and ubiquitinated proteins (Burnett et al., 2003; Donaldson et al., 2003), bringing it into proximity to trim or edit specific linkages within these Ub conjugates (Burnett and Pittman, 2005; Winborn et al., 2008; Scaglione et al., 2011) (**Figure 1A**). Thus, ataxin-3 can bind to and deubiquitinate Ub conjugates, leading us to ask, what are the ubiquitinated substrates ataxin-3 acts upon. Moreover, what are the functional consequences of ataxin-3-mediated deubiquitination and does the expansion of the polyQ tract in MJD affect this normal function?

Ataxin-3 is one of 98 known DUBs, that function to deubiquitinate a wide range of substrates, that includes many E3-Ub ligases. E3s function within the UPS to mediate the covalent attachment of Ub onto lysine residues within target proteins (Shimura et al., 2000) thereby influencing many different cellular pathways. Typically, DUBs oppose this activity of the E3's by mediating the removal of Ub from target proteins. However, many

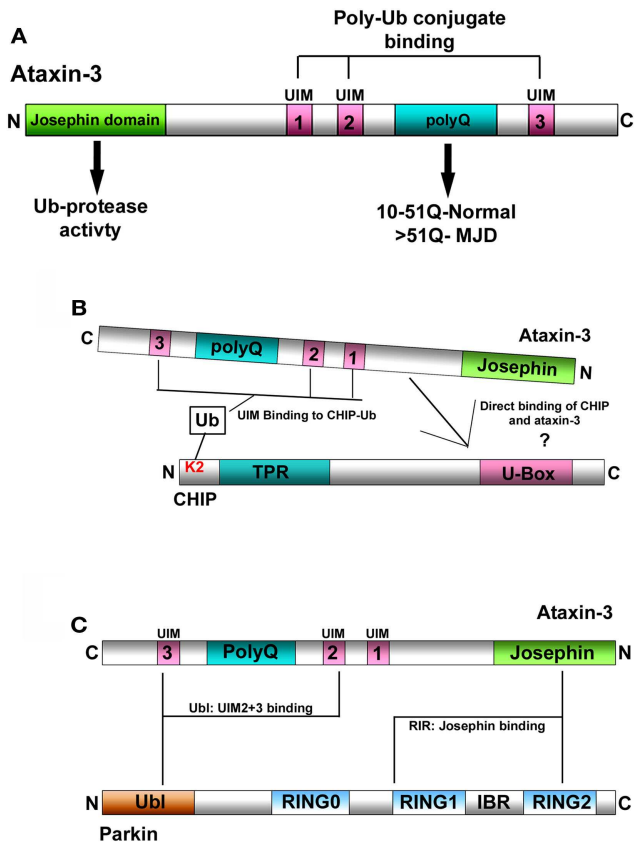

FIGURE 1 | (A) Schematic representation of ataxin-3. Ataxin-3 contains an N-terminal Josephin domain, and three Ub-interacting motifs (UIMs) that flank a polyQ tract of variable length. In normal individuals, this tract can be anywhere from 10 to 51Q. In individuals with MJD, this tract has expanded to now contain >51Q. **(B)** Schematic representation of the interaction between ataxin-3 and CHIP. (1) CHIP contains an N-terminal tetratricopeptide repeat (TPR) domain and a C-terminal *U*-Box domain that confers CHIP with E3-Ub ligase activity. CHIP monoubiquitinates itself at lysine 2 (K2), which in turn facilitates the interaction between CHIP and ataxin-3. CHIP and ataxin-3 also interact directly although the nature of this interaction has yet to be characterized. **(C)** Schematic representation of the interaction between ataxin-3 and parkin. Parkin interacts directly with ataxin-3 via its N-terminal Ub-like domain (Ubl) that binds to the UIMs of ataxin-3, and through its inbetweenring-RING2 (IBR-RING2) domain binding to the Josephin domain of ataxin-3.

E3s regulate their own stability and ability by ubiquitinating themselves. Often, these Ub conjugates target the E3 to the proteasome for degradation (Kao et al., 2000), although destruction of the E3 can be delayed or prevented by one or more DUBs removing these conjugates (Nathan et al., 2008). Paradoxically, DUBs can sometimes promote the degradation of an E3 by removing non-canonical Ub conjugates, which are protecting the E3 from degradation, enabling another E3 to ubiquitinate the E3 in question, thereby promoting its degradation (de Bie et al., 2010). To complicate matters, certain DUBs have been demonstrated to regulate the activity of E3s via deubiquitination (Scaglione et al., 2011). Given that DUBs often deubiquitinate multiple E3s (Daviet and Colland, 2008; Nathan et al., 2008), and since the number of E3s far outnumber DUBs, it is likely that ataxin-3-mediated deubiquitination is regulating the function of multiple E3s.

ATAXIN-3 AND CHIP

Early studies hinted that the *U*-box E3-Ub ligase CHIP might be one such E3 that interacts with ataxin-3. Consistent with this notion, CHIP was first observed to ubiquitinate the expanded form of ataxin-3, thereby directing it for degradation (Jana et al., 2005) In later studies, ataxin-3 was demonstrated to deubiquitinate CHIP, making CHIP the first E3 identified that could be deubiquitinated by ataxin-3 (Winborn et al., 2008). Conversely, CHIP ubiquitinates wild-type ataxin-3, which in turn enhances the overall deubiquitinating activity of ataxin-3 (Todi et al., 2010). Thus, CHIP and ataxin-3 interact and regulate the activity of each other. Of note, unlike other E3: DUB partners in which levels of the E3 are regulated by deubiquitination, changes in ataxin-3 levels do not have any effect on CHIP levels, making it unlikely that ataxin-3-mediated deubiquitination regulates degradation of CHIP (Scaglione et al., 2011). Rather, ataxin-3 appears to regulate the E3 ligase activity of CHIP in the protein quality control pathway.

Typically, E3-Ub ligases mediate the attachment of Ub moieties onto themselves, and CHIP is no exception. Through its interaction with the E2-conjugating enzyme Ube2W, CHIP robustly monoubiquitinates itself on lysine 2 (K2), which in turn enhances its overall E3 ligase activity (Scaglione et al., 2011). When CHIP was unable to self-ubiquitinate, its ability to ubiquitinate a variety of substrates was impaired. Intriguingly, monoubiquitination of CHIP appears to enhance the interaction between ataxin-3 and CHIP, and when bound, ataxin-3 can now deubiquitinate CHIP (**Figure 1B**). Evidence further supporting this notion that the ability of ataxin-3 to deubiquitinate CHIP is coupled to the ligase activity of CHIP, came from assays in which ataxin-3-mediated deubiquitination of CHIP did not occur until after poly-Ub conjugates on substrate proteins had attained a certain length. Once this occurred, ataxin-3 could now deubiquitinate both CHIP and its substrates, trimming the Ub conjugates at the distal ends. This prevented these conjugates from being extended further (Scaglione et al., 2011). Taken together, ataxin-3 opposes the activity of CHIP by deubiquitinating CHIP and by editing the Ub conjugates that it forms on different substrates.

As ataxin-3 tightly regulates the ability of CHIP to ubiquitinate itself and its substrates, why then might this ability of ataxin-3 to edit Ub conjugates be important? With CHIP, ataxin-3 ensures that the Ub conjugates are the appropriate length to efficiently target substrates for proteasomal degradation. Furthermore, by inactivating CHIP via deubiquitination, ataxin-3 tightly regulate the activity of CHIP within the protein quality control pathway, ensuring that it can only ubiquitinate misfolded proteins targeted for degradation. However, if CHIP is unable to remove these proteins, resulting in an accumulation of misfolded proteins, ataxin-3 again utilizes its editing activity to help sequester these proteins into structures termed aggresomes, thereby mitigating the effects of misfolded protein toxicity within the cell (Ouyang et al., 2012; Wang et al., 2012). In this pathway, ataxin-3 edits Ub conjugates on misfolded proteins to generate free Ub C termini that are recognized by HDAC6 and subsequently sequestrated to the aggresomes

(Ouyang et al., 2012). Thus, as an editor of Ub conjugates, ataxin-3 not only tightly regulates CHIP in the protein quality control pathway, but acts independently of CHIP to sequester misfolded proteins into aggresomes, providing the cell with multiple layers of protection from misfolded protein toxicity.

ATAXIN-3-MEDIATED REGULATION OF PARKIN ACTIVITY

Individual DUBs often deubiquitinate and regulate the activity of multiple E3s (Daviet and Colland, 2008; Nathan et al., 2008), making it likely that ataxin-3 can deubiquitinate other E3s. Interestingly, MJD can present with clinical and neuropathological symptoms of Parkinson disease (PD), and this raises the possibility that an interaction between ataxin-3 and a PD-associated protein could be involved in MJD (Gwinn-Hardy et al., 2001; Bettencourt et al., 2011). Similar to CHIP, the PD-associated E3 parkin was demonstrated to ubiquitinate and facilitate the clearance of an expanded ataxin-3 fragment (Tsai et al., 2003). Parkin interacts directly with distinct UIMs in ataxin-3 via its N-terminal Ub-like domain (Ubl^{parkin}) (**Figure 1C**) (Durcan et al., 2011). Through its interaction with parkin, ataxin-3 regulates the ability of parkin to ubiquitinate itself, with ataxin-3 reducing parkin self-ubiquitination both in cells and *in vitro*. Interestingly, although ataxin-3 deubiquitinates parkin, parkin is unable to ubiquitinate ataxin-3 (Durcan et al., 2011, 2012). Finally, ataxin-3-mediated deubiquitination appears to only regulate parkin activity, as the presence or absence of wild-type ataxin-3 had no effect on overall parkin levels (Durcan et al., 2011).

With CHIP, ataxin-3 could trim/edit Ub chains after they had formed on substrates and was able to catalyze the removal of mono-Ub off CHIP (Scaglione et al., 2011). With parkin, ataxin-3 was unable to remove individual Ub moieties or preformed Ub chains after they had formed, suggesting that ataxin-3 was deubiquitinating parkin through a more unconventional mechanism (Durcan et al., 2012). One clue came from *in vitro* assays in which ataxin-3 regulates the formation of Ub conjugates on parkin, only when parkin was actively ubiquitinating. These findings suggest that ataxin-3 actively opposes the ligation of Ub on parkin, thereby impeding the ability of parkin to self-ubiquitinate.

Recently, parkin has been demonstrated to function as a RING/HECT hybrid, with the charged E2-Ub directly transferring the Ub onto cysteine 431 of parkin (Wenzel et al., 2011; Lazarou et al., 2013). This results in the formation of a parkin-Ub thioester intermediate complex, prior to parkin ligating the Ub onto itself (**Figure 2A**). Given that ataxin-3 and parkin interact directly, as demonstrated from *in vitro* binding data, how then is ataxin-3 interfering with the ability of parkin to ubiquitinate itself. Through its cysteine at residue 14, ataxin-3 alone can interact directly with the E2s used by parkin to self-ubiquitinate (Durcan et al., 2012). Remarkably, when the cysteine was mutated to a serine, this interaction was now abolished. When parkin was present, ataxin-3 could not only interact with both parkin and the E2, but it could also promote the transfer of the Ub away from parkin and onto itself. Such E3-like activity is not unprecedented for a DUB, with both UCH-L1 and A20 possessing E3 and DUB activities (Liu et al., 2002; Wertz et al., 2004), although further work is required to ascertain how ataxin-3 might act as an E3-like DUB. From these findings, we propose a model whereby ataxin-3 binds directly to

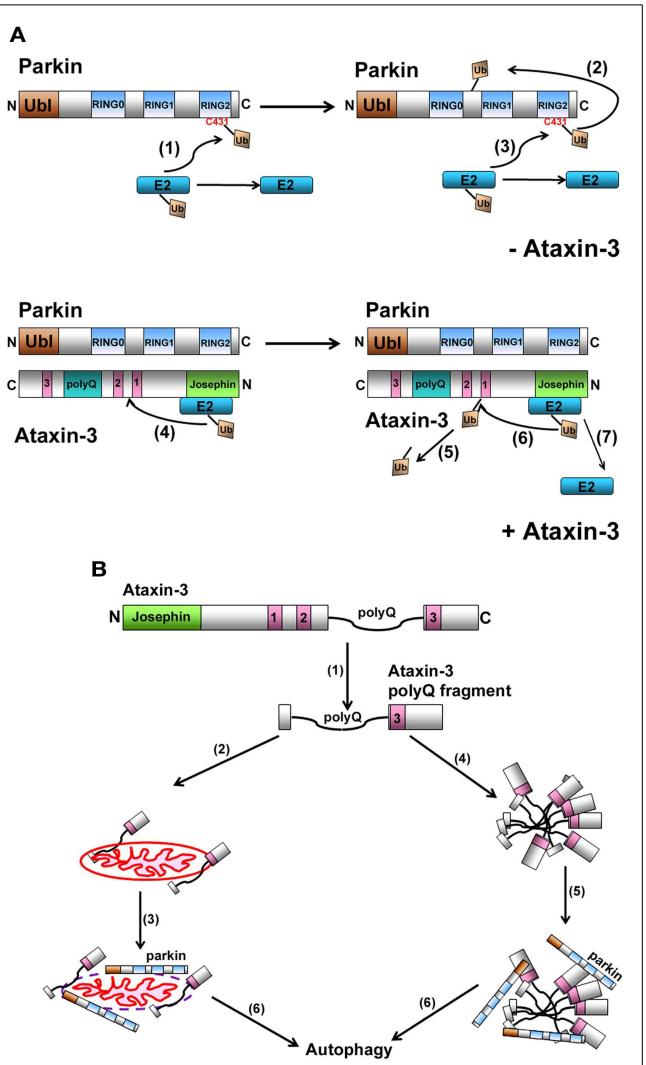

FIGURE 2 | (A) Model representation of how ataxin-3-mediated deubiquitination regulates parkin self-ubiquitination. When parkin ubiquitinates itself, the charged E2-Ub thioester transfers the Ub onto C431 in parkin to form a transient parkin-Ub thioester complex (1). Parkin now transfers this Ub onto one of its own lysines generating an isopeptide linkage (2). Following the transfer of Ub onto itself, parkin is now free to receive a new Ub and this continues the cycle of parkin self-ubiquitination (3). When ataxin-3 is present, we propose that ataxin-3, through its interaction with the IBR-RING2 domain of parkin, blocks the C431 residue, impeding parkin from forming a parkin-Ub thioester. Instead, ataxin-3 now interacts with the E2, directing the transfer of Ub onto a lysine within ataxin-3 (4). This isopeptide linkage is transient as ataxin-3 can catalyze its removal (5) (unpublished data), allowing ataxin-3 to repeat this cycle of self-ubiquitination/deubiquitination, thereby reducing the ability of parkin to ubiquitinate itself (6 and 7). **(B)** Proposed model for how the expanded ataxin-3 can promote the autophagic clearance of parkin. In the presence of calcium, calpains cleave full-length ataxin-3, generating fragments containing the expanded polyQ tract (1). These fragments can associate with mitochondria (2), causing mitochondrial damage, parkin recruitment (3), and ultimately parkin-mediated clearance of itself and the mitochondria via the autophagy pathway (6). Alternatively, these expanded polyQ fragments also have a propensity to aggregate (4), recruiting parkin and CHIP (5), which now act to direct these aggregates and themselves for autophagic clearance (6).

parkin, blocking the E2 from transferring the Ub onto C431 in parkin. As a result, the E2 now transfers the Ub onto ataxin-3 and away from parkin, thereby impeding the activity of parkin to ubiquitinate itself.

MJD, MUTANT ATAXIN-3, AND ITS E3 PARTNERS

From these studies, it is clear that ataxin-3 is involved in tightly regulating the activity of two E3s that function to maintain normal cellular homeostasis. Strikingly, in mouse models of MJD, levels of both CHIP and parkin are significantly reduced when the polyQ tract becomes expanded over 51 glutamines, thereby disrupting the normal cellular homeostasis and promoting neuronal cell loss. Thus, when mutated, ataxin-3 now promotes the destruction of two quality control E3s. Yet, why does the presence of an expanded polyQ tract in ataxin-3 enhance the clearance of both E3s, is this effect dependent on the DUB ability of ataxin-3 or is it caused by the expanded polyQ tract. In the case of CHIP, expansion of the polyQ tract increases the binding between CHIP and ataxin-3, leading to a reduction in CHIP levels in the brains of MJD transgenic mice (Scaglione et al., 2011). One possibility is that the increased affinity of mutant ataxin-3 for CHIP somehow alters their functional relationship, inadvertently causing CHIP to be directed for degradation. Although it is unclear why CHIP is degraded, a reduction in CHIP levels will have an adverse effect on the protein quality control pathway. Consequently, an accumulation of misfolded neurotoxic proteins can be a key contributing factor in the progressive loss of neurons associated with MJD.

Intriguingly, the presence of the mutant ataxin-3 also causes a reduction in parkin levels in the brains of MJD transgenic mice. These findings were further confirmed and extended in cells, with the presence of the expanded form of ataxin-3 promoting clearance of parkin through the autophagy pathway (Durcan et al., 2011). However the mechanism remains unclear. Recent studies have highlighted the role of K27-, K29-, and K63-linked Ub in directing proteins for degradation via the lysosomal and autophagy pathways. Interestingly, parkin assembles Ub conjugates on itself preferentially via these linkages and when compared to wild-type ataxin-3, the polyQ expanded mutant ataxin-3 was more efficient at removing K27- and K29-linked Ub conjugates from parkin. One possibility is that these K27- and K29-linked Ub conjugates protect parkin from autophagic degradation, and their preferential removal enhances parkin turnover via autophagy (Durcan and Fon, 2011).

Alternatively, and a more likely scenario, is that the effect of expanded ataxin-3 on parkin occurs independently of its catalytic activity and is a direct effect of the expanded polyQ tract (**Figure 2B**). Autophagy has been implicated in clearing mutant polyQ protein aggregates in several models of polyQ expansion disorders, including MJD. In this scenario, fragments of the expanded polyQ tract generated from the mutant ataxin-3 cause aggregates to form. By interacting with these inclusions and directing them for destruction, CHIP and parkin may in a sense "just go along for the ride," ultimately resulting in both their clearance along with the aggregates. Thus, over time, extensive clearance of these aggregates can cause levels of CHIP and parkin to diminish. We also cannot exclude a second possibility, that parkin and CHIP are actively involved in the autophagic process, as has been reported for the recently identified role for parkin in targeting damaged mitochondria for autophagic removal (mitophagy) (Narendra et al., 2008). Interestingly, mitochondrial abnormalities have been observed in models of polyQ disorders, including MJD (Ranganathan et al., 2009; Yu et al., 2009; Kazachkova et al., 2012; Laco et al., 2012; Reddy and Shirendeb, 2012). Moreover, polyQ fragments generated from mutant ataxin-3 can interact with and damage mitochondria (Pozzi et al., 2008; Sugiura et al., 2011; Kazachkova et al., 2012). This in turn can now potentially promote the mitochondria recruitment of parkin, triggering the autophagic clearance of parkin-bound mitochondria (Chai et al., 2001; Koch et al., 2011). Taken together, ataxin-3 forms partnerships with two E3s that are essential for maintaining normal cellular homeostasis. In MJD, these partnership are not only disrupted, but the presence of the expanded ataxin-3 now promotes clearance of both parkin and CHIP, which over time can have deleterious consequences on neurons in MJD and PD.

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Mechanisms mediating spinal and bulbar muscular atrophy: investigations into polyglutamine-expanded androgen receptor function and dysfunction

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Spinal and bulbar muscular atrophy (SBMA, Kennedy's disease), a late-onset neuromuscular disorder, is caused by expansion of the polymorphic polyglutamine tract in the androgen receptor (AR). The AR is a ligand-activated transcription factor, but plays roles in other cellular pathways. In SBMA, selective motor neuron degeneration occurs in the brainstem and spinal cord, thus the causes of neuronal dysfunction have been studied. However, pathogenic pathways in muscles may also be involved. Cultured cells, fly and mouse models are used to study the molecular mechanisms leading to SBMA. Both the structure of the polyglutamine-expanded AR (polyQ AR) and its interactions with other proteins are altered relative to the normal AR. The ligand-dependent translocation of the polyQ AR to the nucleus appears to be critical, as are interdomain interactions. The polyQ AR, or fragments thereof, can form nuclear inclusions, but their pathogenic or protective nature is unclear. Other data suggests soluble polyQ AR oligomers can be harmful. Post-translational modifications such as phosphorylation, acetylation, and ubiquitination influence AR function and modulate the deleterious effects of the polyQ AR. Transcriptional dysregulation is highly likely to be a factor in SBMA; deregulation of non-genomic AR signaling may also be involved. Studies on polyQ AR-protein degradation suggest inhibition of the ubiquitin proteasome system and changes to autophagic pathways may be relevant. Mitochondrial function and axonal transport may also be affected by the polyQ AR. Androgens, acting through the AR, can be neurotrophic and are important in muscle development; hence both loss of normal AR functions and gain of novel harmful functions by the polyQ AR can contribute to neurodegeneration and muscular atrophy. Thus investigations into polyQ AR function have shown that multiple complex mechanisms lead to the initiation and progression of SBMA.

Keywords: androgen receptor, spinal and bulbar muscular atrophy, Kennedy's disease, polyglutamine disease, neuromuscular disorder, mouse models, loss-of-function, gain-of-function

INTRODUCTION

Genetic alterations in the X-linked androgen receptor (AR) gene are associated with spinal and bulbar muscular atrophy (SBMA, Kennedy's disease), an adult-onset neuromuscular disease, androgen insensitivity syndrome (AIS), and prostate cancer (Gottlieb et al., 2004, 2012). Although *AR* point mutations, insertions, and deletions lead to varying degrees of AIS, the underlying cause of SBMA is an expansion of the polymorphic *AR* CAG repeat encoding a glutamine tract (La Spada et al., 1991). As a result, the AR protein contains an expanded polyglutamine tract ($n \geq 38$) that alters the AR's normal functions and physiological roles. This article refers to the polyglutamine-expanded AR as the polyQ AR, in order to differentiate it from mutant ARs found in other diseases, and primarily focuses on investigations into the molecular pathogenesis of SBMA published since our last review (Beitel et al., 2005).

SBMA is one of at least nine neurodegenerative diseases caused by expansion of a polyglutamine tract within a specific protein. Others include Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), and six spinocerebellar ataxias [SCA1, SCA2, SCA3 (Machado-Joseph disease), SCA-6, SCA-7, SCA-17 (Truant et al., 2006; Orr and Zoghbi, 2007; La Spada and Taylor, 2010)]. Except for SBMA, which is X-linked, these disorders are inherited in an autosomal-dominant fashion. SBMA has a prevalence of about 1 in 50,000 males, HD, 1 per 15,000 persons worldwide, and, all together, the prevalence of the SCAs has been estimated at ∼3 per 100,000 (Guyenet and La Spada, 2006; Truant et al., 2006). The polyglutamine diseases share several features including late-onset, progressive neurodegeneration, accumulation of misfolded polyQ proteins in the cytoplasm or nucleus of neurons, and a positive correlation between CAG repeat length and disease severity (Parodi and Pennuto, 2011; Tanaka et al., 2012). Despite widespread expression of the polyQ proteins, only specific neuronal populations are affected in each disease. Even so, common mechanisms that disturb neuronal functions in the polyQ disorders may be identified, leading to the development

of effective therapies for these diseases (Pennuto and Fischbeck, 2010; Takahashi et al., 2010).

ANDROGEN RECEPTOR STRUCTURE AND MECHANISMS OF ACTION

The AR is a member of the nuclear receptor superfamily and has been well characterized as a ligand-activated transcription factor (Brinkmann, 2011). It contains an N-terminal domain (NTD) that modulates transcriptional activation, a central DNA-binding domain (DBD) that binds androgen-response elements (AREs), and a C-terminal ligand-binding domain (LBD) (**Figure 1**). Subdomains involved in nuclear localization, dimerization, and interaction with heat-shock proteins (HSPs), co-activators, and other proteins have also been identified (Centenera et al., 2008; Parodi and Pennuto, 2011). Typically, the AR resides in the cytoplasm of the cell in the absence of androgens, complexed with HSP (Brinkmann, 2011). Once the receptor binds testosterone, or its metabolite, 5α-dihydrotestosterone (DHT), the AR undergoes a conformational change that promotes heat-shock protein dissociation, and exposes its nuclear localization signal (NLS), DNA-binding, and dimerization domains. Following nuclear translocation, ARs dimerize at AREs, recruit coregulators, and transactivate AR target genes. The resulting ligand-dependent changes in mRNA and protein expression are thought to be responsible for the differential physiological actions of testosterone and DHT (Brinkmann, 2011). However, a proteomics/systems biology approach to identifying proteins within AR complexes found that the wild-type AR (wt AR) interacts with a wide variety of proteins involved in RNA splicing, protein translation, proteasome/protein ubiquitination, and transcription, suggesting the AR participates in numerous cellular pathways (Paliouras et al., 2011).

ANDROGEN INSENSITIVITY SYNDROME AND SBMA

The AR plays a role in male sexual differentiation, and the development and function of nerves, brain, testes, prostate, and muscles (Jordan and Doncarlos, 2008; MacLean et al., 2008). In XY individuals, the spectrum of AIS ranges from complete androgen insensitivity, which results in a female phenotype, to mild resistance to androgens, which leads to undervirilization, breast enlargement (gynecomastia), and/or infertility in males (Brinkmann, 2001). Conversely, men with SBMA generally develop an adult-onset disorder with progressive muscle cramps, muscle weakness and wasting, muscle twitching (fasciculations), dysarthria (difficulty articulating), and dysphagia (difficulty swallowing) (Rhodes et al., 2009; Katsuno et al., 2010b), in addition to signs of mild AIS. In some cases, these patients are initially misdiagnosed with amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), however, genetic testing for an expanded *AR* CAG repeat ($n \geq 38$) confirms a diagnosis of SBMA (Parboosingh et al., 1997; Bruson et al., 2012). This differential diagnosis is critical, as ∼50% of ALS patients die within 3 years of disease onset, with involvement of both upper and lower motor neurons (Mitchell and Borasio, 2007), whereas the life expectancy for SBMA patients is normal or only minimally reduced (Chahin et al., 2008).

Although men with SBMA may exhibit certain symptoms of AIS [e.g., gynecomastia and reduced fertility (Dejager et al., 2002)], which have been attributed to AR loss-of-function, individuals with AIS do not display the neuromuscular phenotype associated with SBMA. Therefore, the polyQ AR must also gain novel functions that are selectively harmful to motor neurons (Trifiro et al., 1994), and, as shown recently, detrimental to skeletal muscles. SBMA is characterized by loss of lower motor neurons from the brain stem and anterior horn of the spinal cord, which has been presumed to lead to progressive muscle wasting (Ross, 1995). Accordingly, extensive research has focused on mechanisms by which the polyQ AR causes neuronal dysfunction and degeneration (Walcott and Merry, 2002; Beitel et al., 2005; Cary and La Spada, 2008; Finsterer, 2009), and, more recently, pathogenic pathways in muscles (Yu et al., 2006a; Monks et al., 2007; Palazzolo et al., 2009). SBMA is unique among the polyglutamine diseases in that it is a ligand (androgen)-dependent disorder (Katsuno et al., 2002; Orr and Zoghbi, 2007). Thus, men expressing the polyQ AR exhibit progressive neuromuscular signs, while women who are heterozygous for the polyQ AR mutation develop very mild symptoms due to and lower levels of circulating AR ligands (testosterone and

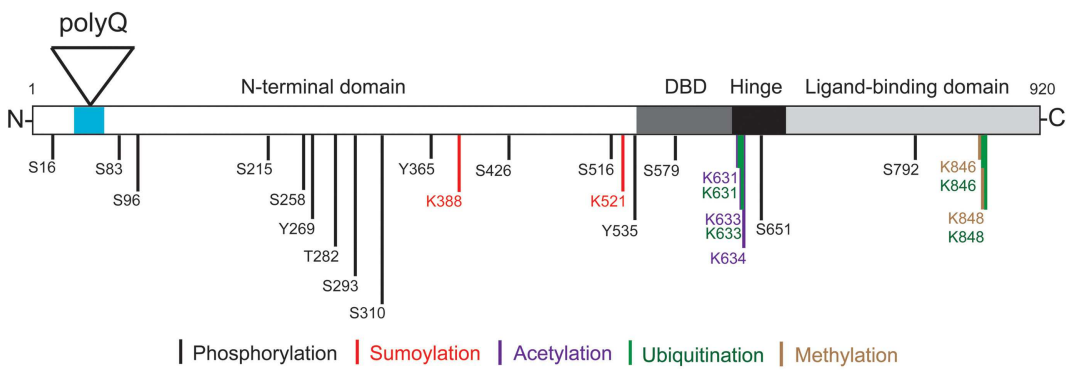

FIGURE 1 | Androgen receptor structure and post-translational modifications. The locations of the AR N-terminal domain (NTD), polyglutamine tract (polyQ), DNA-binding domain (DBD), hinge region, and C-terminal ligand-binding domain (LBD) are shown. Phosphorylation can occur on serine (S), threonine (T), and tyrosine (Y) residues. Specific lysine (K) residues can be acetylated, ubiquitinated, sumoylated, or methylated as indicated. Note the amino acid numbering is based on a 920 amino acid AR (NCBI Reference Sequence: NM_000044.3) (Gottlieb et al., 2012).

Table 1 | Cell and animal models of SBMA.

Cell line	Description	Features	Reference
HEK293	Human embryonic kidney cells		
HeLa	Human cervical cancer cells		
MN AR24; MN AR65	Mouse hybrid motoneuron-neuroblastoma cells	MN-1 cells stably transfected with hAR 24Q or 65Q	Brooks et al. (1997)
NSC34	Mouse neuroblastoma-spinal cord hybrid cell line	Resemble developing motor neurons.	Cashman et al. (1992)
PC12	Rat adrenal pheochromocytoma-derived cell line	Model system for neuronal differentiation	Greene and Tischler (1976)
SK-N-SH; SH-SY5Y	Human neuroblastoma cell lines	General neuron-like phenotype	Ross et al. (1983)

Drosophila	**Description**	**Features**	**Reference**
hAR(Q52)	UAS-hAR(Q52); GMR-GAL4	ARQ52 expressed in eye photoreceptors neuron under control of *GMR* promoter	Takeyama et al. (2002)
ARQ77	UAS-ARQ77; ELAV-GAL4	ARQ77 expressed throughout CNS under control of pan-neuronal *ELAV* promoter	Funderburk et al. (2009)
ARQ65	pUAST-ARQ65; OK371-GAL4	ARQ65 expressed in a defined subset of neurons under control of OK371 promoter	Jochum et al. (2012)

Mouse model	AR/promoter	Neuronal pathology	Nuclear inclusions	Muscle pathology	Other features	Reference
Transgenic AR120Q	Full-length hAR (120Q); CMV promoter	Yes	No	Yes	Muscle weakness & atrophy; testicular atrophy	McManamny et al. (2002)
Transgenic AR97Q	Full-length hAR (97Q); chicken β-actin promoter, CMV enhancer	Yes	Yes, motor neurons, spinal cord, CNS, muscle, etc.	Yes	Muscle weakness & atrophy; neurogenic & myogenic myopathy	Katsuno et al. (2002)
Transgenic AR100	Complete hAR gene (100Q) in YAC	Yes	No, motor neurons; Yes, CNS, liver, muscle	Yes	Muscle weakness & hindlimb atrophy	Sopher et al. (2004)
Transgenic AR112Q	Full-length hAR (112Q); prion protein promoter	No	Yes, spinal cord, brain stem & cortex	No	Hindlimb muscle weakness; some male infertility	Chevalier-Larsen et al. (2004)
Knock-in AR113Q	1340 bp of mAR exon 1 replaced by hAR (113Q) exon 1	Yes	Yes, CNS, muscle	Yes	Motor deficits; testicular atrophy	Albertelli et al. (2006), Yu et al. (2006a)
Transgenic HSA-AR	Rat AR(22Q); HSA promoter	Axonopathy	No	Yes	Overexpress AR only in skeletal muscle	Monks et al. (2007)
Tfm	AR null	No	No	No	Model for AIS	Gaspar et al. (1991)

CMV, cytomegalovirus; CNS, central nervous system; ELAV, embryonic lethal abnormal visual system promoter, hAR, human AR; HSA, human skeletal actin promoter; mAR, mouse AR; YAC, yeast artificial chromosome.

DHT) (Chevalier-Larsen and Merry, 2012). SBMA has been found in men from various ethnic backgrounds throughout the world, although the prevalence is higher in certain regions of Japan and Western Finland (Finsterer, 2010; Katsuno et al., 2010b).

MODEL SYSTEMS

A number of systems have been used to investigate the molecular and cellular mechanisms underlying SBMA, ranging from cultured cells to fly and mouse models [summarized in **Table 1**;

mouse models reviewed in (Merry, 2005; Jordan and Lieberman, 2008; Figiel et al., 2012)]. Cells that lack AR are transiently or stably transfected to examine the effects of the wt or polyQ AR. PC12 cells that inducibly express wt and polyQ ARs have been used extensively (e.g., Montie and Merry, 2009; Ranganathan et al., 2009; Orr et al., 2010; Montie et al., 2011). In *Drosophila*, ARs can be expressed in a tissue-specific fashion, depending on the promoter used to drive AR expression. Most SBMA mouse models, however, are based on overexpression of a polyQ AR protein with more than 90Q; these mice also express endogenous wt AR (Katsuno et al., 2002; McManamny et al., 2002; Chevalier-Larsen et al., 2004; Sopher et al., 2004). The transgenic AR97Q mice express the full-length human AR (hAR) containing 97 CAG repeats under the control of a cytomegalovirus enhancer and a chicken β-actin promoter (Katsuno et al., 2002), while the prion protein promoter was used to drive expression of the hAR with 112Q in the AR112Q transgenic mice (Chevalier-Larsen et al., 2004). In contrast, a yeast artificial chromosome (YAC) genomic fragment containing the 100 kb hAR gene with 100 CAG repeats was used to generate the transgenic AR100 model mice (Sopher et al., 2004) (**Table 1**). SBMA patients, however, express only a polyQ AR protein with 38–62 Gln, and lack wt AR. The presence of the wt AR in transgenic mice models may moderate or compensate for the effects of the polyQ AR. This possibility was highlighted when the transgenic AR100 YAC mice were crossed with AR null testicular feminization mice (*Tfm)* (**Table 1**) (Thomas et al., 2006b), as the AR100Tfm mice exhibited an accelerated neuromuscular disease phenotype relative to the AR100 mice. As well, although the AR20 protein in AR20Tfm mice was able to partially rescue the female-like Tfm phenotype, the external genitalia of the AR100Tfm mice was the same as female or androgen-insensitive Tfm mice, consistent with a loss-of-function of the polyQ AR protein.

A knock-in mouse model for SBMA was made by replacing 1,340 bp of the coding sequence of mouse AR exon 1 with hAR exon 1 sequence containing 113 CAGs (Albertelli et al., 2006; Yu et al., 2006a) (**Table 1**). In contrast to the AR100Tfm mice, in the transgenic AR113Q mice, the polyQ AR was able to effectively masculinize the male mice, likely because the AR113Q is under the regulatory control of the mouse AR gene promoter. The AR113Q knock-in mice show signs of partial androgen insensitivity, including testicular atrophy and decreased fertility, which are seen in SBMA patients (Yu et al., 2006b). A striking age-dependent testicular pathology was observed in the AR113Q, which was distinct from the testicular atrophy seen in *Tfm* mice. These results lead to the conclusion that the abnormalities in testicular morphology in AR113Q were mediated not only by a partial loss of AR function, but also reflect a "toxic" gain-of-function due to AR polyglutamine tract expansion. Unexpectedly, transgenic mice that overexpress the WT rat AR (22 Q) under control of the human skeletal α-actin (HSA) promoter solely in their skeletal muscle fibers reproduced many neuromuscular features of SBMA model mice (Monks et al., 2007). The neuronal and muscular pathology, the localization of nuclear inclusion and notable phenotype features of each mouse model are summarize in **Table 1**.

Testosterone plays a crucial role in progression of symptoms in SBMA mice models. However, raising testosterone levels in male AR112Q transgenic mice for 6 months did not worsen severity or age of onset of their disease, suggesting that the pathogenic mechanism of disease in SBMA saturates at close to endogenous testosterone levels (Chevalier-Larsen and Merry, 2012).

MECHANISMS CONTRIBUTING TO SBMA

Recent investigations have focused on a number of complex, and not necessarily independent, mechanisms that lead to the development of SBMA. These include alterations in AR structure, interaction of the polyQ AR with other proteins, transcriptional dysregulation, formation of harmful polyQ AR oligomers, changes in post-translational modifications, loss of neurotrophic support, and mitochondrial dysfunction. The possibility that polyQ AR expression in skeletal muscles contributes to SBMA is intriguing. More controversial are the role of nuclear inclusions, altered axonal transport, and inhibition of the ubiquitin proteasome system. These topics will be discussed in greater detail below.

ALTERATIONS IN AR STRUCTURE

Investigating polyglutamine repeats in their native context is important for understanding their effects on protein structure and function. In aqueous solution, purified recombinant wt AR NTD (amino acids 1-537) had a relatively limited amount of stable secondary structure. However, for the AR-NTD45Q, a small, but measureable increase in α-helix content and small decrease in the β structures was noted (Davies et al., 2008). The AR-NTD45Q peptide was more sensitive to urea-induced unfolding than the AR-NTD20Q, and limited proteolysis, which was used as a probe for global protein structure, generated a unique pattern of fragments from the polyQ AR NTD. Thus, these spectroscopic and biochemical analyses support the view that the expanded glutamine tract alters the conformation of the AR NTD.

Structural analysis were also performed on full-length AR-proteins expressed using the baculovirus-insect cell system (Jochum et al., 2012). Atomic force microscopy was used to characterize the sub-micrometer scale aggregates of the ARQ22 and ARQ65 proteins purified from DHT-treated Sf9 cells (Jochum et al., 2012). The wt AR formed annular oligomers 120–180 nm in diameter, while the polyQ AR formed oligomeric fibrils up to 300–600 nm in length. Interestingly, ARQ65 purified from DHT- and melatonin-treated Sf9 cells produced annular oligomers, which did not decrease human neuronal SK-N-SH cells viability, unlike the fibrillar oligomer form, suggesting that AR structure modulates pathogenicity.

INTERDOMAIN INTERACTIONS

Dynamic interactions between different AR domains are critical for the receptor's function. DBD-mediated dimerization is essential for the AR to bind DNA and regulate transcription (Centenera et al., 2008). Upon ligand-binding, the AR LBD undergoes a conformational rearrangement that results in formation of a protein interaction surface, the activation function 2 (AF-2) domain, which binds with the ^{23}FQNLF^{27} motif near the AR N-terminus. This intermolecular interaction, known as N/C-terminal interaction, is essential for androgen-dependent activation of specific genes (He et al., 2002). To establish whether N/C-terminal interaction of the polyQ AR played a role in aggregation and cell death, the effects of bicalutamide, a transcriptional antagonist of

the AR, were tested in a cell model of SBMA and motor neurons from transgenic AR112Q mice (Orr et al., 2010). Bicalutamide not only decreased polyQ AR N/C-terminal interactions, but reduced nuclear inclusion formation in PC12 cells expressing AR112Q, even in the presence of DHT. Furthermore, AR112Q PC12 cells and primary neurons were substantially protected from DHT-induced cell death by the addition of bicalutamide. Mutations in the ^{23}FQNLF^{27} motif of AR112Q also reduced DHT-dependent cell toxicity and polyQ AR aggregation, suggesting that N/C-terminal interaction must be maintained in order for the polyQ AR to exhibit its pathogenic gain-of-function properties.

ALTERED PROTEIN INTERACTIONS

The AR NTD contains a transactivation domain that participates in multiple protein–protein interactions with general transcription factors and co-regulatory proteins. Thus changes in the AR NTD induced by polyglutamine expansion (Davies et al., 2008) can potentially strengthen or diminish the interaction of the polyQ AR with these proteins (**Figure 2**). Overall, the wt AR is known to interact directly with more than 236 distinct proteins (Gottlieb et al., 2012). For instance, the SIRT1 deacetylase binds to the AR (Fu et al., 2006), and modulates aggregation and polyQ AR proteotoxicity (Montie et al., 2011). Several proteins differentially interact with the wt or polyQ AR, implicating different pathways in SBMA pathogenesis. While cytochrome c oxidase subunit Vb (COXVb) interacted more strongly with wt AR than polyQ AR in a hormone-dependent manner, COXVb co-localized with polyQ AR, but not wt AR aggregates (Beauchemin et al., 2001), which may contribute to mitochondrial dysfunction in SBMA. In the presence of DHT, retinoblastoma protein (Rb) weakly associated with wt AR, but interacted strongly with the polyQ AR, leading to aberrant E2F1 transcriptional activation (Suzuki et al., 2009). The p23 protein, an essential component of the multichaperone Hsp90 complex, was more highly associated with the polyQ AR than the wt AR, which may be the basis for the preferential degradation of the polyQ AR after 17-AAG treatment (Waza et al., 2005). AR113Q, but not AR10Q, specifically interacted with PTIP (Pax Transactivation-domain-interaction Protein), a protein that functions in DNA repair, suggesting the polyQ AR may dampen the DNA damage response, leading to an accumulation of mutation and cellular dysfunction in SBMA (Xiao et al., 2012). The full impact on cellular functions mediated by novel or altered interactions between the polyQ AR and other proteins is still being explored.

NUCLEAR TRANSLOCATION

The ligand-dependent translocation of AR to the nucleus is required for the receptor to carry out its classical functions as a transcription factor. Transgenic mice expressing a polyQ AR with a deletion within the AR bipartite NLS (ARdNLS112Q) exhibited delayed onset and reduction of motor deficits compared to AR112Q mice. The nuclear accumulation of ARdNLS112Q in the spinal motor neurons of the ARdNLS112Q mice was also deferred relative to the AR112Q mice. Interestingly, in PC12 cells expressing a polyQ AR with a constitutive-acting NLS (NLSX3-AR76Q), although the NLSX3-AR76Q protein localized to the nucleus in the absence of DHT, neither intranuclear inclusions nor cell death were observed. However, upon DHT treatment of the NLSX3-AR76Q cells, inclusions consisting of N-terminal AR fragment formed, and levels of cell death increased.

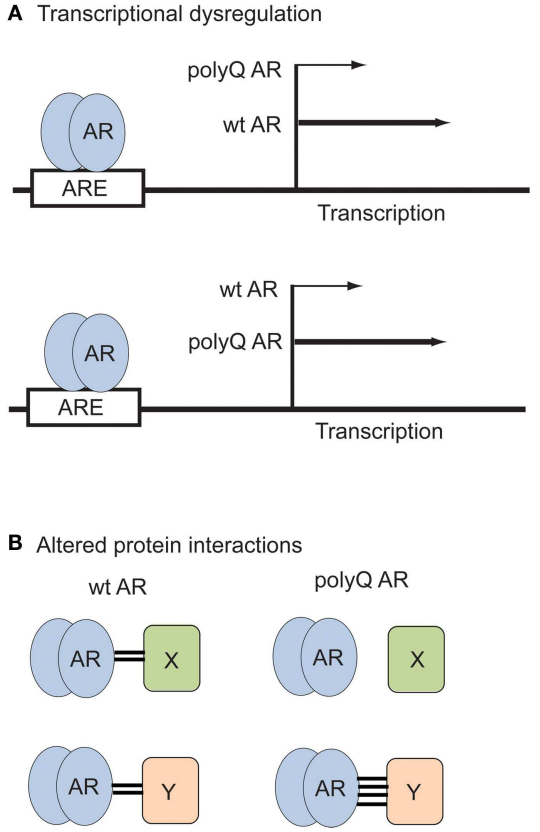

FIGURE 2 | Examples of polyQ AR loss- and gain-of-function in SBMA. **(A)** Transcriptional dysregulation. The polyQ AR may activate expression of certain genes to a lesser (top) or greater (bottom) extent than the wt AR. **(B)** Altered protein interactions. The polyQ AR may fail to associate with proteins (top) or bind more strongly to proteins (bottom) that normally interact with the wt AR.

In a *Drosophila* model, transgenic flies that expressed the AR73Q protein with mutations in the NLS (K633A and K634A; AR73Q KK/AA) were generated (Nedelsky et al., 2010). In addition, a polyQ AR incapable of binding DHT (S215D and S792D mutations; AR65Q SS/DD) was fused to a NLS, to produce NLS-AR65Q SS/DD transgenic flies. *Drosophila* expressing AR52Q or AR65Q-NLS developed an SBMA eye phenotype when fed DHT, however, the AR73Q KK/AA or NLS-AR65Q SS/DD flies showed no signs of photoreceptor degeneration, even when raised on DHT. Overall, these results from these two models indicate that the polyQ AR must be localized within the nucleus, in the presence of its ligand, to induce SBMA disease features.

AGGREGATES AND NUCLEAR INCLUSIONS

The accumulation of misfolded and aggregated proteins is a pathological hallmark of many neurodegenerative diseases, including ALS and polyglutamine diseases (Pandey et al., 2007a). However, immunohistochemical examination of tissues from SBMA

patients with the 1C2 antibody (a monoclonal antibody that specifically recognizes expanded polyglutamine tracts) showed that diffuse nuclear accumulation of the polyQ AR was far more frequent and extensive than nuclear inclusions, was distributed in a wide array of CNS nuclei, and present in more visceral organs than previously identified (Adachi et al., 2005). Cytoplasmic accumulations of the polyQ AR were also detected in neural and non-neural tissues (Adachi et al., 2005). Although there are conflicting views on whether polyQ AR aggregates or nuclear inclusions have a detrimental or a protective effect (Taylor et al., 2003; Beitel et al., 2005), these aggregates may be an indicator of the intracellular inability to remove misfolded proteins (Li et al., 2008). Furthermore, the 1C2-reactive neuronal inclusions can be detected using antibodies directed against specific AR N-terminal regions, but not the DNA- or ligand-binding domains, strongly suggesting that proteolytic cleavage of the polyQ AR generates truncated fragments that are incorporated into the inclusions (Butler et al., 1998; Li et al., 1998; Merry et al., 1998; Chevalier-Larsen et al., 2004). As well, numerous cellular protein have been found to be sequestered into neuronal intranuclear inclusions including HSPs (Hsp40, Hsp70, Hsp90), UPS components (ubiquitin, 19S and 20S proteasome core proteins), and several AR co-activators and transcriptional proteins (CBP, SRC-1) (Rusmini et al., 2011; Schindler et al., 2012).

The first 50 amino acids of the polyQ AR (e.g., filamentous actin-binding sequences) can directly affect aggregation rate, the SDS solubility and the subcellular localization of aggregates (Angeli et al., 2010). Using a FRET assay to measure aggregation of a polypeptide containing the first 127 amino acids of AR, it was determined that coexpression of profilin, an actin-binding protein, inhibited polyQ AR aggregation (Shao et al., 2008). Conversely, cofilin, an F-actin-severing and -depolymerizing factor, increased aggregation, suggesting that the actin cytoskeleton activity may influence polyQ AR aggregation. Disrupting interaction between AR and the ARA70 coactivator in PC12/AR10Q cells with ASC-J9 (5-hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one) promoted AR degradation (Yang et al., 2007). ASC-J9 also reduced aggregate formation and polyQ AR-induced cell death in PC12 AR112Q cells, and in AR97Q transgenic mice reversed muscular atrophy, and improved motor impairment and lifespan. Similar effects were seen in neuronal cells and transgenic mice when genistein, a soy isoflavone, was used to disrupt polyQ AR-ARA70 interactions (Qiang et al., 2013). Thus, decreasing aggregate formation by treatment with compounds that disrupt AR-protein interactions may allow the polyQ AR to be degraded, with beneficial effects.

FORMATION OF SOLUBLE OLIGOMERS

In several SBMA models, the presence of large intracellular inclusions does not correlate with neuronal toxicity or SBMA phenotype (**Table 1**), suggesting other forms of the polyQ AR may be pathogenic. For example, expression of N-terminal truncated polyQ AR fragments in neuronal cells activated c-jun N-terminal kinase (JNK), leading to phosphorylation of c-Jun, which then initiated a Bax-dependent apoptotic cascade (Young et al., 2009). It has also been proposed that soluble polyQ AR NTD fragments can interact with the full-length polyQ AR and impair its transactivation capacity (Schiffer et al., 2008). Although intracellular polyQ AR aggregates are identified using histopathological methods, oligomers have been defined as submacromolecular structures that are soluble after high-speed centrifugation and are comprised of ordered polyglutamine aggregates (Li et al., 2007). In brain extracts from symptomatic male AR112Q mice, the AR112 monomer (∼130 kDa) and an intermediate molecular weight smear (∼250–450 kDa) suggestive of soluble oligomers of polyQ AR protein, were identified by Western blotting (Li et al., 2007). Formic acid treatment has been shown to dissociate SDS-insoluble protein aggregates stabilized by hydrogen bonds, but does not cleave isopeptide bonds. Treatment with formic acid dissociated the polyQ oligomers from SBMA mice and indicated that the oligomers contained a distinct polyQ AR N-terminal fragment of ∼50 kDa. In the male AR112 mice, the oligomers appeared before the onset of motor dysfunction, and did not correlate with the occurrence of nuclear inclusions. However, upon castration, which reversed polyQ-induced pathology in the male AR112 mice, the levels of oligomers decreased rapidly, further implicating soluble polyQ AR oligomers in SBMA pathogenesis.

POST-TRANSLATIONAL MODIFICATIONS

AR function, trafficking, and turnover are dynamically regulated by a variety of post-translational modifications including phosphorylation, acetylation, ubiquitination, sumoylation, and methylation (**Figure 1**) (Montie et al., 2011; Anbalagan et al., 2012; Coffey and Robson, 2012). These modifications can occur through both androgen-independent and androgen-dependent mechanisms. Notably, the polyQ AR was found to be acetylated and phosphorylated in the absence of ligand, while the wt AR underwent these post-translational modifications only in the presence of ligand (Lieberman et al., 2002).

In cells expressing the polyQ AR, phosphorylation of AR112 at S516 via the p44/42 MAP kinase pathway was required to induce cell death (LaFevre-Bernt and Ellerby, 2003). It was proposed that this phosphorylation event enhanced the ability of caspase-3 to cleave the AR112Q and generate cytotoxic polyQ fragments. Akt (also known as protein kinase B) phosphorylates the wt AR at S215 and S792 (Lin et al., 2001). Phosphorylation of AR65Q at the same Akt consensus sites reduced androgen binding and transcriptional activation, and decreased ligand-induced protein stabilization and nuclear translocation (Palazzolo et al., 2007). Insulin-like growth factor 1 (IGF-1) stimulation increased the survival rate of the MN-1 cells stably expressing AR65Q through the phospho-inositol-3-kinase (PI3K) pathway and activation of Akt. IGF-1/Akt signaling also reduced polyQ AR aggregation and increased polyQ AR degradation by the UPS in a phosphorylation-dependent manner (Palazzolo et al., 2009). To study the effects of IGF-1 *in vivo*, transgenic AR97Q mice were crossed with mice overexpressing a rat, non-circulating, muscle-specific isoform of IGF-1. The AR97Q/IGF-1 mice had reduced polyQ AR aggregation in muscles and spinal cord, decreased muscle weakness, less motor neuron loss, improved motor function, and extended life span compared to AR97Q mice, strongly suggesting that augmentation of IGF-1/Akt signaling could counteract the phenotypic and pathological abnormalities caused by the polyQ AR. Further studies showed systemic treatment of symptomatic AR97Q mice with recombinant human IGF-1 and IGF-1 binding protein 3 (rhIGF-IGFBP3) resulted in increased Akt activation, reduced

polyQ AR aggregation and pathology in muscles, improved motor function and increased survival (Rinaldi et al., 2012). Phosphorylation at Akt sites can thus modulate the detrimental effects of the polyQ AR.

Acetylation of wt AR at three lysine residues (K631, K633, and K634) within the AR hinge region is regulated by several acetyltransferases including p300, p300/CBP-associated factor (P/CAF), and TIF60 (Tat-interactive protein). Androgen-induced acetylation of AR can regulate DNA-binding and enhances AR transcriptional activity in a promoter-dependent context (Anbalagan et al., 2012; Coffey and Robson, 2012). Conversely, deacetylation down-regulates AR activity. SIRT1, a member of the sirtuin family of deacetylases, interacts with and deacetylates the wt AR at the conserved lysine motif, inhibiting DHT-dependent wt AR signaling and N/C interaction (Fu et al., 2006). PC12 cells inducibly expressing AR112Q form polyQ length and DHT-dependent nuclear inclusions of proteolyzed polyQ AR, and die in response to DHT (Montie et al., 2011). In the presence of androgens, nuclear-localized AR112Q was hyperacetylated compared to the AR10Q. Stable overexpression of SIRT1 in this model reduced the number of cells with nuclear inclusions and the amount of SDS-insoluble AR112Q seen on Western analysis. SIRT1 overexpression also protected PC12 cells expressing AR112Q, and motor neurons from AR112Q mice, from DHT-dependent death, demonstrating that abnormal acetylation is associated with aberrant polyQ AR function in SBMA.

Sumoylation, the process of covalently attaching small ubiquitin-like modifier (SUMO) to proteins, is mediated by activating, conjugating and ligating enzymes, and reversed by a family of SUMO-specific proteases (SENPs) (Anbalagan et al., 2012). Sumoylation of the wt AR at K388 and K521 in the NTD can inhibit AR transcriptional activity in a promoter context-dependent manner (Mukherjee et al., 2009). Expansion of the polyglutamine tract in the AR does not appear to interfere with AR sumoylation. However, increasing SUMO levels in AR113Q-expressing HeLa cells decreased the fraction of cells containing ligand-induced AR aggregates, without affecting total polyQ AR levels. Thus, sumoylation reduced the formation of polyQ AR aggregates independently of its effect on AR113Q transcription activity.

TRANSCRIPTIONAL DYSREGULATION

Dysregulation of transcriptional events is considered to be a major molecular mechanism through which the polyQ AR contributes to the development of SBMA. The pathogenic polyQ AR accumulates in the cell nucleus in a ligand-dependent manner and may inhibit transcription by interfering with the function of essential transcriptional factors and co-activators (Katsuno et al., 2005). For example, in SBMA cell models, transgenic mice and tissues from SBMA patients, transcriptional co-activators such as CREB-binding protein (CBP) can be sequestered into nuclear inclusions formed by the polyQ AR (McCampbell et al., 2000). AR transcriptional competence has been found to decrease as polyglutamine tract length increases (Kazemi-Esfarjani et al., 1995; Thomas et al., 2006b; Palazzolo et al., 2008). Microarray analysis of MN-1 cells stably expressing 24Q or 65Q AR showed that specific genes that were up- or down-regulated in an androgen-responsive fashion by the wt AR failed to be activated by the polyQ AR, indicative of a partial loss of AR transcriptional activity (Lieberman et al., 2002) (**Figure 2**). In NSC34 cells, the polyQ AR was found to act in DHT-dependent promoter specific context, as ARQ.48 was less effective than AR.Q22 in inducing transcription from classical AREs, but acted similarly to AR.Q22 in repressing transcription from a promoter with a non-classical ARE silencer region (Vismara et al., 2009).

In mouse models of SBMA, decreases in the mRNA levels of vascular endothelial growth factor (VEGF) (Sopher et al., 2004), skeletal muscle chloride channel 1 (CLCN1), skeletal muscle sodium channel α-subunit, neurotrophin-4 (NT-4), glial cell line-derived neurotrophic factor (GDNF) (Yu et al., 2006a), dynactin 1 (Katsuno et al., 2006), genes related to mitochondrial function (Ranganathan et al., 2009), and TGF-β receptor type II (TGF-βRII) (Katsuno et al., 2010a) have been described. Gene expression analysis by qPCR showed muscles from men with SBMA and male AR113Q mice, but not spinal cords or spinal motor neurons, contained significantly higher levels of several mRNAs that are induced in response to endoplasmic reticulum (ER) stress (Yu et al., 2011). Microarray analysis of spinal cords of AR97Q mice showed the gene encoding calcitonin gene-related peptide α (CGRP1) was upregulated, and mediated neuronal damage via activation of the JNK pathway (Minamiyama et al., 2012). Interestingly, although mutations in the FUS (fused in sarcoma) gene are associated with ALS, no evidence of altered FUS expression was found in the spinal cord or motor neurons of AR100 mice (Fratta et al., 2013).

A global characterization of gene expression in three mice models that substantially reproduce an SBMA phenotype was also pursued (Mo et al., 2010). Microarray analyses of the lower hind limb muscles of AR113Q knock-in, AR97Q transgenic, and HSA-AR transgenic mice, relative to wt mice, were carried out. The patterns and levels of transcription of a number of genes were found to be altered in all models. In the polyQ AR models, genes functionally associated with metal ion binding, steroid metabolism, muscle contraction, filamentous actin, endocytosis, chemical homeostasis, and macrophage activation were found to be down-regulated, while genes involved in hydrolase activity, protein binding, and peptidase activity were upregulated. These results support the hypothesis that altered gene expression due to the presence of the polyQ AR may underlie the muscle wasting and motor neuron loss seen in SBMA.

Intriguingly, microRNA (miRNA) expression can also be altered due to the polyQ AR (Miyazaki et al., 2012). Over 500 miRNAs were analyzed using microarrays, and five miRNAs were upregulated more than twofold in the spinal cord of AR97Q mice at an advanced disease stage relative to the AR 24Q mice. At this point, the mechanisms by which the polyQ AR up-regulates miRNAs and the consequences are unknown.

ALTERED RNA SPLICING

Alternative splicing allows for the synthesis of different products from the same gene, increasing the diversity of proteins that can be generated from a limited number of genes. The role of the wt AR in RNA processing and splicing is presently unclear, although the AR has been shown to interact in a ligand-dependent manner with RNA splicing factors PTD-associated (PSF) and p54nrb (Dong

et al., 2007). Hormone- and glutamine length-dependent missplicing of the chloride channel 1 (*Clcn1*) gene and increased expression of the CUGBP1 RNA-binding protein has been demonstrated in SBMA AR113Q knock-in mice (Yu et al., 2009). In AR21Q mice, however, skeletal muscle denervation also induced CUGBP1, but did not alter *Clcn1* RNA splicing, suggesting that a combination of denervation- and polyQ AR-mediated mechanisms is required to alter RNA processing in SBMA model mice (Yu et al., 2009). In *in vitro* studies, the human cardiac troponin T (cTNT) minigene construct was transfected into HeLa cells as a reporter to determine the effect of the wt or polyQ AR on RNA splicing. Expression of the AR24Q in the presence of ligand or the AR112Q in the absence or presence of ligand resulted in increased cTNT exon 5 inclusion. Transcription and RNA processing of the mouse mammary tumor virus (MMTV) driven-calcitonin/calcitonin gene-related peptide (CT/CGRP) minigene is affected by steroid hormone receptors (Auboeuf et al., 2007). Although activation of the AR24Q by ligand slightly increased the CT/CGRP transcript ratios, ligand activation of AR112Q caused a significantly greater increase in this ratio (Yu et al., 2009). Thus, the polyQ AR can influence androgen-regulated RNA processing and splicing events, although the precise effects of altered RNA metabolism on cellular function in SBMA patients remain to be determined.

UBIQUITIN PROTEASOME SYSTEM IMPAIRMENT

Protein homeostasis is regulated in the cell by two principal degradative mechanisms: the ubiquitin proteasome system (UPS), which processes short lived and misfolded proteins for degradation (Ciechanover and Brundin, 2003), and autophagy (Rubinsztein, 2006). Ubiquitination is the enzymatic process that occurs when a ubiquitin moiety is attached to a protein via a highly structured enzymatic cascade that involve an activating enzyme (E1), a conjugating enzyme (E2), and a ubiquitin ligase enzyme (E3) that is substrate specific (Ciechanover and Brundin, 2003). The wt AR-protein undergoes post-translational modifications that modulate its activity, including ubiquitination (Coffey and Robson, 2012; Gioeli and Paschal, 2012) **(Figure 1)**. Mass spectrometry analyses have found two ubiquitination sites in the AR protein, K846, and K848 (Xu et al., 2009). Ubiquitin ligases identified for the AR include MDM2 (Lin et al., 2002), C-terminus of heat-shock cognate protein 70 (Hsc70)-interacting protein (CHIP) (Chymkowitch et al., 2011) and RNF6 (Xu et al., 2009). MDM2 and CHIP target the AR for degradation by polyubiquitinating it, while ubiquitination by RNF6 promotes AR-dependent transcription (Xu et al., 2009). Additional UPS proteins that interact directly with and coactivate the wt AR are an E2 (UbcH7), several E3s (ARA54, E6-AP, hPIRH2/ARNIP), a deubiquitinating enzyme (USP10), and a proteasomal subunit (Rpt5/PSMC3) (Gottlieb et al., 2012). AR function and the UPS may therefore overlap in a number of areas, beyond proteasome-mediated degradation.

One of the characteristic features of the neurodegenerative polyglutamine diseases is the presence of inclusion bodies that colocalize with components of the UPS and the molecular chaperone pathway (Adachi et al., 2001), indicating that the mutant proteins are targeted for degradation (Stenoien et al., 1999). Potentially, sequestration of UPS components or inhibition of the UPS by polyQ proteins could alter protein repair and degradation pathways and thus have been implicated in the pathogenesis of polyglutamine diseases (Bailey et al., 2002; Rusmini et al., 2010). Degradation of an unstable green fluorescent protein reporter (GFP^u) decreased when HEK293-GFP^u cells expressing the polyQ AR were treated with androgens, suggesting that proteasome activity was inhibited by the polyQ AR (Mandrusiak et al., 2003). Androgen-dependent polyQ AR (121Q) UPS impairment was also observed in SBMA flies expressing a reporter for proteasome function (Pandey et al., 2007b). In contrast, in an NSC34 cell model of SBMA, unliganded soluble polyQ impaired the cytoplasmic UPS, and, although testosterone binding induced polyQ AR aggregation, nuclear UPS activity was unaffected (Rusmini et al., 2007). Proteasomal proteolytic activity was also preserved in AR97Q mice, even when they developed a severe phenotype (Tokui et al., 2009). At this point, the complex processes that may lead to proteasomal inhibition and the extent to which UPS dysfunction contribute to SBMA require further investigation.

AUTOPHAGY

Macroautophagy, often simply called autophagy, is the process by which misfolded or damaged proteins are targeted for degradation through the lysosomal system (Rubinsztein, 2006). The role of autophagy has been assessed in several SBMA models. Transgenic *Drosophila* expressing full-length hAR with 52Q or 121Q displayed ligand-dependent, polyQ AR-dependent degeneration of specific neurons, resulting in a rough eye phenotype (Pandey et al., 2007b). This phenotype was associated with UPS impairment and induction of morphological features of autophagy. Inducing autophagy in this fly model, through overexpression of histone deacetylase 6 (HDAC6), accelerated the turnover of the polyQ AR and lowered steady-state levels of monomeric and aggregated polyQ AR. Treatment with the TOR inhibitor rapamycin also suppressed degeneration, suggesting a compensatory relationship between autophagy and the UPS. Activation of autophagy in motor neurons from AR112Q mice also prevented DHT-dependent death induced by nuclear-localized polyQ AR, although, unlike in the fly model, a reduction in the levels of monomeric polyQ AR was not seen (Montie et al., 2009). In NSC34 cells, 17-AAG (17-allylamino, 17-demethoxygeldamycin) treatment promoted the solubility and degradation of the misfolded AR.Q46 through the autophagic pathway, without affecting the UPS (Rusmini et al., 2011).

The unfolded protein response (UPR), an ER protein quality control pathway, was found to be induced in skeletal muscle from SBMA patients and male AR113Q knock-in mice (Yu et al., 2011). Unexpectedly, genetic disruption of the UPR in the AR113Q mice worsened skeletal muscle atrophy, even though autophagy was activated. Conversely, impairing autophagy decreased muscle wasting and prolonged survival of the SBMA mice. The contrasting results seen with the pharmacological and genetic manipulation of autophagy in these SBMA models highlights the complexity of the relationship between the UPR, autophagy, and the UPS, as well as the ability of these systems to clear the polyQ AR from different tissues and subcellular compartments.

HEAT-SHOCK PROTEINS

Heat-shock proteins are molecular chaperones that facilitate the refolding, assembly and intercellular transport of proteins.

The wt AR is normally stabilized by the chaperone activity of Hsp90/Hsp70 complex, which maintains the AR in a conformation that permits androgen binding (Wang et al., 2010). Overexpression of Hsp70, and Hsp40, inhibited accumulation of the polyQ AR protein and suppress cell death in several cells models (discussed in Beitel et al., 2005; Katsuno et al., 2005). Oral treatment of transgenic AR97Q mice with geranylgeranylacetone (GGA), which induced Hsp70, Hsp90, and Hsp105 expression in the central nervous system and skeletal muscle, inhibited the nuclear accumulation of the polyQ AR, and ameliorated the neuromuscular phenotype (Katsuno et al., 2005). Treatment of AR100 mice with arimoclomol, a co-inducer of the heat-shock stress response, upregulated Hsp70 in spinal cord and hindlimb muscle, improved neuromuscular function and motor neuron survival, and delayed disease progression (Malik et al., 2013). Overexpression of Hsp70 interacting protein (Hip), a co-chaperone that stabilizes Hsp70, in AR112Q-expressing HeLa cells enhanced clearance of soluble and ligand-induced insoluble polyQ AR proteins through the UPS (Wang et al., 2013). Similarly, a small molecule (YM-1) that converted Hsp70 to its tight-affinity conformation, increased Hsp70-dependent degradation of hormone-induced AR113Q oligomers in tetracycline-inducible PC12 cells, and partially rescued the DHT-dependent rough eye phenotype in UAS-AR52 flies (Wang et al., 2013). In contrast, inhibition of Hsp70 by methylene blue impaired degradation and enhanced aggregation of full-length AR112Q in HeLa cells (Wang et al., 2010).

The Hsp90/Hsp70-based chaperone machinery is part of the cellular defense against unfolded proteins (Morishima et al., 2008). When Hsp90 heterocomplex assembly is blocked by specific Hsp90 inhibitors like geldanamycin, the client proteins undergo rapid degradation through UPS, assisted by E3 ligases such as CHIP. Indeed, CHIP functions as a negative regulator of wt AR transcriptional activity by promoting AR degradation (He et al., 2004). In SH-SY5Y cells, increasing levels of CHIP effectively ubiquitinated and degraded the monomeric polyQ AR more efficiently than the wt AR, suggesting that the polyQ AR is more sensitive to CHIP than the wt AR (Adachi et al., 2007). Overexpression of CHIP in AR97Q transgenic mice also inhibited neuronal nuclear accumulation of polyQ AR and ameliorated motor symptoms (Adachi et al., 2007). However, the AR112Q protein was degraded at the same rate in CHIP-deficient and CHIP-positive mouse embryonic fibroblasts after treatment with geldanamycin, strongly suggesting that redundant E3 ligases promote polyQ AR degradation through the UPS (Morishima et al., 2008). Further analysis indicated that Hsp90 inhibition by geldanamycin in heat-shock transcription factor (*Hsf1)*-null mouse embryonic fibroblasts did not increase Hsp70 and Hsp40 levels, but still prevented AR112Q aggregation, possibly by inhibiting Hsp90-mediated retrograde trafficking (Thomas et al., 2006a).

A less toxic derivative of geldanamycin, 17-AAG, also shifts the Hsp90 complex toward its proteasome-targeting form, leading to enhanced UPS degradation of Hsp90 client proteins. 17-AAG treatment preferentially degraded the polyQ AR relative to the wt AR in both SH-SY5Y cells, and spinal cord and muscle of transgenic AR97Q mice, thus reducing monomeric and aggregated polyQ AR levels, and decreasing motor impairment in AR97Q mice (Waza et al., 2005). Similar effects were seen in cells and AR97Q mice treated with 17-(dimethylaminoetylamino)-17-demthoxygeldanamycin (17-DMAG), a more potent derivative of 17-AAG (Tokui et al., 2009). Manipulation of Hsp70 and Hsp90 activity can therefore influence poly Q AR stability and degradation.

LOSS OF NEUROTROPHIC SUPPORT

Initially, it was postulated that the polyQ AR gained functions that were harmful to motor neurons, resulting in neuronal loss and denervation-induced muscular atrophy in SBMA. However, another mechanism contributing to neuronal degeneration observed might be the loss of AR-dependent functions that support neuronal cell survival. Although each polyglutamine disease appears to specifically affect different subsets of neurons, SBMA is the only one to cause selective degeneration of motor neurons (Orr and Zoghbi, 2007). As described above, AR100Q mice developed a more severe motor neuron phenotype in the absence of the endogenous wt AR (Thomas et al., 2006b). This implies that the wt AR may play a role in supporting motor neurons and that the polyQ AR is dysfunctional with respect to protecting the neurons from degeneration (Cary and La Spada, 2008). Neurotrophins, including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and GDNF, are small peptide hormones that promote motor neuronal growth and survival (Cary and La Spada, 2008). VEGF and IGF-1 also display motor neuron-specific trophism. Muscles from SBMA patients showed decreased expression of GDNF (Yamamoto et al., 1999). Decreased expression of GDNF, IGF-1, and NT-4 were seen in the AR113Q knock-in mice (Yu et al., 2006a), while lower VEGF levels were found in the AR 100Q YAC mouse model (Sopher et al., 2004). Signaling through the AR itself could also protect specific motor neurons by inducing transcription of genes involved in promoting motor neuron survival (Cary and La Spada, 2008). It is also thought that the BDNF produced by muscle can be transported in a retrograde fashion and thus act as a trophic factor to support motor neurons (Fargo et al., 2008).

The lower motor neurons in the spinal cord and brainstem express high levels of AR, and growth of their elaborate dendritic arbors and extremely long axons is developmentally regulated by androgens (Fargo et al., 2008). In NSC34 cells stably transfected with mouse wt AR, androgens enhanced motor neuron differentiation and neurite outgrowth, likely through upregulation of neuritin (Marron et al., 2005). Motor neurons expressing the polyQ AR, however, developed neurites that were typically short and dystrophic, with abnormal branching patterns (Poletti, 1999). These results suggest that the polyQ AR may prevent the normal pattern of connectivity of motor neurons by interfering with control of their neurite outgrowth. The loss of connectivity with the target musculature could eventually result in death of the motor neurons (Fargo et al., 2008). As well, the polyQ AR may fail to upregulate neuritin, which otherwise might mitigate the detrimental effects of polyQ AR aggregates and allow the motor neurons to maintain or recover normal axonal functionality (Fargo et al., 2008).

MYOGENIC MECHANISMS

The supposition that SBMA is of neurogenic origin (i.e., originates from neuronal degeneration) has been broadened to include

the possibility that this disorder may also have a myogenic contribution. The AR113Q knock-in mice developed early androgen-dependent neuromuscular weakness, and myopathic and neurogenic skeletal muscle pathology was observed before neuronal intranuclear inclusions were seen in the spinal cord (Yu et al., 2006a). Unexpectedly, transgenic mice overexpressing the wt AR in skeletal muscle, but not spinal motor neurons (HSA-AR; **Table 1**), developed an androgen-dependent SBMA-like phenotype (Monks et al., 2007). Although prominent nuclear staining for AR in muscle fibers was observed, there was no evidence for AR-positive aggregates. The male HSA-AR mice exhibited a phenotype similar to other SBMA mouse models, with kyphosis, reduced body weight, weakness, and motor dysfunction. The skeletal muscles of the HSA-AR mice show pathological abnormalities consistent with an SBMA phenotype (myopathy), and signs of motor axon loss, but no loss in the number of motor neuron cell bodies. Myogenin and acetylcholine receptor (AChR) α-subunit mRNA levels were upregulated in HSA-AR muscles, consistent with neurogenic atrophy, while VEGF, a candidate trophic factor for motor neurons, was down-regulated. These results raised the prospect that diseases such as SBMA that have been regarded as "motor neuron diseases" result from processes that originate in muscle, and eventually cause pathology in motor neurons, possibly due to loss of muscle-derived neurotrophic factors (Monks et al., 2007, 2008; Yu et al., 2011).

HSA-AR female transgenic mice were asymptomatic, but rapidly lost motor function when exposed to male levels of testosterone (Johansen et al., 2009). Neither motor neuron nor muscle fiber losses were seen, however, motor deficits were associated with androgen-dependent changes in muscle gene expression. Furthermore, the HSA-AR mice were crossed with *tfm* mice to generate tfm/HSA-AR mice with functional AR only in skeletal muscles (Johansen et al., 2011). The tfm/HSA-AR males had *tfm*-like external genitalia, undescended atrophic testis, low levels of circulating testosterone, but no signs of an SBMA phenotype. Upon testosterone treatment, however, these mice developed a profound neuromuscular phenotype, even though they lacked AR expression in motor neurons. When testosterone treatment was stopped after 9 days, the tfm/HSA-AR males rapidly regained motor function and body weight. Overall, these experiments suggest that cellular dysfunction, rather than loss, underlie the motor deficits triggered by testosterone in the HSA-AR mice.

Investigating the AR113Q mice has also shed light on the role of the polyQ AR in the neuromuscular weakness seen in SBMA (Yu et al., 2011). The male AR113Q mice had less forelimb strength compared to their wt littermates, and morphological changes in skeletal muscle were indicative of both neurogenic and myopathic effects. As in the HSA-AR mice, the skeletal muscles of the AR113Q expressed higher levels of myogenin and AChR α-subunit compared with wt muscle, reflective of denervation. Intranuclear inclusions were detected in muscles of AR113Q males before neuronal intranuclear inclusions developed in their spinal cord. These results suggest that SBMA is initiated by myopathic effects in skeletal muscle, combined with either functional denervation or distal axonopathy that is reflective of motor neuron dysfunction, and that motor neuron loss is a late manifestation of SBMA (Yu et al., 2011).

NON-GENOMIC AR SIGNALING

Androgens can stimulate cell growth and survival through both genomic and non-genomic pathways (Foradori et al., 2008). In the classical view, most androgen action is mediated by an intracellular AR acting at the genomic level as a transcription factor. However, the wt AR is also known to interact with second messenger signaling cascades, such as the mitogen-activated protein kinase (MAPK) pathway (Cary and La Spada, 2008). Investigations into signaling pathways modulated by membrane-associated ARs were performed by inducing expression of AR20Q and AR51Q in NSC34 cells (Schindler et al., 2012). The NSC34/AR51Q cells formed neither insoluble cytoplasmic aggregates nor nuclear inclusions, however, both neurite outgrowth and cell viability were decreased compared to AR20Q-expressing cells. Both the wt AR and polyQ AR localized to the plasma membrane and migrated to lipid rafts upon testosterone treatment, but only the AR20Q activated c-jun, through the JNK pathway. Activated c-jun is involved in neurite outgrowth and cell proliferation, thus these results suggest that the impairment of non-genomic AR signaling may be involved in the development of SBMA.

In addition, an association between polyQ AR-mediated motor neuron damage and disruption of transforming growth factor-β (TGF-β) signaling due to transcriptional dysregulation of TGF-β receptor II (TβRII) has been described. The effects of TGF-β are mediated by transmembrane receptor serine/threonine kinase complex consisting of TβRI and TβRII. TGF-β binding to the complex results in phosphorylation of Smad 2 and 3 (pSmad2/3), which then translocates to the nucleus to regulate gene transcription. Nuclear translocation of pSmad2/3 was suppressed in the spinal cord motor neurons of AR97Q transgenic mice and SBMA patients (Katsuno et al., 2010a). Spinal motor neuron staining and immunoblotting also showed decreased levels of TβRII, but not TβRI, in the SBMA mice and patients. In transfected cells, two factors that regulate TβRII transcription, P/CAF and NF-Y, co-localized with truncated AR97Q in inclusion bodies, potentially causing TβRII down-regulation. As the TGF-β-Smad2/3 signaling pathway has been shown to have potent neuroprotective effects, these results suggest that polyQ-AR-mediated inhibition of TGF-β signaling would result in less effective neuroprotection.

MITOCHONDRIAL DYSFUNCTION

The AR is likely to influence mitochondrial function by regulating transcription of either mitochondrial proteins (encoded by either nuclear or mitochondrial DNA) or transcription factors activating expression of these mitochondrial proteins, or by interacting with proteins that affect mitochondrial function (Gavrilova-Jordan and Price, 2007). Direct AR-mitochondrial interactions are also possible. The wt AR has been localized to mitochondria in human sperm and LNCaP cells (Solakidi et al., 2005), and both the wt AR and, to a greater extent, the polyQ AR associated with mitochondria in cultured MN-1 cells (Ranganathan et al., 2009). The presence of the polyQ AR can alter mitochondrial distribution; cytoplasmic ARQ48 aggregates in transfected HeLa cells sequestered mitochondria, HSPs, proteasome components and steroid receptor coactivator 1 (SRC-1) (Stenoien et al., 1999), and mitochondria were found in polyQ AR aggregates in the NSC34 motor neuron cell line (Piccioni et al., 2002). Both the wt and polyQ AR interacted

with COXVb, a nuclear-encoded mitochondrial enzyme involved in oxidative phosphorylation using a mammalian two-hybrid system (Beauchemin et al., 2001). As well, COXVb co-localized to aggregates formed by the polyQ AR in androgen-treated cells, supporting the proposal that sequestration of mitochondrial proteins may lead to mitochondrial dysfunction in SBMA (Beauchemin et al., 2001).

Proliferator-activated receptor gamma coactivator 1 (PCG-1) is known to regulate mitochondrial biogenesis and function. In ligand-treated AR65Q MN-1 cells, PGC-1β mRNA levels were decreased relative to cells expressing the wt AR (Ranganathan et al., 2009). In addition, mitochondrial transcription factor A, a nuclear-encoded gene controlled by PGC-1β, the antioxidant genes superoxide dismutase 1 (SOD1), SOD2, and catalase, and the mitochondrial protein NADH dehydrogenase 1 gene were down-regulated. PGC-1β and SOD2 mRNA levels were also significantly reduced in male AR113Q knock-in mice. Ligand-dependent increases in mitochondrial membrane depolarization, elevated reactive oxygen species (ROS) levels, activation of the mitochondrial caspase pathway, and increased cell death were observed in cells expressing the polyQ AR (Ranganathan et al., 2009). Thus the polyQ AR likely contributes to mitochondrial dysfunction in SBMA directly though abnormal associations with mitochondria or mitochondrial proteins, or indirectly through ligand-dependent alterations in mitochondrial gene expression, turnover, and respiratory function (Beauchemin et al., 2001; Ranganathan et al., 2009). In addition, it has been proposed that mitochondrial DNA damage may be involved in pathogenesis of SBMA or serve as a useful biomarker to monitor disease progression (Su et al., 2010).

IMPAIRED AXONAL TRANSPORT

Axonal transport moves proteins and organelles between the neuronal cell body and the axon tip (and vice versa), and is essential for the growth and survival of neurons. Abnormalities in axonal transport have been implicated in neurodegenerative diseases (Morfini et al., 2009; Sau et al., 2011; Ikenaka et al., 2012). Early studies in *in vitro* and cell models suggested that the polyQ AR could hinder axonal transport (Piccioni et al., 2002; Szebenyi et al., 2003). It was shown in SH-SY5Y cells and squid axoplasm that the polyQ AR inhibited fast axonal transport through a pathway that involved JNK activation, phosphorylation of kinesin-1 heavy chain subunits by JNK, and inhibition of kinesin-1 function (Morfini et al., 2006). Transgenic AR97Q mice were found to have impaired retrograde axonal transport, even before the onset of muscle weakness, with accumulation of neurofilaments and synaptophysin in the distal motor axon (Katsuno et al., 2006). In addition, expression levels of dynatin 1, an axon motor for retrograde transport, were reduced in these mice.

In another study, deficits in retrograde labeling of spinal motor neurons were seen in both the AR113Q knock-in and HSA-AR myogenic mouse models of SBMA (Kemp et al., 2011). Live imaging of endosomal trafficking in sciatic nerve axons also showed disease-induced defects in the flux and run length of endosomes movement toward the cell body. However, when axonal transport rates were analyzed in cultured primary motor neurons or in sciatic nerves of AR100 mice, no significant axonal transport deficits were observed, implying there was no correlation between impairment of axonal transport and SBMA pathogenesis (Malik et al., 2011). Further investigation into the discrepancies between different models with respect to polyQ AR-mediated effects on axonal transport is warranted.

INSIGHTS FROM *DROSOPHILA* MODELS

The use of genetic models such as *Drosophila melanogaster* has greatly facilitated the study of the underlying molecular mechanisms of SBMA. The development of "humanized" AR transgenic *Drosophila* lines has allowed for the ectopic/tissue-specific expression of the polyQ AR, leading to observable, potentially disease-related phenotypes. Specifically, the wt and polyQ AR genes are cloned into *UAS* expression constructs. Crosses to tissue-specific *GAL4* transgenic fly lines drives the expression of AR in those selected tissues. Although the phenotypes described using such genetic systems are not directly linked to the clinical manifestations of SBMA, the observed phenomena are polyQ tract length-dependent and DHT sensitive. A number of studies have confirmed the working hypothesis of androgen-dependent cellular toxicity of the polyQ AR and have noted the presence of protein aggregates in photoreceptor neurons (Chan et al., 2002; Takeyama et al., 2002; Funderburk et al., 2009; Palazzolo et al., 2010). Interestingly, DHT-dependent reductions in locomotion in larvae expressing the polyQ AR have been described (Funderburk et al., 2009; Nedelsky et al., 2010; Jochum et al., 2012). The role of autophagy and the UPS in a *Drosophila* model of SBMA have also been investigated (Pandey et al., 2007a,b). However, these studies did not address the molecular mechanisms contributing to the observable phenotype or effects on other polyQ AR functions, specifically gene transactivation.

This *Drosophila* genetic system, however, allows for a high-throughput screen of large number of gene sets, and was utilized to screen for genetic interactors that either suppressed or enhanced the polyQ AR/androgen-dependent phenotype (Murata et al., 2008; Suzuki et al., 2009; Nedelsky et al., 2010). Murata et al. (2008) used mutant enhancer trap lines to screen ∼2,000 genes that when co-driven with the polyQ AR would modulate a rough eye phenotype. An RNA-binding protein, *hoip*, was identified that enhanced the polyQ AR-induced eye phenotype (Murata et al., 2008). The homologous gene in yeast has been reported to be involved in rRNA processing (Reichow et al., 2007). By further dissecting the *hoip* protein complex, two more genetic modulators of the polyQ AR phenotype, *nop5* and *nop56* were found. As *nop5* and *nop56* are part of the small nucleolar ribonucleoprotein complex, these results suggest translational regulation may play a role in the neurodegeneration observed in SBMA. Retinoblastoma family protein (*Rbf*), the *Drosophila* homolog of human retinoblastoma protein (Rb), was found to be a neuroprotective factor (Suzuki et al., 2009). Rb is known to function through repressing transcription of genes regulated by the E2F proteins. The androgen-bound polyQ AR, but not the wt AR, appeared to impair transrepressive function of Rb, resulting in aberrant stimulation of E2F-mediated transactivation.

Specific RNAi lines for candidate genes selected from known AR coregulators described in the AR mutations database [http://androgendb.mcgill.ca/; (Gottlieb et al., 2012)] were crossed

Table 2 | PolyQ AR loss- and gain-of-function mechanisms in SBMA.

Mechanism contributing to SBMA	PolyQ AR *versus* wt AR	
	Loss-of-function	Gain-of-function
Alterations in AR structure		✓
Altered protein interactions	✓	✓
Aggregation		✓
Formation of soluble oligomers		✓
Change in post-translational modifications	✓	✓
Transcriptional dysregulation	✓	✓
Altered RNA splicing	?	?
Ubiquitin proteasome system impairment		✓
Induction of autophagy		✓
Loss of neurotrophic support	✓	
Myogenic contributions	✓	✓
Non-genomic AR signaling	✓	✓
Mitochondrial dysfunction	✓	✓
Impaired axonal transport		✓

with AR52Q flies to screen for modifiers of the fly SBMA phenotype. Using this approach, 19 genetic interactors were identified (Nedelsky et al., 2010). Furthermore, microarray analysis was performed on these SBMA flies to identify putative target genes or androgen-regulated genes to account for the observed rough eye phenotype. While a number of differentially expressed genes were observed, neither corresponding AREs nor presumed ecdysone response elements (the sole *Drosophila* steroid hormone receptor) could be identified. Although an interaction of the polyQ AR with a number of genes was proposed, there are a number of post-transcriptional and translation regulatory mechanisms that can alter gene expression profiles. Nevertheless, the approaches taken to carry out high-throughput screens in *Drosophila* are resulting in novel observations of polyQ AR functions that may contribute to SBMA.

THERAPY FOR SBMA

Given that SBMA is a ligand-dependent polyglutamine disease, clinical trials to reduce testosterone or DHT levels in men have been attempted, but showed only limited improvements in certain disease features (Ranganathan and Fischbeck, 2010; Banno et al., 2012). Nonetheless, investigations into the mechanisms underlying the pathogenesis of SBMA have provided indications for other potential therapies. These include: (1) reducing poly Q AR levels by increasing degradation through Hsp-mediated, UPS or autophagic pathways; (2) decreasing formation of aggregates and/or soluble oligomers, or altering their structure (Jochum et al., 2012); (3) normalizing transcriptional dysfunction; (4) altering abnormal polyQ AR post-translational modifications; (5) decreasing polyQ AR mRNA expression using siRNA or miRNAs (Miyazaki et al., 2012); (6) regulating polyQ AR N/C interactions and nuclear localization; (7) modulating AR-coactivator interactions; (8) using antioxidants to reduce ROS levels; (9) inducing IGF-1/Akt signaling; and (10) treatment with serotonin receptor agonists (Minamiyama et al., 2012). Due to the variety of molecular mechanisms involved in the development of SBMA, a combination of therapies may be needed to reduce symptoms and slow disease progression in men. More details on therapeutic approaches to SBMA may be found in several comprehensive reviews (Ranganathan and Fischbeck, 2010; Banno et al., 2012; Fischbeck, 2012; Katsuno et al., 2012; Tanaka et al., 2012; Rocchi and Pennuto, 2013).

CONCLUSION

It is known that androgens can be neurotrophic and are important in muscle development. In retrospect, it is perhaps not surprising that there are both neurodegenerative and neuromuscular components in SBMA, which is caused by polyglutamine tract expansion in the receptor that mediates the actions of androgens. The increase in the length of the polyglutamine tract in the AR is somewhat analogous to the insertion of a new domain through alternative splicing, which generates a novel protein. The polyQ AR retains many of the function of the wt AR, however, it fails to perform as well as the wt AR in certain roles, and gains novel, often deleterious, properties (**Figure 2; Table 2**). Consequently, polyQ AR-mediated loss- and gain-of-function mechanisms can disturb cellular homeostasis, leading to neuronal and muscular dysfunction. Multiple complex and overlapping pathogenic processes have been shown to contribute to the initiation and development of SBMA. The relative contribution of each mechanistic pathway, and thus the potential for therapeutic intervention, will be the subject for future investigations in this field.

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Western Pacific ALS-PDC: a prototypical neurodegenerative disorder linked to DNA damage and aberrant proteogenesis?

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A commentary on

Unraveling 50-year-old clues linking neurodegeneration and cancer to cycad toxins: are microRNAs common mediators?
by Spencer, P., Fry, R. C., and Kisby, G. E. (2012). Front. Gene. 3:192. doi: 10.3389/fgene.2012.00192

The Western Pacific amyotrophic lateral sclerosis and parkinsonism-dementia complex (ALS-PDC) has been described as a Rosetta Stone that bears the essential clue to understanding the etiopathogenesis of related neurodegenerative diseases. The three clinical forms (ALS, atypical parkinsonism with dementia, and dementia alone) have a single pathology (polyproteinopathy, notably tauopathy), just as the Rosetta Stone is inscribed with three distinct scripts bearing a common message. As recently discussed (Kisby and Spencer, 2011), studies of ALS-PDC in the three geographically separate and genetically distinct island populations (Chamorros on Guam; Japanese in Honshu Island's Kii Peninsula; and Papuan New Guineans in Irian Jaya, Indonesia) show:

- ALS-PDC is primarily if not exclusively an environmental disease: no gene mutations identified in related neurodegenerative disorders are found in Guam and Kii-Japan cases, and disease rates have steadily declined in the three affected populations. Emigrants from Guam may develop ALS-PDC years or decades later, but disease risk is absent in their offspring who were born and live abroad. Conversely, Filipino and other immigrants who adopt the Chamorro lifestyle on Guam may acquire the disease.
- As ALS-PDC declined during the twentieth century, the disease changed its clinical face from ALS in the first third of life, to PD in the second, and D in the third, a pattern consistent with a dose-related response to an environmental exposure that waned with modernization. With this hypothesis, those with the highest dose of the putative environmental factor develop fatal ALS (with sub-clinical nigrostriatal damage) relatively shortly after exposure; those with intermediate doses survive with amyotrophy long enough to develop atypical parkinsonism; those with low doses reach old age and display dementia, while others with the lowest exposure have subclinical neurofibrillary disease reminiscent of early aging. Other features of ALS-PDC variably include loss of olfaction, retinal pigment epitheliopathy, and atypical skin cytology.
- The most plausible but unproven trigger decades before the disease surfaces in clinical form is exposure to certain plant-derived neurotoxins in food or medicine, or both. The raw seed of the neurotoxic cycad plant (*Cycas* spp.) was used to heal skin lesions (Guam, Irian Jaya) and as a tonic (Kii). On Guam, processed cycad seed was a Chamorro staple, and the cycad seed-eating flying fox (*Pteropus* sp.) that bioaccumulates the cycad-derived neurotoxin β-*N*-methylamino-L-alanine (L-BMAA) was a delicacy.
- Among the many bioactive chemicals in cycad seed, two with neurotoxic properties are singled out as potential triggers of ALS-PDC: (a) methylazoxymethanol (MAM), a potent genotoxin, carcinogen and developmental neurotoxin that is stored in the plant as an inactive β-glucoside, the concentration of which in cycad flour correlates significantly with incidence rates for ALS and PD in males and females on Guam, unlike the concentration of (b) L-BMAA, a weak excitotoxic amino acid that is taken up by brain tissue and possibly undergoes proteogenesis, resulting in misfolded proteins; daily oral dosing of macaques with L-BMAA for up to 3 months induces a L-dopa-responsive, non-progressive motorsystem disorder with non-excitotoxic cortical and spinal motor neuron pathology. Both L-BMAA and MAM are metabolized to formaldehyde, an established genotoxic agent and human carcinogen.

Whereas, genotoxin-induced DNA damage is rapidly repaired in non-nervous tissue (i.e., cycling cells), this can persist in the brain because some DNA-repair mechanisms are weakly expressed in post-mitotic cells. MAM-induces O^6-methylguanine (O^6-mG) DNA damage, promutagenic lesions that induce uncontrolled mitoses (tumorigenesis) in mouse epithelial tissues and widespread degeneration in the developing murine brain. O^6-mG lesions are clearly responsible for the MAM-induced neuronal loss because the pathology is greater in mice

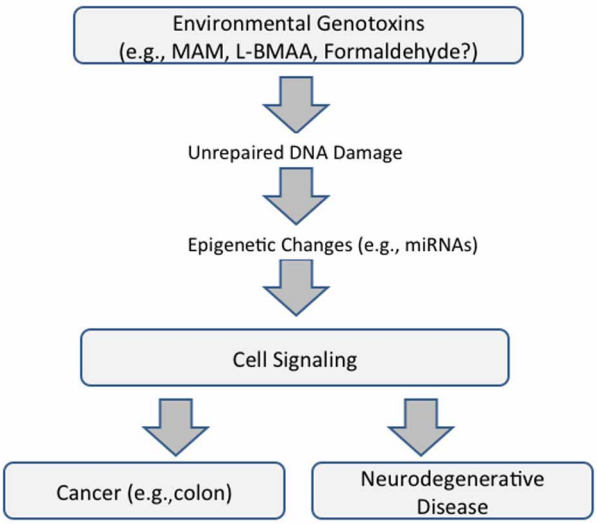

FIGURE 1 | Proposed common pathway underlying cancer and neurodegenerative disease that is mediated by DNA damage and epigenetic changes (modified from Spencer et al., 2012).

that lack the DNA-repair enzyme O^6-mG methyltransferase (*Mgmt*$^{-/-}$) and reduced or absent in mice that overexpress MGMT (Kisby and Spencer, 2011). Motor deficits in these mouse mutants are consistent with the extent of DNA damage. In sum, these findings suggest DNA damage is an initial event that leads to brain pathology (**Figure 1**).

MGMT activity is very low in the young adult human brain and may be absent in mature nerve cells. *Mgmt*$^{-/-}$ mice, an animal model of the young adult human brain, develop persistent brain O^6-mG DNA lesions following a single dose of MAM. In the days following MAM treatment, these DNA lesions modulate key brain cell-signaling pathways that are also perturbed in human neurological disease, notably Alzheimer's disease, Parkinson's disease, and inherited and sporadic forms of ALS. Pathway analysis of MAM-modulated genes that are anchored to DNA damage reveals links with human cancer, genetic disorders, and skin and hair development (Kisby et al., 2011a). Several of these cell-signaling pathways continued to be modulated in the brain of *Mgmt*$^{-/-}$ mice 6 months later, with *de novo* expression changes of numerous genes involving olfaction (Kisby et al., 2011b).

These findings emphasize the relationship between acquired brain tissue DNA damage, modulation of cell-signaling pathways, and the induction of early and persistent molecular changes that lead to neuronal demise. They also reveal important relationships between seemingly disparate diseases—cancer and neurodegeneration—the phenotype of MAM-induced DNA damage being determined, respectively, by the presence or absence of the proliferative capacity of target tissues. The genotoxic properties of MAM and formaldehyde, a common metabolite of MAM and L-BMAA, also involve non-coding RNAs with functional roles in both neurodegeneration and cancer (Spencer et al., 2012) (**Figure 1**).

While proof is lacking that cycad toxin(s) trigger ALS-PDC, the results of recent biochemical and systems biology studies, coupled with the absence of known mutations in related neurodegenerative disorders, encourage further efforts to examine the molecular and cellular actions of MAM and L-BMAA. These studies not only highlight the response of the brain to unrepaired DNA damage-induced by a genotoxin (e.g., alkylating agent) as a potential initiator of a neurodegenerative process, they also provide a foundation for understanding whether such effects can lead to persistent changes at the protein level (e.g., tau and synuclein), including the erroneous incorporation of foreign amino acids, a subject of recent interest. The ability of MAM to perturb synuclein and several other classes of brain proteins (e.g., calcium homeostasis, mitochondrial and RNA processing) is consistent with this hypothesis (Kisby et al., 2006).

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Biophysical insights into how surfaces, including lipid membranes, modulate protein aggregation related to neurodegeneration

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There are a vast number of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), associated with the rearrangement of specific proteins to non-native conformations that promotes aggregation and deposition within tissues and/or cellular compartments. These diseases are commonly classified as protein-misfolding or amyloid diseases. The interaction of these proteins with liquid/surface interfaces is a fundamental phenomenon with potential implications for protein-misfolding diseases. Kinetic and thermodynamic studies indicate that significant conformational changes can be induced in proteins encountering surfaces, which can play a critical role in nucleating aggregate formation or stabilizing specific aggregation states. Surfaces of particular interest in neurodegenerative diseases are cellular and subcellular membranes that are predominately comprised of lipid components. The two-dimensional liquid environments provided by lipid bilayers can profoundly alter protein structure and dynamics by both specific and non-specific interactions. Importantly for misfolding diseases, these bilayer properties can not only modulate protein conformation, but also exert influence on aggregation state. A detailed understanding of the influence of (sub)cellular surfaces in driving protein aggregation and/or stabilizing specific aggregate forms could provide new insights into toxic mechanisms associated with these diseases. Here, we review the influence of surfaces in driving and stabilizing protein aggregation with a specific emphasis on lipid membranes.

Keywords: amyloid disease, lipid membranes, protein aggregation, Alzheimer's disease, Huntington's disease, Parkinson's disease, prion disease

INTRODUCTION

A common motif of several neurodegenerative diseases is the ordered aggregation of specific proteins, leading to their deposition in tissues or cellular compartments (Chiti and Dobson, 2006). Often referred to as protein conformational or misfolding disorders, such diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyloidoses, α1-antitrypsin deficiency, and the prion encephalopathies to name a few. The common structural motif of protein aggregates associated with these diseases is the formation of extended, β-sheet rich fibrils, referred to as amyloid. Despite no apparent correlation between aggregating proteins in size or primary amino acid sequence, the characteristic lesions of each disease typically contain fibrillar structures with common biochemical characteristics (Dobson, 2003; Chiti and Dobson, 2006), indicating the potential for a conserved mechanism of pathogenesis linking these phenotypically diverse diseases. The earliest potential event in the disease process may be the conversion of a protein to a critical abnormal conformation, resulting in toxic gain of function for the monomer, and/or the formation of toxic nanoscale aggregates (**Figure 1**; Naeem and Fazili, 2011). The elusive toxic species, whether monomeric or higher-order, may subsequently initiate a cascade of pathogenic protein–protein interactions that culminate in neuronal dysfunction. The precise timing of such interactions and the mechanisms by which altered protein conformations or aggregates trigger neuronal dysfunction are unclear.

The formation of fibrils often proceeds via a heterogeneous mixture of intermediate aggregate structures, including a variety of protofibrils and oligomers (**Figure 1**). Amyloid formation typically occurs via a nucleation-growth mechanisms that features an initial lag-phase due to a thermodynamically unfavorable nucleation event (Lomakin et al., 1996; Murphy, 2002; Chiti and Dobson, 2006). Once nucleation occurs, aggregation proceeds via an exponential growth phase associated with the addition of monomers into aggregate forms. The initial lag-phase can be circumvented by the presence of pre-existing aggregates that can act as seeds for amyloid formation (Lansbury, 1997; Hu et al., 2009; Langer et al., 2011; Hamaguchi et al., 2012). To further complicate the issue, several aggregates have been identified that may be off-pathway to fibril formation, such as annular structures (Wetzel, 1994; Wacker et al., 2004). While there are often specific mutations or dysfunctional processing that can be directly linked to aggregation, the

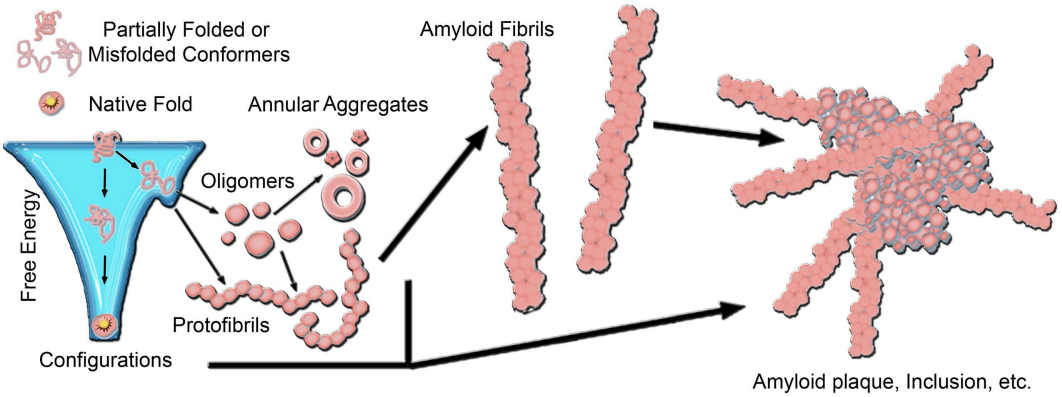

FIGURE 1 | A generic aggregation scheme for amyloid-forming proteins. Proteins fold into their native structure, which is typically a low free energy configuration. However, the energy landscape for protein folding often can have localized minima in which a protein can become trapped into a misfolded conformation, which can lead to aggregation into β-sheet rich amyloid fibrils. The formation of fibrils often proceeds through a heterogeneous mixture of intermediate species, including oligmers and protofibrils. Off-pathway aggregates can also form, such as annular aggregates. These aggregates accumulate into amyloid plaques or inclusions in the diseased brain. The aggregation pathway for any given amyloid-forming protein can vary considerably depending on the protein and its folding environment.

nature, and location of protein aggregates *in vivo* depends on the specific protein associated with disease. The specific protein involved also influences the specific form of the critical aggregation nucleus. For example, synthetic polyglutamine (polyQ) peptides are thought to have a monomeric critical nucleus (Chen et al., 2002a,b; Wetzel, 2012); however the addition of flanking sequences associated with the first exon of the huntingtin (htt) protein can change the size of the critical nucleus to a tetramer (Jayaraman et al., 2012; Wetzel, 2012). This can be further modulated by the addition of β-hairpin motifs within the polyQ domain (Kar et al., 2013). The extent of the lag-phase, and subsequent aggregation of polyQ peptides and htt proteins is dependent on the size of the polyQ domain (Legleiter et al., 2010; Kar et al., 2011). As protein aggregation often progresses from misfolded monomers to oligomeric precursors and finally mature fibrils, intensive research activity has been devoted to determining the most toxically relevant aggregate species in many of these diseases. This is particularly important, as for the vast majority of these diseases, there are no widely effective preventative measures or therapeutic treatments.

Fibril structures associated with several different amyloid-forming proteins have been experimentally resolved, and a common motif of fibrillar aggregates is a cross-β structure (Eanes and Glenner, 1968; Glenner et al., 1971; Kirschner et al., 1986; Sunde et al., 1997; Berriman et al., 2003; Tycko and Ishii, 2003; Tycko, 2004, 2006; Nelson et al., 2005; Fandrich, 2007). While the structural spine of fibrils share this common intermolecular β-sheet structure, a variety of possibilities are available for the packing of protofilaments into the fibril structure, even for the same protein/peptide. This variability can lead to distinct amyloid fibril morphologies. Such variable protofilament arrangements give rise to distinct fibril morphologies, often termed polymorphisms (Kodali and Wetzel, 2007). For example, Aβ has been shown to form a variety of fibril structures *in vitro* dependent on the peptide preparation and aggregation conditions (Kodali et al., 2010). Furthermore, fibril polymorphs have been observed for several other amyloid-forming proteins, such as calcitonin (Bauer et al., 1995), amylin (Goldsbury et al., 1997), glucagon (Pedersen et al., 2006), the SH3 domain of phosphatidylinositol-3′-kinase (Jimenez et al., 1999; Chamberlain et al., 2000; Pedersen et al., 2006), insulin (Bouchard et al., 2000; Jimenez et al., 2002; Dzwolak et al., 2004), and lysozyme (Chamberlain et al., 2000). Polymorphic fibrils can differ in the cross-sectional thickness or helical pitch of the fibril, which can be observed via high resolution imaging techniques like transmission electron microscopy (TEM) and atomic force microscopy (AFM) or distinguished with spectroscopic techniques like circular dichroism (CD; Petkova et al., 2005; Kurouski et al., 2010, 2012; Mossuto et al., 2010; Norlin et al., 2012). While polymorphs are often observed for various *in vitro* aggregation reactions, polymorphs have been observed in amyloid fibrils extracted from tissue as well (Crowther and Goedert, 2000; Jimenez et al., 2001), affirming that *in vivo* aggregation can be heterogeneous and complex. Furthermore, it has been proposed that polymorphic fibrils may result in distinct biological activities and variable toxicity related to the different aggregate structures (Seilheimer et al., 1997; Petkova et al., 2005). These distinct fibril morphologies may also have distinct aggregate intermediates associated with their formation, adding to the heterogeneity of potential protein aggregates and further complicating efforts aimed at elucidating the relative role of discrete aggregates in disease-related toxicity.

While protein preparation and environment influence the structural polymorphs of protein aggregates *in vitro*, determining what environmental factors influence aggregation *in vivo* remains difficult. However, the interaction of proteins at solid interfaces, including cellular membranes comprised of lipid bilayers, may prove to be a fundamental phenomenon with potential implications for protein-misfolding diseases. Solid surfaces, such as mica, graphite, gold, and Teflon, have been shown to heavily influence aggregation kinetics and the resulting aggregate morphology for a variety of amyloid-forming proteins (Goldsbury et al., 1999;

Hoyer et al., 2004; Morriss-Andrews and Shea, 2012). A variety of kinetic and thermodynamic studies point to significant conformational changes being induced in proteins encountering surfaces (Gray, 2004). These surface induced conformational changes in proteins could play a critical role in nucleating amyloid formation or altering aggregate morphology to specific toxic species. Such phenomenon are well demonstrated by a study of immunoglobulin light-chain aggregation on mica (Zhu et al., 2002). Small pieces of mica were incubated in solutions containing a recombinant amyloidogenic light-chain variable domain of smooth muscle actin (SMA) antibody, under conditions in which fibrils normally do not form (i.e., low concentration and no agitation). At short times, amorphous aggregates appeared on mica, and fibrils were observed within 10 h and fibrils were not formed in the solution within the same time frame. The fibrils on the surface of mica grew from the amorphous aggregates and the assemblies of oligomers present on mica. The use of such solid surfaces as model systems provides the opportunity to elucidate how specific surface environment influence protein aggregation.

In regards to disease-related protein aggregation, surfaces of more physiological relevance are cellular and subcellular membranes that are predominately comprised of lipid bilayers. Like solid surfaces, the presence of lipid membranes can alter the aggregation of disease-related proteins by increasing aggregation rates, nucleating aggregation, promoting specific polymorphs, or even stabilizing potentially toxic, transient aggregate intermediates. A significant question remains regarding why amyloid fibrils form *in vivo* at concentrations that are orders of magnitude lower (Seubert et al., 1992) than the critical nucleation concentrations required *in vitro* (Lomakin et al., 1996; Sabate and Estelrich, 2005). A possible answer is the ability to create local concentrations of protein adsorbed onto molecular surfaces, such as cellular and subcellular membranes (Kim et al., 2006; Aisenbrey et al., 2008). Lipid interaction appears to be a common modulator in fibril formation, as studies of α-synuclein (α-syn; Jo et al., 2000, 2004; Necula et al., 2003), islet amyloid polypeptide (IAPP; Knight and Miranker, 2004), and β-amyloid (Aβ; McLaurin and Chakrabartty, 1996, 1997; Choo-Smith et al., 1997; Yip and McLaurin, 2001; Yip et al., 2002) all demonstrate accelerated fibril formation in a membrane environment in comparison to bulk solution. General physicochemical properties of lipid membrane, including phase state, bilayer curvature, elasticity, and modulus, surface charge, and degree of hydration, modulate protein aggregation (Gorbenko and Kinnunen, 2006). The exact chemical composition and lipid constituents of a lipid bilayer can also influence the aggregation process (Evangelisti et al., 2012). Potentially important chemical properties of membrane components include the extent of acyl chain unsaturation, conformation and dynamics of lipid headgroups and acyl chains, and protein–lipid selectivity arising from factors such as the hydrophobic matching at the protein–lipid interface (Jensen and Mouritsen, 2004). Although lipid bilayers may act catalytically to induce aggregation by providing environments that promote protein conformation and orientation conducive to fibril assembly (Thirumalai et al., 2003; Sparr et al., 2004; Zhao et al., 2004), cell membranes may also be targeted by protein aggregates to induce physical changes in the membrane, leading to dysfunction and cell death. This may be due to the ability of amyloid-forming peptides to induce membrane permeabilization by altering bilayer structure via the sequestration of membrane components into fibrils (Michikawa et al., 2001; Lins et al., 2002; Sparr et al., 2004; Zhao et al., 2004; Valincius et al., 2008) or by forming unregulated pore-like structures (Jang et al., 2007). A variety of amyloid-forming proteins, including Aβ, IAPP, and htt, have been show to locally change the rigidity of model lipid bilayers in a generic manner (Burke et al., 2013). Furthermore, the presence of lipid membranes can also influence the ability of small molecules to prevent or destabilize protein aggregates, having a major impact on several therapeutic strategies. Such a scenario has been demonstrated experimentally as (-)-epigallocatechin gallate (EGCG), which has been shown to inhibit the aggregation of several amyloid-forming proteins in the absence of surfaces (Bieschke et al., 2010; Popovych et al., 2012), was less effective at inhibiting aggregation of human IAPP at a phospholipid interface (Engel et al., 2012).

Here, we review the influence of surfaces in driving and stabilizing protein aggregation with a specific emphasis on lipid membranes. We will initially focus on Aβ as an illustrative example, and then quickly review some interesting features of the interaction of other select amyloid-forming proteins with surface interfaces.

THE AGGREGATION OF Aβ ON SOLID SURFACES

The ordered aggregation of Aβ into neuritic plaques is one of the major hallmarks of AD. Aβ is a secreted peptide derived from the endoproteolysis of the amyloid precursor protein (APP), a receptor-like transmembrane protein, and is ubiquitously expressed in neural and non-neural cells. Successive cleavage of APP by β-secretase and γ-secretase results in the release of an intact Aβ peptide. Aβ contains a portion of APP's transmembrane domain, as well as an extracellular portion, resulting in an amphiphilic peptide ∼39–43 residues in length. The Aβ component of amyloid plaques found in the diseased brain consist primarily of two versions of the peptide, which are 40 and 42 amino acids long [Aβ(1–40) and Aβ(1–42) respectively]. Aβ(1–42) aggregates more quickly than Aβ(1–40) and is thought to play a major role in AD (Jarrett and Lansbury, 1993). The amphiphilic nature of Aβ is thought to drive its aggregation and may play an important role in its interaction with solid surfaces and ability to insert and/or penetrate lipid membranes (Lansbury and Lashuel, 2006; Williams and Serpell, 2011). The extra addition of two hydrophobic residues in Aβ(1–42) may also lead to variations in the interaction of this peptide with surfaces compared to Aβ(1–40).

Hydrophobic Teflon surfaces can be considered mimics of the non-polar plane of lipid membranes. While both Teflon and Aβ carry a negative charge at physiological pH, protein dehydration effects lead to substantial adsorption of Aβ at pH 7(Giacomelli and Norde, 2003). Aβ(1–40) and Aβ(1–42) adsorption to Teflon particles increased aggregation and fibrillogenesis (Linse et al., 2007). Adsorption of Aβ to another hydrophobic surface, highly ordered pyrolytic graphite, results in extended aggregate formation in a nucleation dependent manner (Kowalewski and Holtzman, 1999). Using a variety of surfaces with tunable hydrophobicity or hydrophilicity (as well as supported lipid bilayers), weakly adsorbed peptides with two-dimensional diffusivity were found

to be critical precursors to surface growth of Aβ(1–42) fibrils (Shen et al., 2012). As the adsorption of Aβ on highly hydrophilic surfaces was negligible, fibril growth was inhibited on such surfaces. On highly hydrophobic surfaces, the two-dimensional diffusion of Aβ along the surface was too low, also inhibiting fibril formation. It appears that surface properties that promote weak adsorption of Aβ to the surface and maintain translational mobility result in local concentrations of Aβ due to confinement within the plane of the surface, allowing for fibril formation at a concentration far below the critical concentration observed in bulk solutions. The adsorption of Aβ to hydrophilic silica surfaces is pH dependent, occurring at pH 4 and 7 when Aβ has an overall positive charge (Giacomelli and Norde, 2005), suggesting a vital role of electrostatics on Aβ's adsorption to surfaces.

Due to the ability of AFM to be operated in solution and track the formation and fate of individual aggregates with time on surfaces (Goldsbury et al., 1999), the impact of surface chemistry on the morphology of Aβ aggregates has been extensively studied with this technique. On mica, a hydrophilic surface, Aβ(1–40) (Blackley et al., 2000) and Aβ(1–42) (Kowalewski and Holtzman, 1999) form small, highly mobile oligomeric aggregates that organize into extended pre-fibrillar aggregates that continually elongate with time (**Figure 2A**). These aggregate structures are similar in morphology to those formed in bulk solution from similarly prepped Aβ stocks (Kowalewski and Holtzman, 1999;

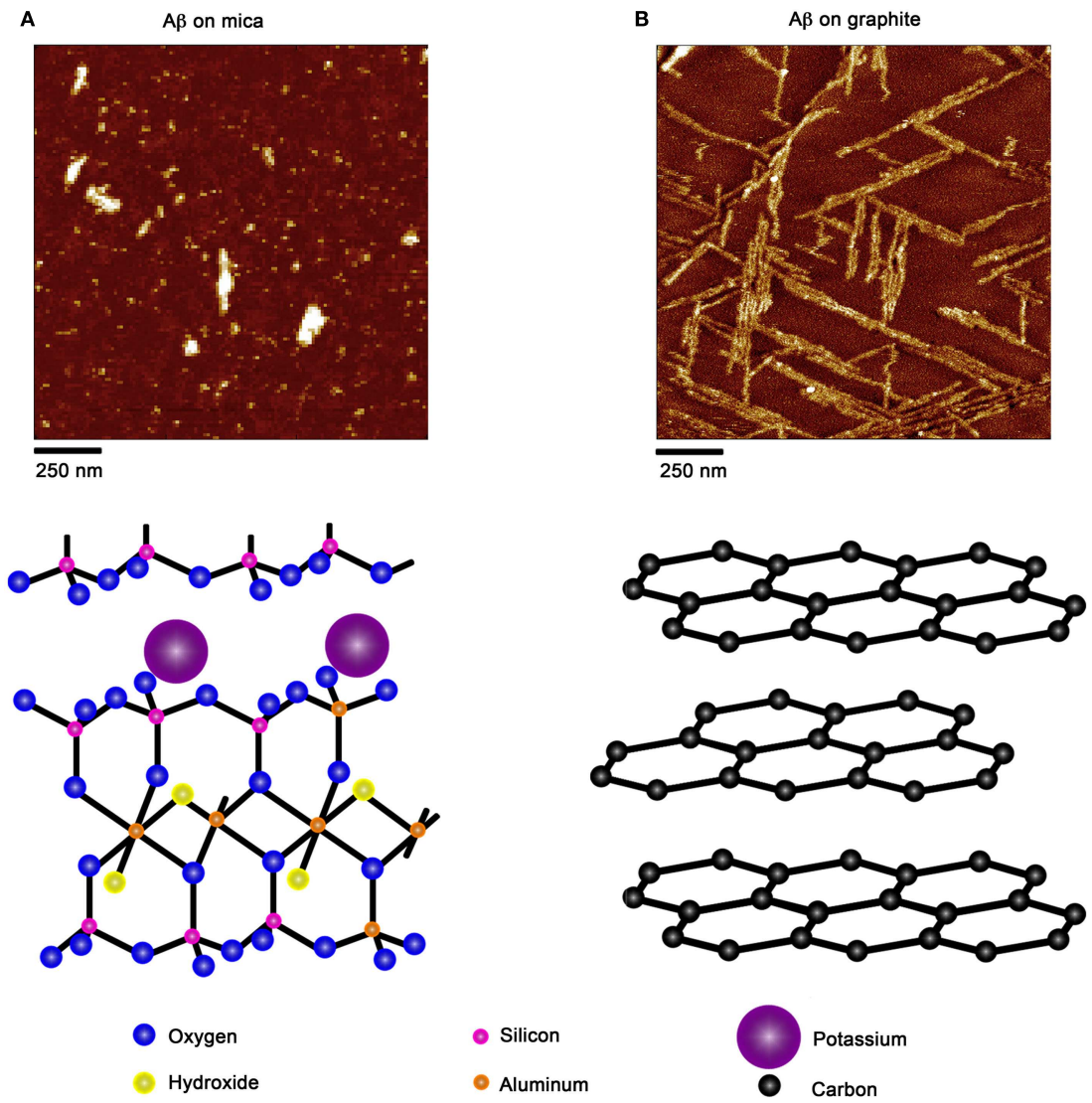

FIGURE 2 | Aβ aggregation is modulated by the presence of chemically distinct solid surfaces. (A) On highly ordered pyrolytic graphite, Aβ aggregates into extended nanoribbons that are epitaxially ordered on the surface. The distinct orientation of Aβ aggregates on graphite is attributed to the optimization of the contact between the peptide and underlying hydrophobic carbon lattice. **(B)** On a negatively charged, hydrophilic mica surface, Aβ forms discrete oligomers that maintained some lateral mobility along the plane of the surface. These oligomers could organize into elongated protofibrillar structures. Schematic representations of the structure of each surface (graphite and mica) are provided under each image.

Legleiter and Kowalewski, 2004). However, Aβ(1–42) aggregates into morphologically distinct structures on a graphite surface (**Figure 2B**), forming extended nanoribbons with heights of ∼1–1.2 nm and widths of ∼18 nm (Kowalewski and Holtzman, 1999). These dimensions suggest that Aβ adopts a fully extended β-sheet conformation perpendicular to the long axis of the nanoribbons. These nanoribbons elongated with time, organize themselves into parallel, raft-like structures with a preferential alignment along the graphite lattice. Aβ adsorbs to and aggregates on surfaces functionalized with methyl, carboxyl, or amine groups; however, aggregate morphology and surface affinity is dependent on the specific surface chemistries (Moores et al., 2011). Hydrophobic surfaces promote formation of spherical amorphous clusters; charged surfaces promote the formation of protofibrils (Moores et al., 2011). Studies of the aggregation of Aβ peptides containing single point mutations on mica further support the notion that electrostatics play an important role in Aβ adsorption and aggregation on surfaces (Yates et al., 2011). These mutations are clustered around the central hydrophobic core of Aβ (E22G Arctic mutation, E22K Italian mutation, D23N Iowa mutation, and A21G Flemish mutation) and are associated with familial forms of AD. In bulk solution and under identical preparatory conditions, these Aβ mutants form aggregated species that were morphologically similar to those of Wild Type Aβ; however, on a mica surface the aggregates differ in morphology (**Figure 3**). While Wild Type Aβ forms oligomers and putative protofibrils on mica similar to other previously described studies, Arctic Aβ aggregate into extended, fibrillar aggregates on mica that orient on the surface similar to the previously described Wild Type Aβ aggregates on graphite. However, the dimensions of the Arctic Aβ aggregates on mica indicate they most likely contain a β-turn as opposed to the fully elongated Wild Type Aβ nanoribbons on graphite. Italian Aβ, which replaces a negatively charged residue with a positive one, adsorbs quickly to mica and predominantly forms oligomeric aggregates reminiscent of those formed by wild type Aβ on mica. However, there was a small percentage of Italian Aβ aggregates similar in morphology to those formed by Arctic Aβ on mica.

THE INTERACTION OF Aβ WITH LIPID SURFACES

While studies on model surfaces can provide mechanistic detail on how solid interfaces alter and/or promote Aβ aggregation, ultimately, pathological protein aggregation occurs in a cellular environment, dictating the need to study protein-misfolding and aggregation on more physiologically relevant surfaces. This is not to say that studies on solid surfaces are irrelevant. For example, the aforementioned dependence on lateral mobility of Aβ on a surface being critical in fibril formation was directly extended to lipid surfaces as well (Shen et al., 2012). This phenomenon could be important in light of single molecule studies demonstrating that Aβ inserted into anionic lipid membranes demonstrate high lateral mobility until aggregating into oligomers (King et al., 2012).

It has been hypothesized that a potential pathway for Aβ toxicity may lie in its ability to modulate lipid membrane function. This hypothesis is based on the observation that Aβ bears a portion of the APP transmembrane domain. Thus, elucidating the interaction between Aβ and membrane lipids could be critical in understanding potential pathways of Aβ toxicity, especially given the results of studies that demonstrate that changes in membrane composition occur in AD along with the association with plaques, tangles, and neuritic dystrophy. Importantly, it has often been observed that exogenously added Aβ will selectively bind a subset of cells in an apparent homogenous population of cells in culture (Lacor et al., 2004; De Felice et al., 2008). Such an initial cellular binding event may play a critical role in toxic mechanisms and cell to cell propagation of disease. This cell selectivity may be influenced by the presence of specific lipid components or membrane properties (Okada et al., 2007; Wakabayashi and Matsuzaki, 2007; Lin et al., 2008). Once Aβ aggregation begins in or near a membrane, the potential toxic mechanism include disruption of the bilayer structure, changes in bilayer curvature, and/or the creation of membrane pores or channels (Arispe et al., 1993a,b;

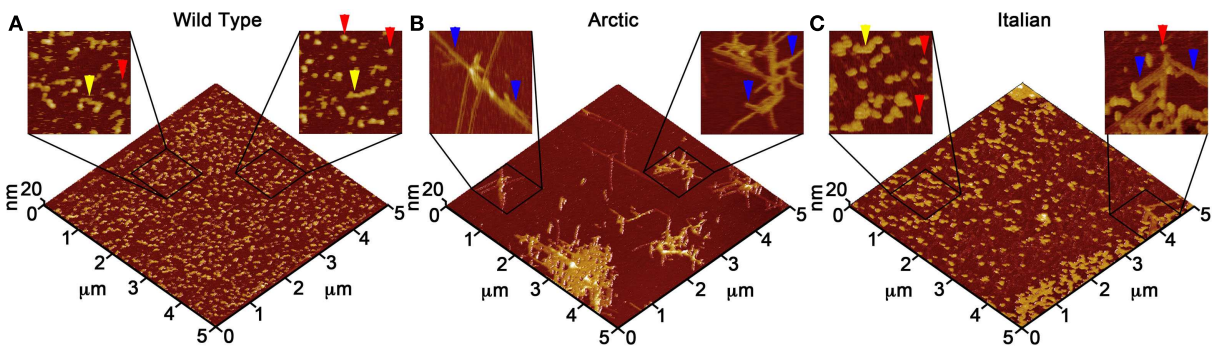

FIGURE 3 | Point mutations in Aβ(1–40) modulate aggregate morphology in the presence of a mica surface. Using solution AFM, the aggregation of Wild Type, Arctic (E22G), and Italian (E22K) Aβ was monitored on a mica surface (Aβ concentration was 20 μM for all experiments). 5 μm × 5 μm images are presented in 3D with indicated zoomed in areas of 1 μm × 1 μm shown in 2D. **(A)** Wild Type Aβ formed a large population of oligomers (red arrows) and highly curved, elongated protofibrils (yellow arrows) with aggregate heights of ∼3–5 nm similar to presented in **Figure 2**. **(B)** Arctic Aβ formed rigid, branched, and highly ordered fibrillar aggregates (blue arrows) along the crystallographic lattice of mica with aggregate heights of ∼2–5 nm along the contour. These Arctic Aβ aggregates morphologically distinct from those formed by Wild Type Aβ. **(C)** Italian Aβ predominately aggregated into small oligomers (2–3 nm tall, red arrows) that coalesced into larger protofibrils (yellow arrows), in a similar fashion to Wild Type Aβ; however, a small number of rigid, elongated "Arctic-like" fibrillar aggregates of Italian Aβ also formed (blue arrow).

McLaurin and Chakrabartty, 1996, 1997; Mirzabekov et al., 1996; Gorbenko and Kinnunen, 2006; **Figure 4**). The majority of studies on membrane-mediated fibrillogenesis have been undertaken with model systems including amyloidogenic peptides or proteins and lipid vesicles or supported bilayers of varying composition (Terzi et al., 1997; Lindstrom et al., 2002; Bokvist et al., 2004; Sparr et al., 2004). These studies often point to the importance of the chemical nature of membrane lipids and the mode of protein–lipid interactions in determining fibrillogenic properties of membrane bound Aβ. Lipids can also stabilize toxic protofibrils and even revert mature fibrils into such toxic species (Martins et al., 2008), providing another potential role for lipid surfaces in toxicity.

A large number of biophysical techniques have been applied in understanding the specific interactions between lipid membranes and Aβ. Due to the ability to control bilayer composition, biomimetic unilamellar vesicles have been extensively used to elucidate the interaction between Aβ and membranes (Williams et al., 2010). Simple vesicles comprised of a single lipid component, soybean PC, have been used to demonstrate that the presence of neutral PC delays the characteristic lag time to initiate Aβ aggregation in a lipid concentration-dependent manner (Sabate et al., 2005). Lipids can also induce changes in the secondary structure of Aβ, as CD studies demonstrated that a variety of lipids induce a transition from an α-helical to β-sheet structure in Aβ(McLaurin and Chakrabartty, 1997). As with solid surfaces, the charge of the lipid membrane surfaces, determined by the headgroups of phospholipids, dictate the extent of Aβ/membrane association due to electrostatic considerations. For example, similarly prepared Aβ(1-40) displays a stronger affinity to liposomes comprised of POPG compared to those comprised of POPC, with only the association with POPG enhancing the rate of Aβ aggregation (Kremer and Murphy, 2003). Freshly prepared Aβ(1–40) preferentially binds negatively charged PG membranes and composite membranes containing negatively charged lipids in comparison to neutral membranes; however, the relative affinity for fibril aggregates of Aβ with these lipid membranes is altered (Lin et al., 2007). Allowing Aβ(1–40) to form fibrils causes the affinity for negatively charged membranes to be smaller compared to the affinity for neutral membranes, suggesting that Aβ aggregation state can further modulate the interaction with lipid surfaces.

A potential mechanism for amyloid-forming proteins, such as Aβ, is their ability to alter membrane structure and integrity, leading to permeation of cellular membranes (**Figure 4**). Detergent-like effects arise from the amphiphilic nature of Aβ, leading to reduced membrane surface tension leading to membrane thinning and hole formation (Hebda and Miranker, 2009). Several AFM studies performed in solution have provided valuable insight into the aggregation of Aβ on a variety of model lipid membranes, leading to altered membrane morphology. The interaction of Aβ(1–40) with bilayers formed from total brain lipid extract (TBLE) revealed that Aβ(1–40) will partially insert into bilayers, growing into small fibers (Yip and McLaurin, 2001). In the same study, larger fiber-like structures associated with disruption of the bilayer morphology and integrity were observed as measured by increased surface roughness and formation of holes, respectively. Large fibrils were often highly branched and associated with edges of disrupted bilayer. The TBLE bilayers also aided in nucleation and enhancement of fibril growth. Interestingly, preformed fibrils were not capable of disrupting the TBLE bilayers, which may indicate that the act of aggregation, that is pre-fibrillar aggregates, may be key in Aβ-induced membrane disruption. Similar experiments exposing DMPC bilayers to Aβ(1–40) resulted in the

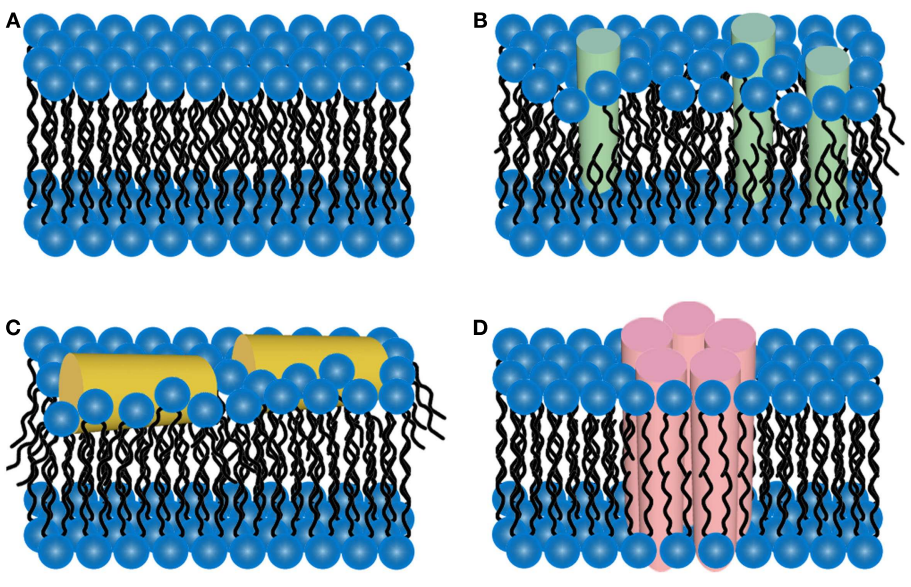

FIGURE 4 | Schematic representations of potential mechanisms of amyloid/lipid association. (A) A schematic representation of simplified, undisrupted bilayer is presented. This bilayer structure can be perturbed by **(B)** amyloid-protein insertion or **(C)** association of amphiphilic α-helices lipid-binding domains. Such scenarios could lead to membrane thinning and non-specific membrane leakage. **(D)** Many amyloid-forming proteins have been shown to form pore-like structures that can act as unregulated ion-selective channels.

formation of globular aggregates that were associated with small holes in the bilayer, whereas, fibril growth and/or extensive bilayer disruption was not observed. Aβ(1–42) demonstrated a different interaction/aggregation pattern on TBLE bilayers (Yip et al., 2002). Discrete molecules of Aβ(1–42) could be detected on the surface that were replaced by distinctly larger aggregates with time. However, bilayer defects were rarely detected upon exposure to Aβ(1–42). Point mutations in Aβ(1–40) also altered the aggregation on and ability to disrupt TBLE bilayers (**Figure 5**; Pifer et al., 2011). These same point mutations were shown to cause polymorphic aggregation of Aβ on mica. Aggregation in the presence of TBLE bilayers resulted in a variety of polymorphic aggregates in a mutation dependent manner and a variable ability to disrupt bilayer morphology/integrity. Such results highlight the potential role electrostatic and hydrophobic properties of Aβ play in its ability to bind, insert, and potentially disrupt lipid membranes.

Another proposed toxic mechanism points to Aβ's ability to alter cellular ion concentrations, calcium in particular, through the formation of membrane pores (**Figure 4D**). Initial evidence for this scenario came from the observation that PS bilayers that had Aβ(1–40) directly incorporated into them displayed linear current/voltage relationships in symmetrical solutions (Arispe et al., 1993a). Further evidence for this scenario was provided by studies on phospholipid vesicles that had either Aβ(1–42) (Rhee et al., 1998) or Aβ(1–40) (Lin et al., 1999) directly incorporated into them. In both cases, these vesicles stiffened in the presence of calcium, due to calcium ion-induced charge–charge repulsion inside the vesicles, binding of calcium to lipids and proteins, and an enhanced efficiency of lipid–protein interactions. This increased stiffness of the vesicles could be blocked by pretreatment with anti-Aβ antibodies, Tris, or zinc, all of which would block putative calcium channels. Reconstituting Aβ(1–42) with a planar lipid bilayer resulted in the formation of multimeric channel-like structures with symmetries suggesting tetramer or hexamer pore-like structures of Aβ(Lin et al., 2001; Quist et al., 2005). The formation of a variety of similar aggregate structures in lipid membranes have also been demonstrated computationally (Capone et al., 2012; Tofoleanu and Buchete, 2012).

Similar impacts on membranes due to exposure to Aβ have been detected in cellular models. Cells exposed to Aβ(1–40), Aβ(1–42), and Aβ(25–35) on endothelial cells undergo morphological changes and cell disruption, with the highest sensitivity to Aβ(1–42) (Zhu et al., 2000). While cell disruption was induced by nanomolar concentrations of Aβ(1–42), micromolar concentrations of Aβ(1–40) were required to trigger similar effects. Similar observations were reported for fibroblasts in the presence of nanomolar Aβ(1–42), as morphological changes along the periphery of the cell were observed that could be blocked by anti-Aβ antibodies, zinc, and the removal of calcium (Zhu et al., 2000). Protofibrils and low molecular weight oligomers of Aβ can alter the electrical activity of neurons and reproducibly induced toxicity in mixed brain cultures in a time- and concentration-dependent manner, suggesting changes in membrane integrity and depolarization (Hartley et al., 1999). Aβ peptides induce ion channel-like ion flux in model lipid membranes and neuronal membranes independent from the ability of Aβ to modulate intrinsic cellular ion channels or transporter proteins (Capone et al., 2009) Even intracellular forms of Aβ can alter the electrophysiological properties of cultured human primary neurons (Hou et al., 2009). Aβ(1–42) oligomers form single ion channel permeable to Ca^{2+} in oocytes are highly toxic and not attributable to endogenous oocyte channels (Demuro et al., 2011).

The discussed potential mechanism of Aβ toxicity associated with lipid membranes are not exhaustive, nor are they mutually exclusive. Aβ-induced membrane disruption of POPC, POPOC/POPS/gangliosides, and TBLE systems occurred in a two-step process (Sciacca et al., 2012). The initial step involved the formation of ion-selective pores, followed by non-specific fragmentation of the lipid membrane due to fibrillization. This demonstrates that different mechanism of membrane disruption may be associated with specific stages of aggregation. Large unilamellar lipid vesicles (LUVs) encapsulating self-quenching fluorescent dyes can

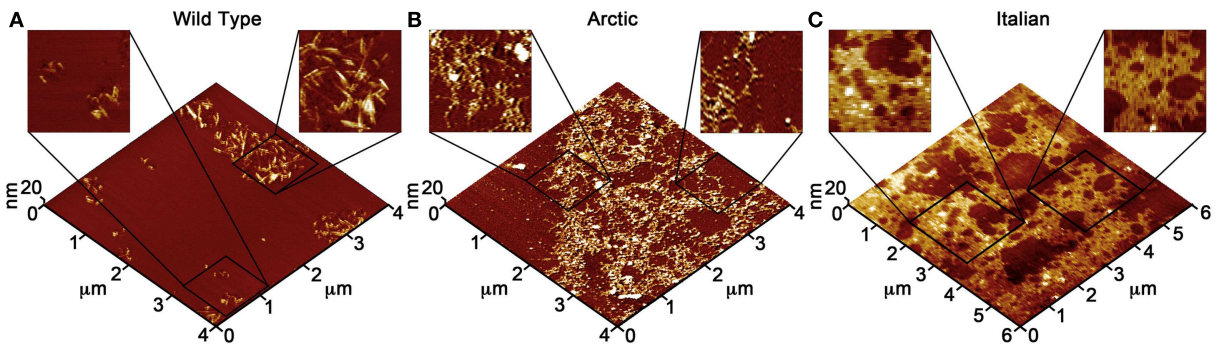

FIGURE 5 | Point mutations in Aβ influence peptide aggregation in the presence of total brain lipid bilayers. Using solution AFM, aggregation of Wild Type, Arctic (E22G), or Italian (E22K) Aβ in the presence of supported TBLE bilayers was monitored (Aβ concentration was 20 μM for all experiments). 3D images are presented (4 μm × 4 μm and 6 μm × 6 μm) with indicated zoomed in areas of 1 μm × 1 μm and 2 μm × 2 μm shown in 2D. **(A)** With time, Wild Type Aβ aggregated into discrete oligomers and fibrils that were associated with regions of the bilayer with perturbed morphology (an increase in surface roughness). **(B)** While many small oligomers of Arctic Aβ were observed on the bilayer, highly curved fibrils that were associated with membrane disruption were the dominant aggregate species. These Arctic Aβ fibrils were morphologically distinct from fibrils observed for Wild Type Aβ. **(C)** While Italian Aβ also formed similar oligomers compared Wild Type and Arctic Aβ, large patches of disrupted bilayer morphology developed that may be associated with distinct fibril aggregates.

be used as reporters of membrane disruption and leakage. Such systems have been used to elucidate the ability of Aβ to disrupt membrane integrity. Upon exposure of LUVs comprised of DMPC and containing calcein to Aβ(1–42) oligomers, the LUV structure is disrupted, allowing leakage of the dye, but preformed fibrils have a decreased ability to disrupt LUVs (Williams et al., 2010). However, the oligomers that interacted with the DMPC LUVs formed fibrils, suggesting that the aggregation process may actually play a role in membrane disruption.

A variety of studies indicate the ability of Aβ to bind membranes is highly dependent on the presence of specific lipid components, i.e., cholesterol (Yip et al., 2001; Reiss et al., 2004; Yu and Zheng, 2012), sphingolipids (van Echten-Deckert and Walter, 2012), gangliosides (McLaurin and Chakrabartty, 1996), and neutral or charged phospholipids (McLaurin and Chakrabartty, 1997; Sabate et al., 2005, 2012). This may be due to specific chemical/electrostatic interactions between membrane components and Aβ and/or the mechanical properties of the bilayer associated with their specific composition. For example, altering the cholesterol content of supported TBLE bilayers changes Aβ aggregation on membranes. Aβ(1–40) induced bilayer disruption and its ability to form fibrils on the bilayer was strongly dependent on cholesterol content of the supported bilayers (Yip et al., 2001). Cholesterol depletion of bilayers inhibited the ability of Aβ(1–40) to perturb bilayer structure. When Aβ(1–40) was added to TBLE bilayers that had been enriched with 10% exogenous cholesterol, discrete Aβ(1–40) peptides appeared on the bilayer within ∼30 min. Eventually, ring-like Aβ(1–40) structures with diameters of 55–80 nm as well as short fibrils and small aggregates were observed on the cholesterol enriched bilayer, but no membrane disruption was observed. At higher cholesterol content (30% of the total lipid), these Aβ(1–40) aggregates were not observed. The ability of Aβ(1–40) to disrupt the TBLE bilayers with varying amounts of cholesterol correlated with bilayer fluidity, indicating that decreased fluidity (modulated by cholesterol content) of the membrane somehow enhanced the interaction between the bilayer and Aβ. Simulation of POPC bilayers containing different mole fractions of cholesterol demonstrate that cholesterol induces changes in bilayer properties, i.e., membrane structure, dynamics, and surface chemistry, that cause increased bilayer thickness, ordering of hydrophobic chains, surface hydrophobicity, and decreased lipid mobility (Yu and Zheng, 2012). These effects promoted the binding of Aβ(1–42) to the model POPC lipid bilayers.

Cholesterol is also critical in the insertion of oligomeric forms of Aβ(1–42) into POPC membranes (Ashley et al., 2006). With DOPC model bilayers, the addition of cholesterol acts as a target for the binding of Aβ to the membrane (Drolle et al., 2012). AFM studies further illustrate that the alteration of bilayer mechanical properties induced by lipid composition impact the ability of Aβ to bind membranes, by demonstrating that astrocyte secreted lipoprotein particles containing different isoforms of apolipoprotein E (apoE), of which the apoE4 allele is a major risk factor for the development of AD, protect TBLE bilayers from Aβ(1–40) induced disruption (Legleiter et al., 2011). The apoE4 allele was less effective in protecting these bilayers from Aβ(1–40) compared with their apoE3 counterparts, and further analysis revealed that this was due to the varying ability of the lipoprotein particles containing different alleles of apoE to modulate the fluidity of bilayers by acquiring bilayer components (most likely cholesterol and/or oxidatively damaged lipids). There is evidence that peptide/membrane affinity in vascular cells can also be related to the ability of cholesterol to modulate membrane fluidity and structure (Subasinghe et al., 2003). Other cell culture assays, using PC-12 and SH-SY5Y cells, demonstrated that depleting cells of cholesterol increased the cellular binding of Aβ(1–40) (Yip et al., 2001).

While the mechanical properties of bilayers can influence their susceptibility to Aβ binding, once Aβ binds a membrane, this association may also alter the mechanical properties of the membrane, leading to dysfunction. Such a scenario is plausible considering the observed morphological changes associated with lipid membranes exposed to Aβ(Yip et al., 2002; Legleiter et al., 2011; Pifer et al., 2011). Anisotropy studies with POPC and POPG lipid membranes demonstrated that monomeric Aβ had initially little impact on bilayer fluidity; however, oligomers were able to decrease bilayer fluidity (Kremer et al., 2000). Furthermore, oligomers prepared at pH 6 had a larger impact on bilayer fluidity compared to oligomers that formed at a neutral pH, suggesting that distinct, polymorphic oligomers were formed under the different conditions (Kremer et al., 2000). Studies performed on supported phospholipid membranes revealed that exposure to Aβ modifies morphology and local mechanical properties of bilayers, reducing the force required to break through the membrane with an AFM probe (Dante et al., 2011). The lysis tension of unilamellar vesicles containing oxysterols are altered by exposure to nanomolar concentration of Aβ peptides (Kim and Frangos, 2008). Collectively, these results suggest Aβ can negatively impact the mechanical integrity of lipid membranes.

The major risk factor associated with AD is age. Age-related changes in membrane composition and/or physical properties may facilitate an increased cellular susceptibility to Aβ cytotoxicity. For example, both enhanced cellular cholesterol content (Wood et al., 2002; Cutler et al., 2004; Panchal et al., 2010) and oxidative damage (Chen and Yu, 1994; Choe et al., 1995) are associated with aging, decreased fluidity of membranes, and AD. Oxidative damage of polyunsaturated fatty acids, in general, increase lipid bilayer rigidity as a result of increased steric hindrance restricting the movement of lipid acyl chains (Choe et al., 1995; Choi and Yu, 1995). Furthermore, Aβ oligomers display preferential accumulate at oxidatively damaged plasma membranes of cells (Cecchi et al., 2007), and there is evidence of enhanced oxidative damage in AD brains (Williamson et al., 2008; Ansari and Scheff, 2010). Such studies suggest that altered membrane mechanics play a role in facilitating Aβ/lipid interactions.

SURFACE AGGREGATION OF OTHER AMYLOID-FORMING PROTEINS

Surfaces can also modulate the aggregation of other amyloid-forming proteins associated with neurodegenerative diseases. Specifically, these proteins may also alter membrane homeostasis, presumably via similar mechanisms as described for Aβ. Here we will briefly discuss some features of the interaction of other amyloid-forming proteins: htt, α-syn, apolipoprotein C-II, and prions. Many studies of the aggregation of these two proteins

have focused on lipid vesicles or organelles, which have membranes comprised predominately of lipids, and these studies further highlight how aggregation can be modulated by membrane composition.

THE INTERACTION OF α-syn WITH SURFACES

Parkinson's disease is a neurodegenerative disease caused by the sporadic misfolding and aggregation of the protein α-synuclein (α-syn) leading to the appearance of inclusions termed Lewy bodies. Electron microscopy (EM) and *ex situ* AFM have shown that, while a heterogeneous population of oligomers, protofibrils, and annular aggregates exists (Conway et al., 2000; Apetri et al., 2006), over time the predominant aggregate species are fibrillar (Conway et al., 1998; Narhi et al., 1999; Apetri et al., 2006). While Lewy bodies have long been known to be comprised of fibrils (Duffy and Tennyson, 1965) it is now widely believed that pre-fibrillar and pre-Lewy body inclusions aggregates are responsible for disease. The fragmentation of the Golgi apparatus, for example, corresponds to the appearance of protofibrils, rather than fibrils (Gosavi et al., 2002). This notion that pre-fibrillar aggregates are the cause of disease is supported by dementia with Lewy bodies patient brains lysates containing elevated levels of α-syn oligomers (Paleologou et al., 2009) compared to control and AD patient brains. Toxicity in cell models is usually displayed without fibrillar or protofibrillar species, but rather a 54–83 kDa aggregate, perhaps comprised of 17 kDa oligomers, that appears to mediate neurotoxicity (Xu et al., 2002). Transgenic mice, unlike human patients, exhibited neurodegeneration and inclusions comprised of fine granular material and clear vacuoles, not fibrils (Masliah et al., 2000). Furthermore, appearance of Thioflavin T (ThT) reactive aggregates have been shown to correspond with decreased fluidity of lipid acyl chains in membranes (Smith et al., 2008).

Surface stabilized α-syn aggregates have been observed by in solution AFM studies, where fibrillar sheets grew in length along two directions 120° from each other reflecting the pseudo-hexagonal surface geometry of muscovite mica. Altering the surface substrate from mica, a hydrophilic surface, to highly order pyrolytic graphite, a hydrophobic surface, impeded sheet formation, demonstrating a specific surface dependent growth mechanism (Hoyer et al., 2004). This is contrary to what has been seen in EM and *ex situ* AFM studies in which α-syn is aggregated in bulk solution. In these studies, α-syn forms oligomers and fibrils without any discernible directionality (Conway et al., 2000).

In pre-synaptic termini, α-syn exists in both free and plasma membrane or vesicle bound states (McLean et al., 2000). Densitometric analysis of rat brain fractionation demonstrated that ∼15% α-syn in the supernatant is membrane bound (Lee et al., 2002). Homozygous deletions of α-syn in mouse models and overexpression of α-syn in a neuronal cell line corresponded with changes in membrane fluidity and cellular fatty acid uptake and metabolism (Sharon et al., 2003; Castagnet et al., 2005; Golovko et al., 2005). Similarly, α-syn has been shown to have a strong interaction with synthetic anionic phospholipid vesicles (Davidson et al., 1998; Jo et al., 2000; Ramakrishnan et al., 2003), crude brain vesicles, cellular membranes, lipid rafts, and lipid droplets (Jensen et al., 1998; McLean et al., 2000; Cole et al., 2002; Fortin et al., 2004). EPR studies have demonstrated that the α-syn helix extends parallel to the curved lipid (Jao et al., 2008), while electron microscopy experiments note α-syn's ability to tubulate vesicles (Varkey et al., 2010). *Ex situ* AFM studies of PG vesicles exposed to α-syn lead to membrane fragmentation (Volles et al., 2001) and in solution AFM experiments of α-syn aggregation on mica supported lipid bilayers demonstrate that α-syn association leads to bilayer disruption and eventual fibril formation on the exposed mica surface (Jo et al., 2000). This interaction with lipid structures is believed to be directed by the first N-terminal 60 amino acids of α-syn, which contains an amphipathic α-helix structurally similar to apolipoproteins-binding domains (Clayton and George, 1998).

Thus, the first 60 amino acids of α-syn causing subcellular localization may lead to 1) an increase in local α-syn concentration and nucleation sites or 2) the α-helical structure of the membrane bound α-syn might impede misfolding into high-ordered aggregates. Supporting the first hypothesis, FTIR and far-UV CD studies demonstrate that aggregation of α-syn depends on the proximity of the membrane; amorphous aggregates were formed on or close to membranes whereas fibrillar aggregates were formed distant to membranes (Munishkina et al., 2003). Fluorescence and AFM experiments with polytetrafluroethylene balls and α-syn also highlight that aggregate formation is dominated by reactions at hydrophobic interfaces, like lipid membranes (Pronchik et al., 2010). Similarly, fluorescence studies on supported lipid bilayers demonstrate that α-syn clustering on membranes is a function of anionic lipid and/or protein concentration (Pandey et al., 2009). Double electron–electron resonance studies reveal well-defined α-syn aggregates with lipids that could form part of larger aggregates and serve as nucleation sites (Drescher et al., 2010). The second hypothesis that α-helical membrane bound α-syn impedes aggregation into higher ordered aggregates, is supported a fluorescence resonance energy transfer study, where membrane binding alters the tertiary conformation of α-syn such that oligomerization is inhibited (Narayanan and Scarlata, 2001). However, it is important to note that the two hypotheses may not be mutually exclusive. It is possible that α-syn binding to a membrane stabilizes and nucleates a toxic aggregate specie.

Circular dichroism studies have also hinted at surface altered aggregate species as α-syn in PBS is in a random coiled secondary structure, whereas α-syn in the presence of POPC/POPS small unilamellar vesicles (SUVs) formed an α-helical structure. These studies further demonstrated that α-syn aggregation was not an effect of surface curvature as POPC/POPS multilamellar vesicles (MLVs), POPC/POPI, and POPC/POPA SUVs do not result in α-syn α-helical structure, whereas with the addition of PE to POPC/PI and POPC/POPA SUVs α-syn's α-helical content increased (Jo et al., 2000). These studies suggest that surface membrane composition plays a role in stabilizing aggregates. Stabilized annular aggregates have been found in *in vitro* studies and human brain samples. This stabilized pore-like structure is hypothesized to lead to membrane ion leakage (Lashuel et al., 2002; Pountney et al., 2004). Thus, surface stabilized aggregates, such the membrane stabilized annular aggregates, may be one key toward understanding the mechanism of toxicity in PD.

THE INTERACTION OF HUNTINGTIN WITH SURFACES

Huntington's disease is another neurodegenerative disease caused by a polyQ expansion within exon1 htt. The length of the polyQ domain is intimately correlated to age of onset and severity of

disease (Snell et al., 1993; Penney et al., 1997; Tobin and Signer, 2000). Inclusion bodies, the hallmark of disease, once thought to be the toxic species, have been shown by a survival analysis to potentially have a beneficial rather than pathogenic response to htt aggregation (Arrasate et al., 2004). AFM experiments with both GST-fusion htt exon1 proteins and synthetic polyQ peptides demonstrate a heterogeneous and complex aggregation mechanism, including oligomers, fibrils, annular aggregates, and inclusions, in which antibodies detect numerous different conformations of these aggregates (Legleiter et al., 2009, 2010). Analytical size exclusion chromatography experiments have demonstrated that flanking sequences of the polyQ domain alter aggregation rates considerably. Specifically, in bulk solution the first 17 N-terminal amino acids accelerate aggregation while the a C-terminal polyproline (polyP) domain retards aggregation rates (Thakur et al., 2009). Different types of HD models have shown that in neurons, both normal and mutant htt proteins localize to several subcellular compartments, such as endosomes, pre-synaptic, and clathrin-coated vesicles, and dendritic plasma membrane (Harjes and Wanker, 2003). Furthermore, htt inclusion bodies developed in cell lines expressing large N-terminal htt fragments incorporate multi-vesicular membranes, autophagosomes, and mitochondria into their surfaces (Kegel et al., 2000; Qin et al., 2004).

Immunohistochemical studies and subcellular fractions have also highlighted the fact the htt is enriched in membrane-containing fractions (Gutekunst et al., 1995). In fact, ∼50% of endogenous htt distributes with membranes after subcellular fractionation of neuron-like clonal striatal cells (Kegel et al., 2005). Thus, the wide subcellular localization and membrane-incorporated aggregates suggest that there is a strong htt interaction with lipid bilayers, which may be directed by the first 17 amino acids on the N-terminus of htt exon1. This domain appears to adopt a highly conserved amphipathic α-helix with membrane binding properties (Atwal et al., 2007), which may be facilitated by the polyP domain on the C-terminal side of the polyQ domain (Qin et al., 2004). Similar to PD, subcellular localization of htt may lead to a local increase in htt concentration creating aggregation nucleation sites or stabilization of the α-helical conformation may actually stabilize specific aggregate species that are transiently formed in bulk solution. It is possible that htt association to membranes nucleates some types of aggregation while potentially stabilizing specific intermediates along that aggregation pathway.

Surface stabilized aggregates of simple polyQ peptides have been observed via in solution AFM studies that demonstrated that, while the majority of peptide formed extensive fibrillar networks, discrete oligomers formed on a mica surface (Legleiter et al., 2010; Burke et al., 2011). These studies are contrary to previous assumptions based on bulk solution experiments that aggregation of pure polyQ peptides proceeded directly from monomer to fibril without oligomeric intermediates (Chen et al., 2002a,b). Similar to α-syn, htt has also been observed by CD to alter its structure in the presence of POPC and POPS:POPC SUVs, both compositions of endoplasmic reticulum (ER) and ER derived vesicles (Atwal et al., 2007). These studies were able to show that while htt does have α-helical content in free solution, α-helical content is altered in the presence of SUVs. Interestingly, although no structural data was provided, densitometry data from Western blots were able to demonstrate that htt/lipid interaction is modulated by membrane composition and polyQ length (Kegel et al., 2009). Here, increased polyQ length had a preferential association with multivalent phospholipids. Stabilized oligomers have been identified to be associated with mitochondrial structural proteins in HD patient brains. Here, it is believed that the oligomeric species lead to mitochondrial fragmentation, abnormal mitochondrial dynamics, and oxidative DNA damage (Shirendeb et al., 2011). Surface stabilized htt aggregates, such as mitochondrial stabilized oligomeric species, may lead to understanding potential toxic mechanisms and therefore therapeutic targets.

Posttranslational modifications of htt further modify its trafficking and interaction with membranous cellular surfaces. Sumoylation of the first 17 N-terminal amino acids in htt exon1 leads to its being trafficked to the nucleus (Steffan et al., 2004). This sumoylation of mutant htt also increases soluble diffuse aggregates that elicit greater cytotoxicity and neurotoxicity in HD *Drosphila* models (Steffan et al., 2004). More specifically, when Rhes, a protein selectively localized in the striatum that increases sumoylation in transgenic mice, is overexpressed in mutant htt knock-in striatal cells, cell survival is reduced by 60% whereas there is no effect with wild type htt (Subramaniam et al., 2009). Similarly, phosphomimetic mutations at serine 13 and 16 have been shown to alter the kinetics of aggregation by reducing fibrillization while accumulating alternative aggregates (Gu et al., 2009). YAC128 mouse models demonstrated that ganglioside GM1 treatment induced phosphorylation at serines 13 and 16 resulting in a restoration of normal motor behavior (Di Pardo et al., 2012). Furthermore, structural studies have determined that phosphorylation of serines 13 and 16 inhibit the first 17 N-terminal amino acids' amphipathic α-helix, altering the localization of htt within cells (Atwal et al., 2011). Collectively, these posttranslational modifications of the N-terminal domain modulate its lipid-binding properties and the cellular trafficking of htt.

THE INTERACTION OF apoC-II WITH SURFACES

Not all amyloid diseases are neurodegenerative in nature, and insights into the ability of lipid association to promote specific aggregation pathways and structure can be gleaned from these systems. One such illustrative system is amyloid deposition associated with aortic atherosclerotic lesions (Westermark et al., 1995; Mucchiano et al., 2001; Rocken et al., 2006), which contain numerous plasma apolipoproteins, such as apolipoprotein C-II (apoC-II; Medeiros et al., 2004). Lipid stabilized conformations of apoC-II have long been observed. CD studies have shown apoC-II exists in a highly disordered conformation in bulk solution (Tajima et al., 1982), whereas, in the presence of sodium dodecylsulfate, trifluoroethanol, and phosphatidylcholine vesicles apoC-II adopts a helical structure (Tajima et al., 1982). Furthermore, in the absence of lipid, TEM, and AFM studies have revealed that apoC-II forms stable fibrillar ribbons (Hatters et al., 2000; Teoh et al., 2011) with increased β-sheet content as measured by CD (Hatters et al., 2000), whereas TEM and turbidity assays have demonstrated that DHPC micelles inhibit amyloid formation while inducing α-helical formation believed to be amphipathic (Hatters et al., 2001). ThT fluorescence assays have even demonstrated that a 1:4 apoC-$II_{60–70}$ peptide to D5PC lipid ratio is sufficient to inhibit fibril formation

up to 24 h (Hung et al., 2008). In the presence of sub-micellar phospholipid concentrations, apoC-II forms a tetrameric structure that when seeded forms apoC-II fibrils, thus indicating that the tetramer specie is on-pathway to fibril formation (Ryan et al., 2008).

Intriguingly, TEM and CD studies have observed apoC-II polymorphisms by altering the lipid environment present during fibrillization. Under low-lipid concentrations, two apoC-II populations are observed in solution, which are believed to have competing fibril assembly pathways resulting in two distinct fibril structures. One fibril structure is believed to occur via the same pathway as "lipid-free" conditions, resulting in the rapid formation of ribbonlike fibrils. The second fibril structure results in a slower development of straight fibrils and is believed to form from the remaining population of lipid-associated apoC-II. Furthermore, the population of ribbonlike fibrils appears to decline as the straight fibrils are assembling, thus it is believed that apoC-II is able to transition from a mature ribbonlike fibril into the straight fibrillar assembly pathway (Griffin et al., 2008). Therefore, the lipid stabilized straight fibrils may be key toward understanding the toxic mechanism associated with atherosclerosis. The mechanisms by which lipids trigger specific aggregate forms of apoC-II may inform us concerning similar phenomena in neurodegenerative diseases.

THE INTERACTION OF PRIONS WITH SURFACES AND PARALLELS WITH CELL TO CELL TRANSPORT OF AMYLOID-FORMING PROTEINS

Of all of the neurodegenerative diseases, prion diseases (or transmissible spongiform encephalopathies) have long been considered unique due to their infectious nature (Prusiner and Hsiao, 1994; Prusiner, 1998; Aguzzi and Calella, 2009). Prion diseases are caused by the posttranslational misfolding of the benign, α-helical prion protein cellular isoform (PrP^{C}) into an infectious disease-related, β-sheet rich form (PrP^{Sc}; Caughey et al., 1991; Pan et al., 1993). AFM and EM experiments using PrP proteins and fragments demonstrate a complex aggregation mechanism involving oligomers (Serio et al., 2000), polymorphic fibrils (Anderson et al., 2006), and amorphous aggregates (Pan et al., 1993). Prions replicate by forcing PrP^{C} of the host animal to adopt the PrP^{Sc} form, and this infectious, protein-only mechanism is now widely accepted (Soto, 2011).

As exposure to the PrP^{Sc} form occurs extracellular, the interaction of prions with the exterior surface of cells may play an important role in a variety of toxic or infectious mechanisms. Several studies point to a role for lipid membranes in the conversion of PrP^{C} to PrP^{Sc} that leads to aggregation (Stahl et al., 1990; Sanghera and Pinheiro, 2002; Robinson and Pinheiro, 2010). The model prion protein fragment (PrP118-135) undergoes conformational and orientational changes in model POPG lipid bilayers (Li et al., 2012). Furthermore, the interaction between prions and cellular membranes lead directly to liposome fusion and apoptotic cell death (Pillot et al., 1997, 2000). SDS-PAGE and subcellular fractionation studies demonstrated that PrP^{C} is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein (Oesch et al., 1985; Meyer et al., 1986), and fluorescence studies have indicated that membrane environment alters the conformation of recombinant PrP lacking a GPI anchor (Morillas et al., 1999), playing an important role in the initial formation of the PrP^{Sc} form. Furthermore, lipid rafts or caveola-like domains are believed to be involved in the conformational transition of PrP (Gorodinsky and Harris, 1995; Vey et al., 1996). FTIR studies of PrP^{C} binding to lipid membranes composed of DMPC, sphingomyelin, cerebroside, and cholesterol observed PrP^{C} forming β-sheets at the membrane interface as the concentration of PrP^{C} reached a concentration threshold (Elfrink et al., 2008). Studies using CD spectroscopy also demonstrated that β-sheet formation in PrP106-126 fragments is induced by the clustered negative surface charges on a lipid membrane surface (Miura et al., 2007). Recently, an immunofluorescence analysis of MYC-tagged PrP^{Sc} exposed to Rocky Mountain Laboratory mouse prions was able to demonstrate that the infectious isoform PrP^{Sc} was present primarily located at the plasma membrane within 1 min of exposure (Goold et al., 2011). Collectively, these studies suggest that the exterior surfaces of cell may play a role in the initial formation of PrP^{Sc} and its subsequent propagation.

Recently, several other amyloid-forming proteins have been shown to have prion-like infectious properties. The ability to circumvent the lag-phase of amyloid formation by adding preformed aggregates in a process called seeding (Jarrett and Lansbury, 1993; Lansbury, 1997; Paravastu et al., 2006; Nonaka et al., 2010; Jucker and Walker, 2011; Serem et al., 2011), demonstrating that such seeds can impose aberrant structure on other proteins. Such a phenomenon appears to play a role in the cell to cell translation of the disease state across specific regions of the brain (Vonsattel and DiFiglia, 1998; Braak et al., 2003; Ravits et al., 2007; Braak and Del Tredici, 2011), and this is reminiscent of the infectious nature of prions. Acceleration of AD has been observed in several transgenic mouse studies by the injection of preformed Aβ aggregates, suggesting that Aβ may have self-propagating conformations that can seed aggregation *in vivo* (Kane et al., 2000; Meyer-Luehmann et al., 2006; Stohr et al., 2012). A similar phenomenon has been observed for tau (Clavaguera et al., 2009) and α-syn (Mougenot et al., 2012). While cellular membranes may still represent a target in many toxic mechanisms, for predominately extracellular Aβ, the ability of misfolded conformers to induce/seed aggregation does not necessarily depend on cellular uptake. However, for seeding of protein aggregation associated in neurodegenerative diseases associated with intracellular inclusions/deposits, uptake of the self-propagating conformers is necessary, and this may be facilitated by the interaction with the cell membrane or other lipid-containing structures. Cellular uptake of aggregates of several amyloid-forming proteins has been demonstrated. Aggregates superoxide dismutase-1 associated with ALS can penetrate cells by macropinocytosis and seed further aggregation (Munch and Bertolotti, 2012). Pure polyQ and htt exon1 aggregates have both been shown to penetrate mammalian cells, inducing aggregation (Ren et al., 2009; Trevino et al., 2012). Experiments with cultured cells have demonstrated that extracellular aggregates of tau are endocytosed by cells, inducing the aggregation of intracellular tau (Frost et al., 2009; Nonaka et al., 2010; Guo and Lee, 2011), and the propagation of tau aggregates within the brain of a mouse model via a prion-like mechanism has been demonstrated (de Calignon et al., 2012). The ability of α-syn aggregates to seed intracellular aggregation in a variety of cellular systems (Danzer et al., 2009; Hansen et al., 2011) has also been demonstrated and mouse models (Mougenot et al., 2012) has been demonstrated. The interaction of amyloid-forming proteins

with cellular surfaces may also stabilize aggregates with seeding capabilities. Such a scenario has been demonstrated for α-syn (Lee et al., 2002).

CONCLUSION

While the aggregation of amyloid-forming proteins in bulk solution has been extensively studied, there is still much to understand at the molecular level about protein aggregation associated with surfaces. Of particular interest are lipid membrane surfaces, which cannot only mediate and influence protein aggregation, but also may be directly targeted by toxic protein aggregates. Due to the transient nature of several aggregate species and the continuing debate concerning specific toxic species, the mechanisms associated with the ability of surfaces, like lipid membranes, to potentially stabilize (Drescher et al., 2010) or promote (Martins et al., 2008) specific aggregates need to be further elucidated. Understanding these phenomenon may prove crucial in the effectiveness of therapeutic strategies based on manipulating the aggregation pathways of amyloid-forming proteins, as has been demonstrated for EGCG (Engel et al., 2012). The exact mechanisms associated with amyloid-forming proteins leading to cellular dysfunction and death have not fully been elucidated. The ability of such proteins to perturb membrane integrity via a variety of scenarios could directly lead to membrane dysfunction, disrupting organelles, or cellular homeostasis. Still, the specific aggregate species that cause membrane destabilization are not entirely clear, and it could be that the aggregation process itself occurring at lipid surfaces may play a critical role in damaging membranes. It is an intriguing possibility that induced changes in lipid membranes may represent a common toxic motif. Continued research into the mechanism of interaction between specific conformers capable of seeding aggregation with cellular membranes is needed to fully understand how amyloid propagates from cell to cell (Munch and Bertolotti, 2012). How specific changes in cellular properties, such as membrane mechanics, influence the susceptibility of specific cells to the prion-like propagation of these protein aggregates remain unclear (Cecchi et al., 2007). Here, we highlighted some specific features of amyloid aggregation at model surfaces and lipid membranes. While the studies reviewed here are not exhaustive, we hope that collectively they offer a compelling argument that such surface induced aggregation may play a role in a variety of toxic mechanisms associated with these diseases.

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Comprehensive method for culturing embryonic dorsal root ganglion neurons for Seahorse Extracellular Flux XF24 analysis

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Changes in mitochondrial dynamics and function contribute to progression of multiple neurodegenerative diseases including peripheral neuropathies. The Seahorse Extracellular Flux XF24 analyzer provides a comprehensive assessment of the relative state of glycolytic and aerobic metabolism in live cells making this method instrumental in assessing mitochondrial function. One of the most important steps in the analysis of mitochondrial respiration using the Seahorse XF24 analyzer is plating a uniform monolayer of firmly attached cells. However, culturing of primary dorsal root ganglion (DRG) neurons is associated with multiple challenges, including their propensity to form clumps and detach from the culture plate. This could significantly interfere with proper analysis and interpretation of data. We have tested multiple cell culture parameters including coating substrates, culture medium, XF24 microplate plastics, and plating techniques in order to optimize plating conditions. Here we describe a highly reproducible method to obtain neuron-enriched monolayers of securely attached dissociated primary embryonic (E15) rat DRG neurons suitable for analysis with the Seahorse XF24 platform.

Keywords: Seahorse XF24 Extracellular Flux analysis, mitochondrial respiration, oxygen consumption rate, extracellular acidification rate, embryonic dorsal root ganglion neurons

INTRODUCTION

Genetic and environmental stressors associated with various neurodegenerative disorders alter the metabolic state of the cell causing a quick remodeling of catabolic and anabolic pathways in order to manage and/or adapt to the new environment. Bioenergetic deficits are often coupled with abnormal cellular pathologies and phenotypic changes, which exhibit unique profiles (Chen and Chan, 2006; Finsterer, 2006; Scaglia, 2010; Zheng et al., 2011; Saxena, 2012). Thus, the relative state of aerobic and glycolytic (anaerobic) metabolism is indicative of the overall health of the cell, and analysis of nutrient and biomolecular flow in live cells is essential to understanding the relevant cellular responses to disease pathology and/or changes in the extracellular environment. The Seahorse XF24 Extracellular Flux analyzer (hereafter referred to as the Seahorse XF24 analyzer) allows non-destructive, non-invasive, and sensitive analysis of the bioenergetic state of live intact cells in real-time providing invaluable assessments of mitochondrial function (Wu et al., 2007; Ferrick et al., 2008).

Recent data demonstrate that mitochondrial dysfunction is an early event underlying the development and progression of multiple neurodegenerative disorders including drug-induced peripheral neuropathies (Trushina and McMurray, 2007; Yao et al., 2009; Podratz et al., 2011; Xiao et al., 2011; Liu et al., 2012). In particular, altered mitochondrial fission, fusion, and motility were found in Charcot–Marie Tooth disease, as well as optical and diabetic neuropathies (Chen and Chan, 2006; Frank, 2006; Chowdhury et al., 2012; Saxena, 2012; Schapira, 2012). These are novel observations with limited mechanistic understanding. Cultures of adult and embryonic dorsal root ganglion (DRG) neurons represent the most relevant *in vitro* model to study peripheral sensory neuropathies (Melli and Hoke, 2009), and utilization of the Seahorse XF24 analyzer could significantly add to the understanding of disease mechanisms in order to find efficient therapeutic approaches.

Contrary to neurons from the central nervous system, DRG neuronal culture is associated with multiple challenges. One of the major obstacles in obtaining monolayers of DRG neurons, an absolute requirement for a reproducible and reliable analysis using the Seahorse XF24 analyzer, is their propensity to aggregate and detach from culture plates. Additionally, DRG neurons are sensitive to seeding density, culture media, coating substrate, and type of XF24 microplate plastic, all of which could contribute to detachment from the culture plate. These factors could potentially lead to an underestimation of cell number and significantly influence the interpretation of results. In the present work, we describe a comprehensive method that allows obtaining neuron-enriched cultures of primary embryonic (E15) rat DRG neurons that form monolayers of firmly attached cells at the optimum seeding density necessary for analysis of mitochondrial respiration with the Seahorse XF24 analyzer.

MATERIALS AND METHODS

MATERIALS

Chemicals

Ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), NaCl, sodium deoxycholate, boric acid, Triton X-100, sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride

(PMSF), and phosphatase inhibitor cocktails 2 and 3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Complete EDTA-free protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN, USA). Tris-HCl was purchased from Bio-Rad (Hercules, CA, USA). XF calibrant solution, dimethyl sulfoxide (DMSO), oligomycin A, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), rotenone, and antimycin A were purchased from Seahorse Bioscience (Billerica, MA, USA).

Culture reagents

5-Fluoro-2′-deoxyuridine (FUDR), 1-β-D-ribofuranosyluracil (uridine), and D-(+)-glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Minimal Essential Medium (MEM), Leibovitz's L-15 media, B-27, Neurobasal media, Dulbecco's phosphate buffered saline (DPBS), L-glutamine, and Pen-Strep were purchased from Gibco Life Technologies by Invitrogen (Grand Island, NY, USA). Hank's Buffered Salt Solution (HBSS) and sodium pyruvate were purchased from Cellgro by Mediatech (Manassas, VA, USA). Bovine calf serum (BCS) of US origin was purchased from Hyclone by Thermo Scientific (Logan, UT, USA). Trypsin was purchased from Worthington Biochemical (Lakewood, NJ, USA). Nerve growth factor (NGF) was purchased from Bioproducts for Sciences, a division of Harlan Sprague-Dawley (Indianapolis, IN, USA). Thy 1.1 (CD90) rat anti-mouse antibody was purchased from ProSpec-Tany TechnoGene Ltd. (Ness Ziona, Israel). XF assay medium was purchased from Seahorse Bioscience (Billerica, MA, USA).

Culture dishes

All polyethylene terephthalate and polystyrene 24-well Seahorse XF24 microplates and cartridges were purchased from Seahorse Bioscience (Billerica, MA, USA). All other culture dishes (standard 96-well microplates, 60 mm dishes) were purchased from BD Biosciences (San Jose, CA, USA).

Animals

The Mayo Clinic Institutional Animal Care and Use Committee (IACUC) approved all animal studies involving DRG neurons extracted from wild-type embryonic day 15 (E15) Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI, USA).

METHODS

Microplate substrate coating and plastic

It should be noted that the surface area of each well of 24-well Seahorse XF24 microplates is identical to that of standard 96-well microplates (0.32 cm^2). The following coating substrates were tested on XF24 microplates: rat tail collagen Type I (5–50% in sterile deionized water; 30–40 μl/well) was purchased from BD Biosciences (Bedford, MA, USA); natural mouse laminin (2 μg/ml in sterile DPBS; 30–40 μl/well) was purchased from Gibco Life Technologies by Invitrogen (Grand Island, NY, USA); poly-L-lysine (0.1 mg/ml in sterile deionized water; 500 μl/well) and poly-L-ornithine (0.5 mg/ml in sterile deionized water or borate buffer [pH 8.4]; 500 μl/well) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Coating procedures used for Seahorse XF24 and standard 96-well microplates were as follows: microplates covered with poly-lysine were incubated overnight at 37°C, 5% CO_2, and 95% humidity. The substrate solution was then removed, and the microplates were dried without rinsing under a sterile laminar flow hood. Poly-ornithine (dissolved in borate buffer) was added to the microplates, which were covered with foil and incubated overnight at room temperature (RT) under a sterile laminar flow hood. The substrate solution was then removed, wells were rinsed twice with sterile deionized water, and the microplates were dried under a sterile laminar flow hood. Laminin was only used in a combination with poly-lysine or poly-ornithine. Laminin was combined with poly-lysine and co-incubated according to the above poly-lysine coating procedure. Following poly-ornithine coating, laminin was applied to each well of the microplate and allowed to dry (unrinsed) at RT under a sterile laminar flow hood. Collagen was applied alone or in a combination with either poly-lysine or poly-ornithine. When applied alone, collagen was added to each microplate well and allowed to dry (unrinsed) at RT under a sterile laminar flow hood. When used in combination, collagen was added following the above poly-lysine or poly-ornithine coating procedures, and allowed to dry (unrinsed) at RT under a sterile laminar flow hood. Application of collagen and laminin involved adding enough solution to cover the bottom of the microplate well, while avoiding excessive capillary action from well edges, which can cause uneven distribution of these particular substrates. In addition, DRG attachment and distribution was evaluated using polystyrene and polyethylene terephthalate XF24 microplates to test the effect(s) of variations in the physical properties of each plastic on attachment and distribution of DRG neuronal culture.

Dissociated DRG neuronal culture and plating

Dissociated DRG neurons were isolated as described previously (Wood, 1976; McDonald et al., 2005), with modifications described below. Whole DRG explants were removed by aseptic microdissection from E15 Sprague-Dawley rats and dissociated with 0.25% trypsin in HBSS for 30 min, followed by mechanical dissociation by glass Pasteur pipet, flamed to reduce opening to <200 μm. Cells were re-suspended at 2 ml per 10 spines in 15% serum AN_2 media (MEM, 15% BCS, 39 mM glucose, 1.2 mM L-glutamine, and fresh 10 ng/ml NGF). Cells were pre-plated on either uncoated or Thy 1.1-coated culture dishes (1:100 in L-15 or MEM for at least 4 h at RT or overnight at 4°C) at 10 embryo spines per 60 mm dish for 1.5–2 h in a humidified incubator at 37°C and 5% CO_2. Pre-plating was used to remove non-neuronal cells in order to increase accuracy of cell counting and seeding, as well as to reduce the amount of time cells would spend in anti-mitotic media. The amount of spines allotted per 60 mm dish was optimized to avoid over-crowding, while the pre-plating incubation time was optimized to allow support cells to adhere but not DRG neurons.

Following pre-plating, the cell suspension was transferred to a 15 ml tube, the dishes were gently rinsed with 15% serum AN_2 medium, and the rinse added to the cell suspension. Cells were concentrated in a Beckman TJ-6 centrifuge (Fullerton, CA, USA) at 3,000 rpm for 5 min at RT, and re-suspended in an appropriate volume of 15% serum AN_2 in order to seed each well of a 24-well Seahorse XF24 microplate or standard 96-well microplates at 25,000–100,000 cells/well using a volume of 150 μl or less. After

seeding, plates remained at RT for 45 min to 1 h before bringing the total volume of each well to 500 µl (XF24 microplates) or 200 µl (96-well microplates) with 15% serum AN_2, followed by overnight incubation at 37˚C, 5% CO_2, and 95% humidity. Leaving plates at RT for up to 1 h promotes even distribution and adhesion of cells and avoids edge-effect within wells and on the plate as a whole (Burt et al., 1979; Oliver et al., 1981; Lundholt et al., 2003). Cultures were then treated with 20 µM FUDR and 20 µM uridine in 15% serum AN_2 for 3–5 days with media changed every other day. This method eliminates ∼99% of non-neuronal supporting cells. DRG neuron-enriched cultures were incubated in either 15% serum AN_2 without FUDR/uridine or Neurobasal media supplemented with B-27 (a serum substitute),1.2 mM L-glutamine, fresh 10 ng/ml NGF, and Pen-Strep (without FUDR/uridine) for 24 h prior to experiments (see Optimized Step-by-Step Protocol in Appendix).

Cell proliferation assay

The Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Madison, WI, USA), or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, was completed according to the manufacturer's instructions. The amount of formazan product measured at an absorbance of 490 nm is directly proportional to the number of living cells in culture, and thereby provides a method to determine the percentage of cell death that occurs from original plating to the time of assay. After plating DRG neurons onto poly-lysine-coated 96-well microplates, cells were maintained in 15% serum AN_2 media (with FUDR/uridine) for 3 days and then placed in Neurobasal media (without Pen-Strep or FUDR/uridine) for 3 days. The MTS assay was completed at 7 days in culture (DIC). DRG neuron suspensions from each pre-plating condition (uncoated or Thy 1.1-coated 60 mm dishes) were seeded at densities of 25,000; 50,000; 75,000; and 100,000 cells/well of a standard 96-well microplate. PC12 cells were used to create a standard curve to calculate DRG neuronal densities in all wells of the 96-well microplates. PC12 cells were plated 3 h prior to the assay and seeded in triplicate at 0; 6,250; 12,500; 25,000; 50,000; 100,000; 150,000; and 200,000 cells/well of a standard 96-well microplates.

Seahorse XF24 mitochondrial stress analysis

Uptake and secretion of metabolic end-products such as oxygen and protons to and from the extracellular milieu allows the Seahorse XF24 Extracellular Flux analyzer to conduct real-time measurements of oxygen consumption and extracellular acidification of the surrounding microenvironment using solid-state fluorescent oxygen and pH biosensors coupled to a fiber-optic waveguide (Wu et al., 2007). The consumption and flux of oxygen and protons causes rapid and measurable changes in oxygen tension and pH within a transient microchamber created by the sensor cartridge and well plate while in the measurement position (see Figure 1 of Wu et al., 2007). Mitochondrial complex inhibitors are preloaded in the injection ports and are subsequently injected into the well media. After a short period of mixing, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements are made using highly sensitive photodetectors specific for the excitation and emission of oxygen (532/650 nm) and protons (470/530 nm; Wu et al., 2007). Additional details relating to the Seahorse XF24 analyzer can also be found at http://www.seahorsebio.com/.

Prior to the Seahorse XF24 mitochondrial stress analysis, representative wells from each substrate and DRG neuron seeding density were imaged on a Zeiss Axiovert 35 inverted light microscope (Thornwood, NY, USA) using 5× phase objective lens at 1 DIC and on the day of flux analysis (all wells were observed for continuity in plating, cell density, and distribution). The Seahorse XF24 mitochondrial stress test was conducted as described previously, with exceptions noted (Wu et al., 2007; Ferrick et al., 2008). Specifically, DRG neurons on XF24 microplates were rinsed once and re-suspended in 500–675 µl of XF assay buffer (DMEM without $NaHCO_3$, 7 mg/ml D-glucose, 2 mM glutamax; pH 7.4), then equilibrated for 1 h at 37˚C in a non-CO_2 incubator. All medium and solutions of mitochondrial complex inhibitors were adjusted to pH 7.4 on the day of assay. Following three baseline measurements of OCR and ECAR, mitochondrial complex inhibitors were sequentially injected into each well. Three OCR and ECAR readings were taken after addition of each inhibitor and before automated injection of the subsequent inhibitor. Mitochondrial complex inhibitors, in order of injection, included oligomycin (1.5 µM) to inhibit complex V (i.e., ATP synthase), FCCP (0.75 µM) to uncouple the proton gradient, antimycin A (1.0 µM) to inhibit complex III, and rotenone (1.0 µM) to inhibit complex I. Optimization of cell density and working concentration titers for each individual inhibitor was completed prior to the Seahorse XF24 mitochondrial stress analysis according to the Seahorse XF24 User's Manual (Seahorse Bioscience, Billerica, MA, USA). OCR and ECAR were automatically calculated, recorded, and plotted by Seahorse XF24 software version 1.8 (Seahorse Bioscience, Billerica, MA, USA). At the end of each assay, cells were washed once with an excess of RT DPBS, lysed with ice-cold RIPA buffer (0.15 M NaCl, 1 mM EDTA [pH 8], 1 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris-HCl [pH 8], and protease and phosphatase inhibitor cocktails), and the protein content estimated by Bio-Rad D_c protein assay (Bio-Rad, Hercules, CA, USA) using a Molecular Devices Softmax M3 microplate reader (Sunnyvale, CA, USA). Data was normalized for total protein content per well.

Statistical analysis

Normalized Seahorse XF24 measurements were averaged per density group per plate via Seahorse XF24 software version 1.8 (Seahorse Bioscience, Billerica, MA, USA) as mean ± SEM.

RESULTS

CELL PROLIFERATION ASSAY

Pre-plating was used to remove non-neuronal cells in order to increase accuracy of cell counting and seeding, as well as to reduce the amount of time cells would spend in anti-mitotic media. The amount of spines allotted per 60 mm dish was optimized to avoid over-crowding, while the pre-plating incubation time was optimized to allow support cells to adhere but not DRG neurons. Results of the MTS assay demonstrated that pre-plating DRG neuron suspensions for 1.5–2 h on dishes pre-coated with Thy 1.1, followed by re-plating of unattached cells onto 96-well microplates

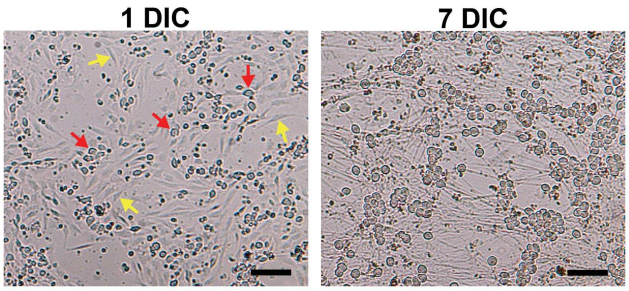

FIGURE 1 | Elimination of non-neuronal cells in DRG neuronal cultures. Although Thy 1.1 pre-plating alone did not completely remove non-neuronal cells (1 DIC, yellow arrows; red arrows, DRG neurons), it significantly reduced the amount of time DRG neurons spent in anti-mitotic media. Dissociated DRG neurons seeded at 50,000 cells/well in a 24-well Seahorse XF24 polystyrene microplate at 1 and 7 DIC at 5× phase objective. Scale bar: 200 μm.

and treating with FUDR/uridine for 3–5 days eliminates ∼99% of support cells from culture (**Figure 1**; **Table 1**). Thy 1.1 pre-plating also significantly improved the accuracy of DRG neuron counting using Trypan blue exclusion. However, the length of FUDR/uridine treatment was dependent on initial seeding density and plate surface area. Live cell counts based on absorbance intensity demonstrated an average of 38% cell loss in those wells that contained neuronal suspensions originally pre-plated on Thy 1.1-coated dishes, which is attributed to the removal of support cells. The absorbance intensity in wells containing neuronal suspensions pre-plated on uncoated 60 mm plates increased up to 61%, suggesting inefficient removal and proliferation of support cells (**Table 1**). The continued presence of non-neuronal cells despite FUDR/uridine treatment in wells that contained cells originally pre-plated on uncoated dishes was confirmed by daily monitoring cell growth and morphology in culture up to the day of MTS assay (7 DIC). Additionally, data demonstrate that the optimum lowest DRG neuron seeding density for Seahorse XF24 analysis was 50,000 cells/well, as DRG neurons could not survive seeding densities at or below 25,000 cells regardless of pre-plating method.

Table 1 | Efficiency of pre-plating DRG neuron suspensions on coated and uncoated 60 mm culture dishes at support cell removal.

Pre-plating coating†	Seeding density (cells/well)†	% Cell loss*	% Cell gain/loss*	Avg% cell gain/loss**
Anti-Thy 1.1	25,000	−90.02**		−38.76
	50,000	−38.27		
	75,000	−42.08		
	100,000	−35.93		
Uncoated	25,000		−35.56**	+61.77
	50,000		+87.85	
	75,000		+64.99	
	100,000		+32.48	

†After a 1.5–2 h pre-plating incubation, cell suspensions were collected and seeded onto standard 96-well microplates, where the surface area of each well is identical to that of Seahorse XF24 microplates (0.32 cm²/well).

**% Gain/loss based on the number of live cells calculated at 7 DIC via Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (i.e., MTS assay).*

***Average (Avg)% gain/loss excludes the 25,000 seeding density group, as values from both pre-plating methods were statistical outliers via rejection quotient Q with ≥95% confidence. Thus, seeding XF24 microplates at 25,000 cells/well was not a viable test group.*

SUBSTRATE COATING

Seahorse XF24 microplates coated with ≥40% collagen maintained healthy and well-attached DRG neurons at 50,000 cells/well for up to 10 DIC. In addition, DRG neurons showed no evidence of detachment after addition of all four mitochondrial complex inhibitors during the Seahorse XF24 mitochondrial stress assay (∼2 h) regardless of XF24 microplate plastic type. DRG neurons seeded at densities below 50,000 cells/well tended to lift by 8–9 DIC on both XF24 microplate plastic types coated with collagen at concentrations ≤30%. Cells also exhibited significant detachment after addition of just one mitochondrial complex inhibitor. Conditions were not improved by UV cross-linking of the collagen coating on microplates prior to cell culture (known to improve durability and life of collagen coatings; Caruso and Dunn, 2005). Despite the strength of collagen as a coating substrate, DRG neurons tended to aggregate and clump at all concentrations tested (**Figure 2A**).

Poly-L-lysine and poly-L-ornithine substrates provided equally satisfactory DRG neuron monolayers on both XF24 microplate plastic types (**Figure 2B**). However, DRG neurons plated on these substrates at densities below 75,000 cells/well must be analyzed within 6 DIC to avoid significant neuronal detachment before the Seahorse XF24 assay can even be attempted, regardless of XF24 microplate plastic type (**Figure 2C**). Combinations of laminin and poly-lysine or poly-ornithine did not improve the adhesive qualities of either substrate beyond that which they already possessed on either XF24 microplate plastic type.

Optimal DRG neuron culture was achieved when 10% collagen was added to poly-lysine and poly-ornithine substrates. This combination produced firmly attached, non-aggregated DRG neuronal cultures at 50,000 cells/well on both XF24 microplate plastic types (**Figure 2B**; **Table 2**) that can withstand the Seahorse XF24 mitochondrial stress analysis (∼2 h) without lifting at 8 DIC (**Figure 3**). This allowed for subsequent estimation of protein concentration for normalization and further data analysis. Collagen concentrations ≥20% used in combination with poly-lysine or poly-ornithine increased the tendency of DRG neurons to aggregate and clump.

CULTURE MEDIUM

Within 24 h after the addition of supplemented Neurobasal media, DRG neurons migrated into large clumps and began to rapidly detach and die, regardless of the coating substrate and XF24 microplate plastic (**Figures 2A,C**). By contrast, 15% serum AN_2 medium allowed preservation of healthy, non-aggregated DRG neuronal cultures (**Figure 2B**). These data suggest 15% serum-supplemented AN_2 medium is optimal for culture of healthy DRG neuron cultures at the optimum lowest seeding density (50,000 cells/well) for Seahorse XF24 analysis.

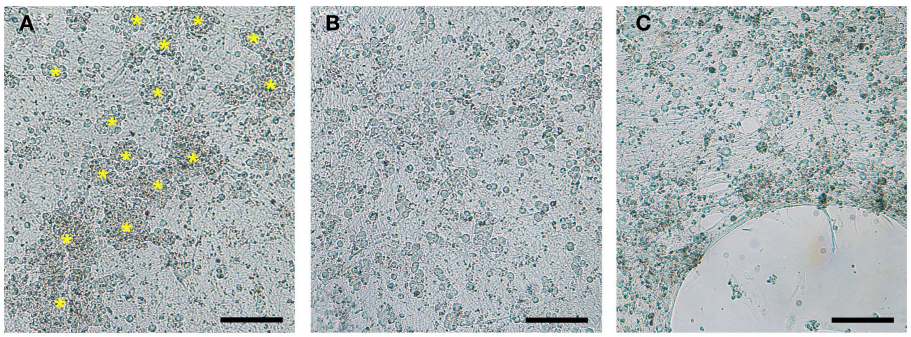

FIGURE 2 | Optimization of coating substrates for DRG neuronal culture. (A) 24-well Seahorse XF24 microplates coated with collagen promoted aggregation and clumping of DRG neurons (yellow asterisks). **(B)** Poly-lysine, poly-ornithine, and the optimal substrate combination of poly-lysine + 10% collagen provided equally satisfactory DRG neuron monolayers. **(C)** Detachment of DRG neurons cultured on poly-lysine and poly-ornithine alone or in combination with laminin observed after 6 DIC. DRG neurons seeded at 50,000 cells/well in a XF24 polystyrene microplate imaged at 7 DIC with a 5× phase objective. Scale bar: 200 μm.

Table 2 | Optimal conditions for plating DRG neurons for Seahorse XF24 Extracellular Flux analysis[§].

Pre-plating method[†]	Growth media	Coating substrate	XF24 microplate plastic type	Seeding density
Coating: anti-Thy 1.1 Dishes: standard 60 mm, plastic Incubation: 1.5–2 h; 37°C, 5% CO_2, 95% humidity	15% serum AN_2 media	Poly-l-lysine (0.1 mg/ml) + collagen (10%)	Polyethylene terephthalate or polystyrene	50,000 cells/well*

[§] *See the detailed step-by-step protocol for culturing embryonic DRG neurons.*

[†] *Following incubation, cell suspensions were collected, counted, and seeded onto Seahorse XF24 microplates.*

**Seeding density per well of an XF24 microplate.*

SEAHORSE XF24 CELL DENSITY TITRATION

Seahorse XF24 cell density titration was completed to determine the optimum lowest DRG neuronal seeding density that produced optimal OCR and ECAR readouts. Increasing cell density resulted in linear increases in both OCR and ECAR (**Figures 4A–D**). When neurons were seeded at 25,000 cells/well, OCR and ECAR values were negligible due to the detachment of cells either before or during the analysis (**Figures 4A–D**). ECAR spectra show that the increase in acidification rate with increasing cell number does not significantly change between 40,000 and 50,000 cells/well (**Figure 4D**). This indicated that 40,000 cells/well was the optimum lowest seeding density where optimal increases and decreases in both OCR and ECAR were obtained within the linear response range. However, following initial Seahorse XF24 mitochondrial stress analysis, neurons seeded at 40,000 cells/well showed minor detachment at well edges, whereas those seeded at 50,000 cells/well did not. Therefore, 50,000 cells/well was confirmed as the optimum lowest DRG neuronal seeding density for Seahorse XF24 analysis.

MICROPLATE TEMPERATURE AND PLASTIC TYPE

Results demonstrated that when using the optimal substrate (poly-lysine + 10% collagen) and seeding density (50,000 cells/well), DRG neuron attachment and growth were similar on both polyethylene terephthalate and polystyrene Seahorse XF24 microplates. However, it was found that regardless of substrate and XF24 microplate plastic type, DRG neurons were particularly sensitive to temperature. "Edge-effect" within each well and on the microplate as a whole was observed when newly seeded DRG neuronal cultures at RT were directly transferred to a humidified incubator at 37°C and 5% CO_2 (**Figure 5**). This effect did not correct itself

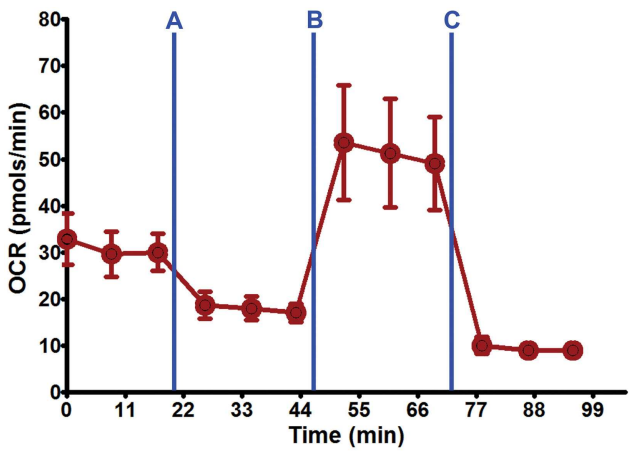

FIGURE 3 | Seahorse XF24 mitochondrial stress analysis. Using the optimal seeding density (50,000 cells/well) and coating substrate combination (poly-lysine + 10% collagen) on 24-well Seahorse XF24 polystyrene microplate, well-adhered, and dispersed DRG neuronal cultures were maintained throughout the culture process and Seahorse XF24 analysis (7 DIC). Each data point is presented as mean ± SEM. Injection series: **(A)** Oligomycin (1.5 μM); **(B)** FCCP (0.75 μM); **(C)** rotenone (1.0 μM) and antimycin A (1.0 μM).

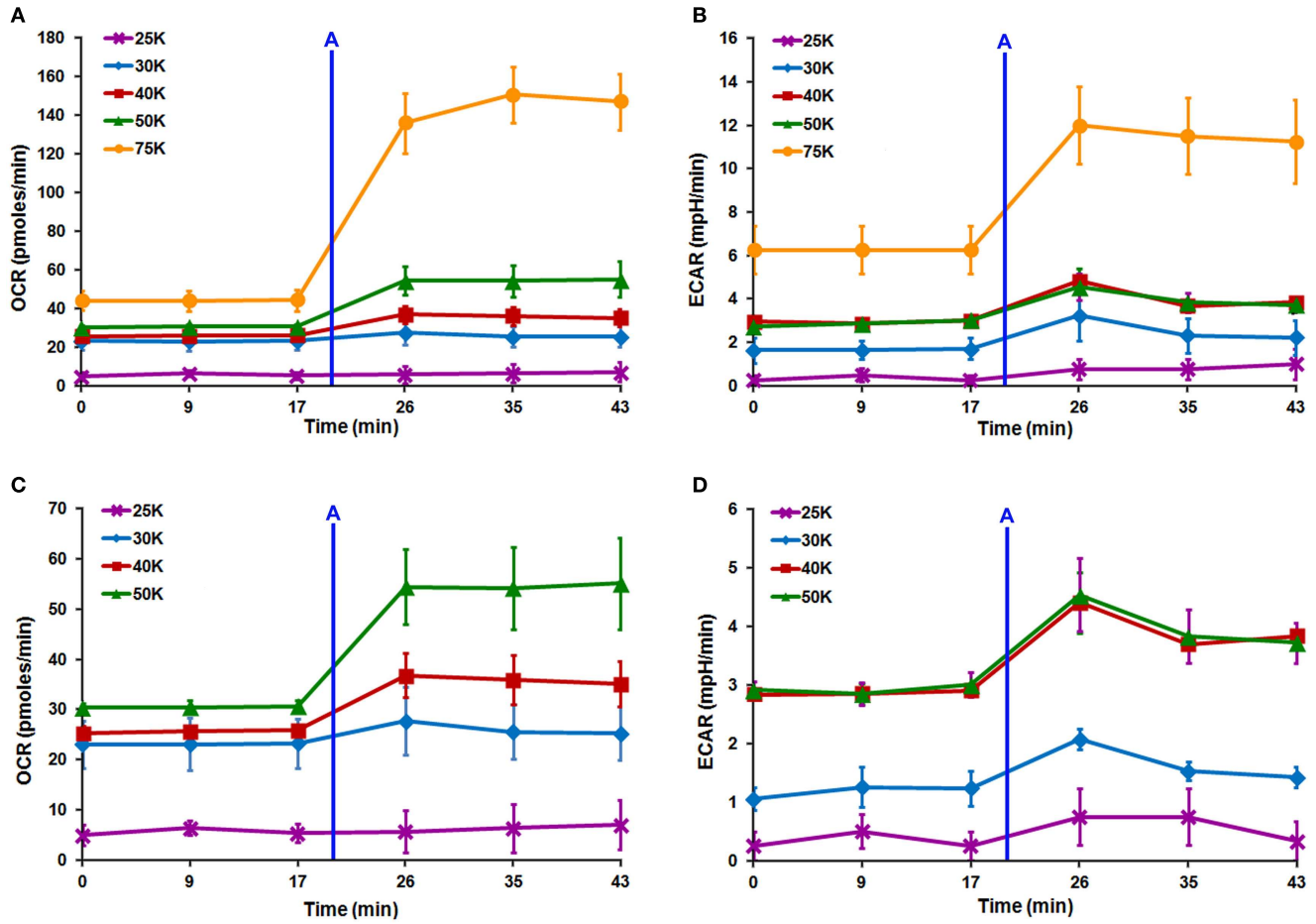

FIGURE 4 | Seahorse XF24 optimization of DRG neuron seeding density. Titration of DRG neuron seeding density with the XF24 platform maximizes increases and decreases in OCR and ECAR. Linear increases are seen in both **(A)** OCR and **(B)** ECAR spectra with increasing cell number. **(A–D)** XF24 microplates seeded at 25,000 cells/well (purple line) produced negligible OCR and ECAR due to detachment either before or during analysis. **(D)** ECAR values plateau between 40,000 (red line) and 50,000 (green line) cells/well indicating the optimal seeding density for Seahorse XF24 analysis is likely 40,000 cells/well. After Seahorse XF24 mitochondrial stress analysis, XF24 microplates seeded at 40,000 cells/well showed minor detachment, thus, 50,000 cells/well was selected as the optimal density. Each data point is presented as mean ± SEM. All readings completed on polystyrene XF24 microplates pre-coated with 0.1 mg/ml poly-lysine + 10% collagen. Injection **(A)** FCCP (0.5 μM).

with time. "Edge-effect" was prevented when cultures were left to equilibrate at RT for at least 45 min to 1 h before being placed in a 37˚C, 5% CO_2 humidified incubator.

DISCUSSION

The major requirements for obtaining reproducible and reliable data with the Seahorse XF24 analyzer is working with a consistently uniform population of target cells that are evenly distributed and firmly attached to the culture dish. We first optimized the conditions that would allow obtaining enriched cultures of primary rat DRG neurons using 24-well Seahorse XF24 microplates. A significant obstacle in obtaining pure DRG neuronal cultures in dishes with diameter less than 35 mm is the presence of highly proliferative support cells. While a previous study by Delree et al. (1989) demonstrated that dissociated DRG neurons can be purified by Percoll gradient centrifugation, this protocol is time-consuming, uses abrasive enzymatic dissociative treatment that can contribute to neuronal cell loss, and does not allow for complete removal of Percoll reagent after plating. Alternatively, many studies have shown the effectiveness and ease of use of FUDR/uridine treatment that applied at low concentrations and for the period of up to 5 days does not cause cellular toxicity (Wallace and Johnson, 1989; Fex Svenningsen et al., 2003). Therefore, we examined different pre-plating conditions in conjunction with FUDR/uridine anti-mitotic treatment.

Pre-plating cells onto uncoated or coated culture dishes is a relatively fast, effective, and cell stress-free technique commonly used to separate cells based on differential adhesive properties (Banker and Goslin, 1998; Yang et al., 2010). The MTS assay was used to determine the efficacy of pre-plating on uncoated and Thy 1.1-coated 60 mm plastic culture dishes to remove non-neuronal cells prior to culturing DRG neurons on dishes with a surface area of 0.32 cm^2 (Seahorse XF24 and standard 96-well microplates). Results of the MTS assay demonstrated that pre-plating DRG

neuron suspensions for on Thy 1.1 pre-coated dishes, followed by re-plating unattached cell suspensions onto microplates and treating with FUDR/uridine for 3–5 days eliminates ∼99% of support cells from culture (**Figure 1**; **Table 1**). Although Thy 1.1 pre-plating alone did not remove 100% of non-neuronal cells, it did significantly reduce the amount of time DRG neurons needed to spend in anti-mitotic media (**Table 1**). Furthermore, these data suggested that the optimal lowest seeding density for Seahorse XF24 analysis was between 50,000 and 100,000 cells/well.

In order to further validate MTS assay results, multiple DRG neuronal densities were assessed via Seahorse XF24 analyzer to determine the lowest optimal cell number that would produce optimal OCR and ECAR values. Following injection of low dose of FCCP after three baseline measurements, linear increases in both OCR and ECAR were observed with increasing cell density (**Figures 4A–D**). Further examination of the ECAR spectra demonstrated that the increase in ECAR with increasing cell number began to plateau between 40,000 and 50,000 cells/well (**Figure 4D**). According to the Seahorse XF24 User's Manual, this result indicated that the optimum lowest DRG neuronal seeding density was 40,000 cells/well, as it produced an optimal increase and decrease in both OCR and ECAR values and was within the linear response range for DRG neurons. However, following preliminary Seahorse XF24 mitochondrial stress analysis, wherein all four mitochondrial complex inhibitors were sequentially injected within a ∼2 h time period, XF24 microplates seeded at 40,000 cells/well showed minor detachment at the edges of each well, whereas those seeded at 50,000 cells/well did not. This detachment was exacerbated upon rinsing with PBS while preparing cells for protein estimation, thereby potentially introducing error into post-normalization analysis of the data. Therefore, 50,000 cells/well was selected as the optimum lowest DRG neuronal seeding density for Seahorse XF24 analysis.

Having determined the optimum lowest DRG neuron seeding density for Seahorse XF24 analysis, we then optimized the coating substrate, which would extend the life, health, and dispersion of DRG neurons in culture. Plating and maintaining monolayers of neuron-enriched DRG cultures is essential to accurately measure their metabolic state using the Seahorse XF24 analyzer. The durability of coating substrates is crucial to applications in which DRG neurons are to be treated with experimental drugs, which cannot occur until the cells have been established in culture and resting in anti-mitotic-free media for at least 24 h (a minimum 4–5 DIC period). The simple increase of DIC with experimental drug treatments lasting more than 12 h could lead to cell lifting before Seahorse XF24 analysis can be completed. Moreover, the experimental drug itself could weaken the culture and cause premature DRG neuron detachment.

We determined that combining a positively charged coating substrate (i.e., poly-lysine) with the extracellular matrix (ECM) coating molecule collagen (10%) produced firmly attached, non-aggregated DRG neuronal cultures that can withstand the Seahorse XF24 mitochondrial stress analysis without lifting (at 8 DIC; **Table 2**). The need for an ECM-based coating substrate to increase DRG neuron adhesion has been well documented, establishing collagen-based substrates as optimal for the culture

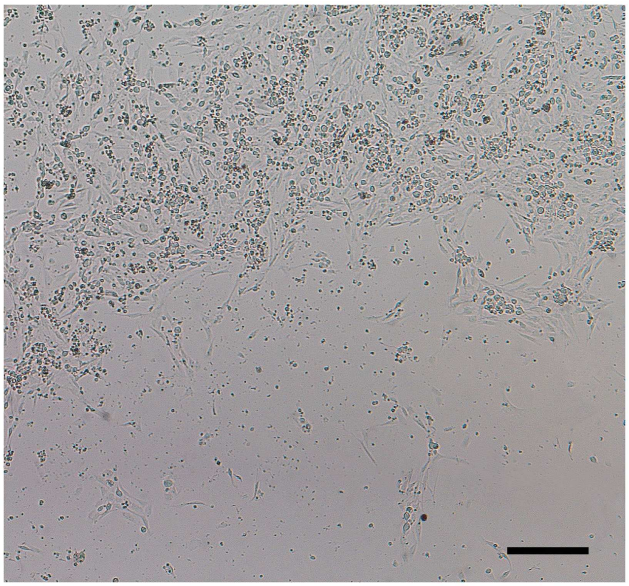

FIGURE 5 | "Edge-effect." DRG neurons cultured in 24-well Seahorse XF24 microplates at RT display uneven distribution when placed directly in a humidified incubator at 37°C and 5% CO_2. This "edge-effect" is caused by a thermal gradient between the periphery and center of each well. DRG neuronal culture at 1 DIC seeded at 50,000 cells/well of an XF24 polystyrene microplate at 5× phase objective. Scale bar: 200 μm.

of primary DRG neurons (Letourneau, 1975; Gundersen and Barrett, 1984; Gundersen, 1988; Sango et al., 1993). Although, the presence of the positive charge afforded by poly-lysine and poly-ornithine is known to be sufficient for dissociated DRG neuronal cultures (Letourneau, 1975; Gundersen and Barrett, 1984; Gundersen, 1988; Delree et al., 1989), the current study demonstrates that at seeding densities below 75,000 cells/well on surface areas $\geq$0.32 cm^2, these substrates alone are not able to maintain DRG neurons over 6 DIC.

This is not surprising *in lieu* of previous reports which show that the presence of trophic support factors other than NGF in media conditioned by glial cells is vital to the adhesion and survival of purified DRG neurons seeded at low density *in vitro* (Gundersen and Park, 1984; Delree et al., 1989). Our results also illustrate the speed and effectiveness with which Thy 1.1 pre-plating in combination with anti-mitotic treatment removes support cells, as this procedure precluded either the time required to condition media and/or the presence of enough glial cells with which to effectively condition the media. Furthermore, since the mere presence of support cells would provide a surface with which to securely attach DRG neurons (Letourneau, 1975; Gundersen and Barrett, 1984; Fallon, 1985), their absence, in the case of poly-lysine and poly-ornithine substrates, would leave DRG neuron attachment at the mercy of a single positive charge.

To ensure the healthiest cultures possible for analysis with the Seahorse XF24 analyzer, we next evaluated the best culture medium in which to grow embryonic DRG neurons at 50,000 cells/well on an XF24 microplate. The most commonly used medias to culture neurons are Neurobasal media (Banker

and Goslin, 1998) and 15% serum AN_2. Neurobasal medium was specifically designed to maintain healthy neuronal cultures while promoting removal of non-neuronal cells (Brewer et al., 1993). However, it was found that supplemented Neurobasal media caused DRG neurons to migrate into large clumps and rapidly detach (**Figures 2A,C**), while 15% serum AN_2 medium maintained healthy, non-aggregated DRG neuronal cultures (**Figure 2B**). Thus, 15% serum-supplemented AN_2 medium was found to be optimal for maintaining healthy DRG neuron cultures at the optimum lowest seeding density for Seahorse XF24 analysis (50,000 cells/well).

Finally, we determined that DRG neurons could be plated and maintained equally well on both polyethylene terephthalate and polystyrene plastic XF24 microplates, assuming seeding number, media, and coating substrates are optimized. Furthermore, DRG neurons were particularly sensitive to temperature. Studies have shown that when microplate cultures are transferred from RT to 37°C, a thermal gradient develops between the periphery and center of each well, as well as between the peripheral and center wells of the plate producing an attachment pattern known as the "edge-effect" (**Figure 5**) that does not correct itself with time (Burt et al., 1979; Oliver et al., 1981; Lundholt et al., 2003). "Edge-effect" can be eliminated by allowing newly seeded DRG neuronal cultures to stay at RT for at least 45 min to 1 h before being transferred to a humidified incubator at 37°C and 5% CO_2.

In conclusion, we have developed a comprehensive protocol that allows reproducible and reliable preparation of DRG neurons suitable for the analysis of mitochondrial respiration using the Seahorse XF24 analyzer. Optimal conditions are summarized in **Table 2** and the optimized step-by-step protocol for culturing E15 DRG neurons for XF24 analysis can be found below. This method will be instrumental for research involved in the investigation of mitochondrial dysfunction underlying peripheral neuropathies, among other neurodegenerative diseases.

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APPENDIX

OPTIMIZED STEP-BY-STEP PROTOCOL

All culture materials, equipment, and reagents should be used sterile unless otherwise indicated.

Day 0: culture plate preparation (day before dissection)

(1) Coat Seahorse XF24 microplates by adding 500 µl of poly-lysine to each well and incubate overnight in a humidified incubator at 37˚C and 5% CO_2.
Coat 3–4 microplates per litter when seeding at 50,000 cells/well.
(2) Prepare anti-Thy 1.1 solution and add 2 ml per 60 mm dish and incubate:
   i. Overnight at 4˚C.
   – OR –
   ii. At room temperature (RT) under a sterile laminar flow hood for ≥4 h.
Coat two 60 mm plastic dishes per litter.

Day 1: dissection and plating

(1) Remove poly-lysine and allow XF24 microplates to dry under a sterile laminar flow hood.
(2) Add 30–40 µl of 10% collagen to each well and let XF24 microplates dry under a sterile laminar flow hood.
This amount supplies enough solution to cover the well bottom, while avoiding excessive capillary action from well edges, which can cause uneven distribution of collagen as it dries.
(3) Dissect and isolate E15 DRG whole explants as described previously (Wood, 1976; McDonald et al., 2005).
(4) Transfer all DRG dissected from one litter to a plastic 15 ml conical tube using a glass Pasteur pipette.
Wash the pipette with 15% serum AN_2 to prevent DRG from sticking.
(5) Centrifuge the conical tube at 800 rpm for 5 min at RT and discard supernatant.
(6) Add 1–2 ml of 0.25% trypsin solution to the conical tube and incubate for 30 min in a 37˚C water bath with constant gentle agitation.
(7) Centrifuge the cell suspension at 800 rpm for 5–10 min at RT and discard supernatant.
(8) Re-suspend DRG in warm 15% serum AN_2 at 2 ml per 10 embryo spines.
Do not exceed this volume (see step 10, Day 1).
(9) Flame a few glass Pasteur pipettes to reduce the opening to <200 µm and mechanically dissociate DRG until the suspension is completely homogenous.
Wash the pipette with 15% serum AN_2 to prevent DRG from sticking.
(10) Remove the anti-Thy 1.1 solution from the 60 mm plates, add 2 ml of the dissociated DRG suspension to each 60 mm plate, and incubate suspensions on plates for 1.5–2 h in a humidified incubator at 37˚C and 5% CO_2.
Do not add more or less than 2–2.5 ml per 60 mm plate. More interferes with cell settling and plate adhesion; less results in drying of plates during incubation.
(11) After 1.5 h, examine plates under an inverted light microscope at 10× phase objective to note the efficiency of non-neuronal cell binding to the culture plate and determine whether additional incubation time is needed.
Do not incubate longer than 2.5 h; DRG neurons will begin to adhere and cultures may dry out.
(12) Transfer cells in suspension from each 60 mm plate to a plastic 15 ml conical tube, *gently* rinse the 60 mm dish with 1–2 ml of warm 15% serum AN_2, and add the rinse to the cell suspension.
Both Thy 1.1 plates from each litter can be combined.
(13) Repeat homogenization of step 9, Day 1, if necessary.
(14) Count cells using Trypan blue exclusion and a hemacytometer.
Cell types can usually be differentiated as follows: DRG neurons are typically largest, round, and phase-bright; non-neuronal cells (i.e., fibroblasts and Schwann cells) are smaller, flat, non-spherical, and phase-bright.
   a. If cells need to be concentrated:
      i. Centrifuge cells in the plastic 15 ml conical tube at 3,000 rpm for 10 min at RT and discard supernatant.
      ii. Re-suspend cells in an appropriate volume of warm 15% serum AN_2 in order to seed each well of the XF24 microplate using a volume of 50–150 µl or less
      iii. Repeat homogenization of step 9, Day 1.
(15) Seed cells at 50,000 cell/well on a XF24 microplate (pre-coated with poly-lysine + 10% collagen) using a volume of 50–150 µl or less per well.
Do not add more or less than 50–150 µl/well. More solution interferes with cell settling and plate adhesion; less results in drying of plates during incubation in step 16, Day 1.
(16) Incubate cells on XF24 microplates at RT under a laminar flow hood for 45 min to 1 h before bringing the total volume of each well to 500 µl with RT 15% serum AN_2 media.
The total volume of each well is arbitrary, as long as there is enough media to keep cultures hydrated for 24 h (≥200 µl) and the volume is known.
Leaving plates at RT for 45 min to 1 h promotes even distribution and adhesion of cells and avoids edge-effect within wells and on the plate as a whole.
(17) Incubate DRG neuronal cultures overnight in a humidified incubator at 37˚C and 5% CO_2.

Day 2: media exchange

(1) Examine XF24 microplates under an inverted light microscope at 5–10× phase objective to note neuronal health, distribution, etc.
(2) Remove media from each well, leaving at least 50 µl of media to cover cells, and replace with 450 µl (or appropriate total volume desired) of warm 15% serum AN_2 containing 20 µM FUDR and 20 µM Uridine.
The total volume of each well is arbitrary, as long as there is enough media to keep cultures hydrated for 24–48 h (≥200 µl) and the volume is known.
(3) Continue incubation of cultures in a humidified incubator at 37˚C and 5% CO_2.

Day 3 – day of assay: DRG neuronal culture and maintenance

(1) Repeat step 2, Day 2, replacing media every other day with fresh warm 15% serum AN_2 containing FUDR/uridine until ∼99% of the non-neuronal cells are eliminated.
DRG neurons should not spend more than 5 days in culture in FUDR/uridine-containing media, as it will become toxic.
(2) Repeat step 2, Day 2, using fresh warm FUDR/uridine-*free* 15% serum AN_2 media for at least 24 h before cells are treated with experimental drugs or analyzed with the Seahorse XF24 platform.

RECIPES

All culture materials, equipment, and reagents should be used sterile unless otherwise indicated.

15% serum AN_2 media ([final] provided)

(1) 15% bovine calf serum
(2) 1.2 mM L-glutamine
(3) 7 mg/ml D-(+)-glucose
(4) Dilute in MEM (+Earl's salts)
(5) Aliquot and store at −20˚C
∗Add NGF (10 ng/ml) fresh with each 15% AN_2 media exchange.

2 mM FUDR (5-fluoro-2′-deoxyuridine) stock (10 ml)

(1) 4.924 mg FUDR
(2) Dilute to 10 ml in ddH_2O
(3) Store covered at 4˚C for up to 2 weeks
∗Add fresh to 15% AN_2 with each media exchange at 1:100 dilution (20 μM [final]).

2 mM Uridine (1-β-D-ribofuranosyluracil) stock (10 ml)

(1) 4.884 mg uridine
(2) Dilute to 10 ml in ddH_2O
(3) Store covered at 4˚C for up to 2 weeks
∗Add fresh to 15% AN_2 with each media exchange at 1:100 dilution (20 μM [final]).

0.25% Trypsin (10 ml)

(1) 0.025 g Trypsin (216 U/mg protein)
(2) Dilute in HBSS (Ca^{2+} and Mg^{2+}-free)
∗Always make fresh the day of use.

Nerve growth factor

(1) Use at 10 ng/ml in 2 mg/ml cytochrome-*c* as a carrier protein in MEM.
(2) Aliquot and store at −80˚C.

Poly-L-lysine hydrobromide (50 ml)

(1) 5 mg of poly-L-Lysine Hydrobromide.
(2) Dilute in ddH_2O.
(3) Excess solution can be stored at −20˚C, but avoid multiple freeze-thawing.

Thy 1.1 (CD90) rat anti-mouse monoclonal antibody solution

(1) Rehydrate anti-Thy 1.1 to 1 mg/ml in DPBS.
(2) Aliquot and store at −20˚C.
(3) Dilute 1:100 in MEM or L-15.
Use of MEM or L-15 does not appear to effect outcome.
∗Add fresh to 15% AN_2 with each media exchange at 1:100 dilution (20 μM [final]).

TIPS

– Estimate ∼30–40 DRG tissues dissected per embryo spine (dependent on dissection efficiency; always estimate low).
– One DRG tissue contains ∼1.0×10^4 total cells; ∼60% support cells, ∼40% DRG neurons.
– Estimate 10 embryos per litter.