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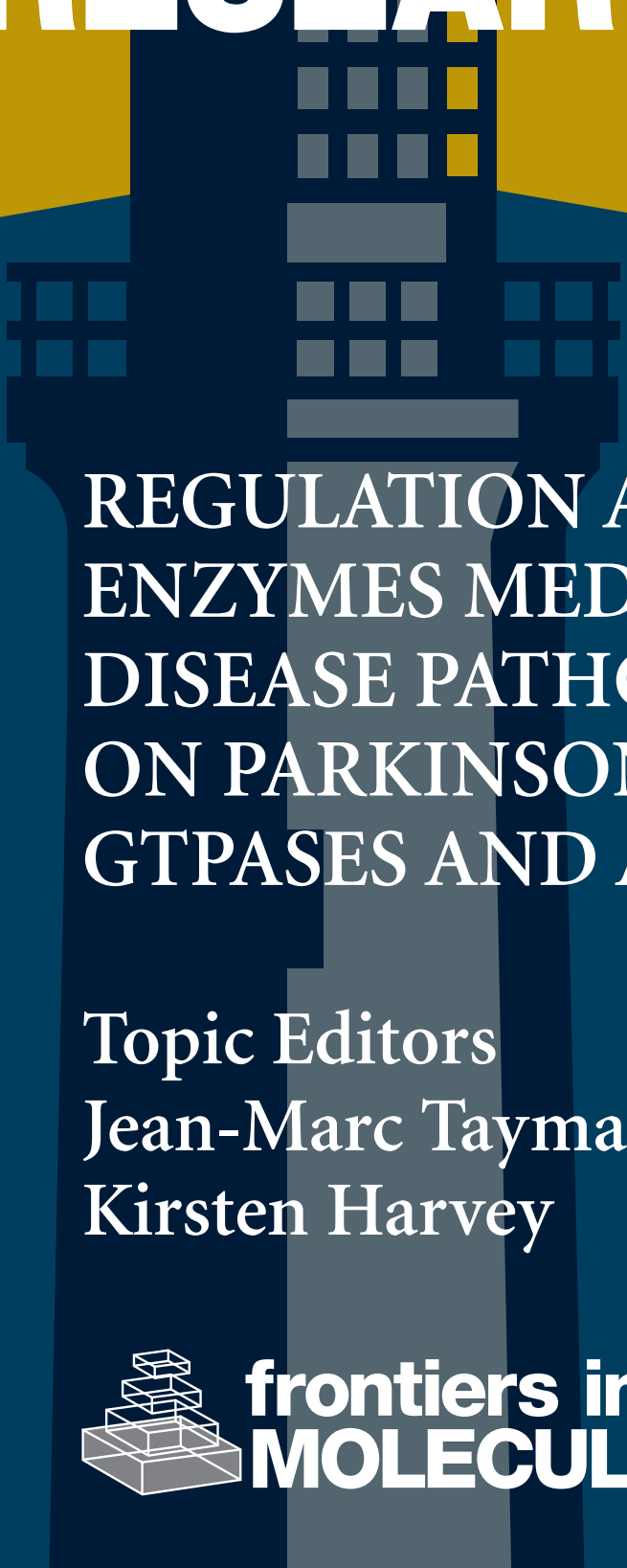

REGULATION AND TARGETING OF ENZYMES MEDIATING PARKINSON'S DISEASE PATHOGENESIS: FOCUS ON PARKINSON'S DISEASE KINASES, GTPASES AND ATPASES

Topic Editors
Jean-Marc Taymans, Veerle Baekelandt and Kirsten Harvey

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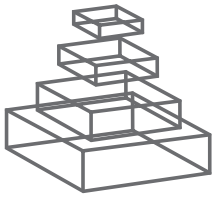

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REGULATION AND TARGETING OF ENZYMES MEDIATING PARKINSON'S DISEASE PATHOGENESIS: FOCUS ON PARKINSON'S DISEASE KINASES, GTPASES AND ATPASES

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Understanding the molecular pathogenesis of Parkinson's disease (PD) is a priority in biomedical research and a pre-requisite to improve early disease diagnosis and ultimately to developing disease-modifying strategies. In the past decade and a half, geneticists have identified several genes that are involved in the molecular pathogenesis of PD. They not only identified gene variants segregating with familial forms of PD but also genetic risk factors of sporadic PD via genome-wide association studies (GWAS). Understanding how PD genes and their gene products function holds the promise of unraveling key PD pathogenic processes. Therefore the precise cellular role of PD proteins is currently the subject of intense investigation.

Interestingly, a number of PD proteins have enzymatic functions, including kinase, GTPase or ATPase functions. In the context of understanding disease pathogenesis or developing disease-modifying therapies, enzymes possess several useful features. Firstly, enzymes are often key elements of cellular signaling networks, acting as on-off switches to determine signaling intensity. For instance, kinases mediate phosphorylation events, which activate or inactivate their substrates, while GTPases modulate activity of their effector proteins via direct interaction in a GDP/GTP dependent manner. ATPases also control cellular processes through their involvement in cellular energy production and/or in transmembrane transport. Secondly, enzymes are attractive targets for therapeutics development. This is exemplified

by the growing number of kinase inhibitors approved for clinical use, while compounds modulating GTPases or ATPases have also been proposed as potential therapeutics. Finally, as elements in cellular signaling networks, enzymes are not generally constitutively active but subject to further regulation through additional signaling components. Knowledge of how PD kinases, GTPases and ATPases are activated or inactivated can aid in understanding how PD signaling networks are deregulated in disease and point to new possibilities in targeting pathological signaling processes.

The objective of this research topic is to provide an overview of current knowledge on the regulation of cellular signaling networks of PD kinases, GTPases and ATPases. Both upstream and downstream signaling events will be covered, with a focus on molecular events that can readily be monitored (relevance as disease biomarkers) and have a potential to be modulated (relevance as potential therapeutic target).

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EDITORIAL
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Regulation and targeting of enzymes mediating Parkinson's disease pathogenesis: focus on Parkinson's disease kinases, GTPases, and ATPases

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Understanding the molecular pathogenesis of Parkinson's disease (PD) is a priority area in biomedical research. It is a pre-requisite to developing disease-modifying strategies and to improve early diagnosis of disease. Over the last two decades, geneticists have identified several genes underlying PD. Some of these gene changes segregate with Mendelian forms of the disease. Others were identified as genetic risk factors of sporadic PD via genome-wide association studies (GWAS).

Interestingly, a number of PD proteins have enzymatic functions, including kinase, GTPase or ATPase activity. As enzymes are often key elements in the regulation of cellular signaling networks, they themselves or additional pathway components might provide new therapeutic targets for disease modifying therapies. For instance, kinases mediate phosphorylation events, which activate or inactivate their substrates, while GTPases modulate activity of their effector proteins via direct interaction in a GDP/GTP dependent manner. ATPases also control cellular processes through their involvement in cellular energy production and/or in transmembrane transport. Finally, as is typical in cellular signaling networks, enzymes are themselves not generally constitutively active, but rather they are subject to regulation. Knowledge of how PD kinases, GTPases, and ATPases are activated or inactivated can aid in understanding how signaling networks are deregulated in PD and can also point to new therapeutic targets. This special Research Topic of *Frontiers in Molecular Neuroscience* discusses new key knowledge on the regulation and targeting of kinases, GTPases and ATPases in PD and presents new original research in this field.

The PD enzymes discussed in this Research Topic were discovered either in genetic linkage or genetic association studies (www.genenames.org/genefamilies/PARK, www.pdgene.org, Lill et al., 2012) or in biological studies revealing a strong functional link with one of the PD proteins. In this context, PD enzymes are the ATPase ATP13A2 encoded by a gene at the PARK9 locus, several GTPases, including the small GTPase RAB7L1, and leucine-rich repeat kinase 2 (LRRK2) which functions as a kinase and GTPase (Greggio, 2012; Taymans, 2012). Several PD proteins are kinases including Pten-induced kinase 1 (PINK1) and LRRK2, discovered in genetic linkage studies in familial PD patients. In addition, LRRK2 and cyclin G associated kinase (GAK) were identified in genetic association studies in sporadic PD sufferers. Importantly, the pathogenicity of some gene products of PD genes is also regulated by phosphorylation by upstream kinases, such as polo-like kinase 2 (PLK2), which phosphorylates the PD protein α-synuclein. There are also multiple kinases phosphorylating the microtubule associated protein tau (MAPT), a protein known to be involved in Alzheimer's disease and associated to PD in GWAS (Lill et al., 2012).

Van Veen et al. review the cellular function and pathological role of ATP13A2 and related P-type transport ATPases in PD and other neurological disorders (Van Veen et al., 2014). P-type ATPases are transmembrane transporters, which use energy from ATP hydrolysis to transport substrates across membranes and against their concentration gradients. The authors provide an overview of this family of proteins and their molecular and biochemical features. An overview is also given of the involvement of P-type ATPases in multiple neurological disorders. They specifically elaborate on ATP13A2, which causes a severe form of early onset Parkinsonism known as Kufor-Rakeb syndrome.

Rivero-Ríos et al. review the evidence pointing to the upstream deregulation of calcium signaling in Parkinson's disease (Rivero-Ríos et al., 2014). Ca^{2+} is not only an important secondary messenger in cellular signaling, but is also essential for the proper functioning of multiple cellular organelles. The authors discuss the role of PD genes in the regulation of Ca^{2+} homeostasis as well as the potential therapeutic strategies to slow PD progression based on counteracting abnormal Ca^{2+} handling.

When studying kinases involved in disease processes, a common pathomechanism is hyperphosphorylation of substrates. In their review, Tenreiro et al. explore the phosphorylation of two important proteins in PD, namely α-synuclein and tau (Tenreiro et al., 2014). A-synuclein is a key protein in PD, as it is a main component of the defining pathological feature of PD, Lewy bodies, and genetic variants of α-synuclein are linked with familial PD and associated with sporadic PD. Interestingly, phosphorylation of one key residue (serine 129) is associated with PD pathology.

By contrast, tau contains multiple phosphorylation sites associated with disease, and is known to be involved in AD, although it also contributes to some forms of Parkinsonism. Another common feature of cellular functioning of kinases is that often multiple kinases functionally interact with each other in signaling networks. Related to this, Matenia and Mandelkow discuss recent work revealing an association of PINK1 with the microtubule associated kinase MARK2 which is involved in axonal transport (Matenia and Mandelkow, 2014).

Arguably, LRRK2 is one of the most important proteins involved in PD, and this is reflected by the number of papers on LRRK2 in this Research Topic. Gilsbach and Kortholt review the available structural biology data on homologs of LRRK2 (Gilsbach and Kortholt, 2014). The structure of other members of this Ras of complex proteins (ROCO) family has potential implications for LRRK2 functioning. Indeed, ROCO proteins from lower organisms such as bacteria and slime molds have yielded atomic structure data, which has begun to reveal mechanistic insight in the functioning of this poorly known family of proteins. The authors focus on structures of the ROCO GTPase and kinase domains. Although there is a good degree of structural and functional heterogeneity between different ROCO proteins, the recently elucidated ROCO structures are suggestive of common mechanisms for ROCO proteins, which can be tested for LRRK2 in the future. In the absence of structures for human LRRK2, the combination of these data with molecular models of LRRK2 (such as the optimized human LRRK2 kinase domain model presented in another paper of this issue, Vancraenenbroeck et al., 2014) can provide insight into the structural underpinnings of LRRK2 functions.

Boon et al. (2014) discuss aspects of LRRK2 as a regulator of cellular signaling, with a focus on reported interactions of LRRK2 with MAPK signaling cascades, GTPases and GTPase regulating proteins. This paper also discusses the key issues in LRRK2 biology to link biochemical activity of LRRK2 to cell biological effects (Boon et al., 2014). Related to this, Cirnaru et al. report original research showing that LRRK2 kinase activity regulates synaptic vesicle trafficking and neurotransmitter release through modulation of LRRK2 macro-molecular complexes (Cirnaru et al., 2014). The LRRK2 kinase activity is currently regarded as a potential therapeutic target, however several issues remain, most specifically in how to monitor this activity in cell culture. Related to this is the fact that LRRK2 is itself a phosphorylated protein (Gloeckner et al., 2010; Lobbestael et al., 2012). The LRRK2 phosphorylation sites are potential correlates for LRRK2 activity. Reynolds et al. (2014) provide an extensive study characterizing in parallel multiple autophosphorylation and cellular phosphorylation sites and explore how the regulation of these sites correlates to LRRK2 kinase activity and biology. Vancraenenbroeck et al. studied another aspect of LRRK2 cellular phosphorylation by using a kinome-wide panel of kinase inhibitors to identify compounds that regulate LRRK2 phosphorylation levels. This type of approach is designed to inhibit upstream kinases phosphorylating LRRK2 and compounds that lead to dephosphorylation are expected to point to these upstream kinases. The correlation of cellular activity with the *in silico* and *in vitro* analysis of the kinase inhibitors shows that the strongest dephosphorylation of LRRK2 is induced by compounds which act on LRRK2 itself, without excluding a role for upstream kinases (Vancraenenbroeck et al., 2014).

Kinases are a very attractive class of drug targets in general and much expertise has been built up from the development of kinase inhibitors for the treatment of cancers. Kinase inhibitors have a relatively low attrition rate in development compared to compounds directed toward other classes of targets and several kinase inhibitors are now in routine clinical practice, mostly in the field of oncology. Developing small compounds to modulate GTPase activities on the other hand is a path much less traveled. Given the number of potential GTPase targets for the treatment of PD, including LRRK2 (Taymans, 2012) or RAB7L1 (Beilina et al., 2014), approaches to discover and develop drugs modulating GTPases merit further investigation. In their review, Hong and Sklar (2014) discuss several GTPases which may be targeted for PD therapy and suggest approaches for GTPase drug discovery. Finally, to have a full overview of kinases involved in PD, it is important to have an understanding of their brain expression patterns. This subject is extensively reviewed by Dzamko et al. (2014) for a number of PD kinases, including LRRK2, GAK, and PLK2.

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We are grateful to all authors and reviewers for their efforts in producing high quality contributions on key enzymes mediating PD, an area that likely holds the key to future PD diagnostics and treatments.

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Cellular function and pathological role of ATP13A2 and related P-type transport ATPases in Parkinson's disease and other neurological disorders

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Mutations in *ATP13A2* lead to Kufor-Rakeb syndrome, a parkinsonism with dementia. ATP13A2 belongs to the P-type transport ATPases, a large family of primary active transporters that exert vital cellular functions. However, the cellular function and transported substrate of ATP13A2 remain unknown. To discuss the role of ATP13A2 in neurodegeneration, we first provide a short description of the architecture and transport mechanism of P-type transport ATPases. Then, we briefly highlight key P-type ATPases involved in neuronal disorders such as the copper transporters ATP7A (Menkes disease), ATP7B (Wilson disease), the Na^+/K^+-ATPases ATP1A2 (familial hemiplegic migraine) and ATP1A3 (rapid-onset dystonia parkinsonism). Finally, we review the recent literature of ATP13A2 and discuss ATP13A2's putative cellular function in the light of what is known concerning the functions of other, better-studied P-type ATPases. We critically review the available data concerning the role of ATP13A2 in heavy metal transport and propose a possible alternative hypothesis that ATP13A2 might be a flippase. As a flippase, ATP13A2 may transport an organic molecule, such as a lipid or a peptide, from one membrane leaflet to the other. A flippase might control local lipid dynamics during vesicle formation and membrane fusion events.

Keywords: alpha-synuclein, mitochondria, mitophagy, lysosome, dystonia, parkinsonism, flippase, heavy metal toxicity

INTRODUCTION

Neurodegenerative diseases, the fourth leading cause of death in developed countries, are characterized by progressive loss of neurons within the central nervous system leading to motor and cognitive dysfunction. Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative disorders (Lees et al., 2009; Tolleson and Fang, 2013). Their prevalence is increasing as a consequence of the ageing population and lack of successful treatments. PD is a progressive movement disorder characterized by severe loss of dopaminergic neurons in the *substantia nigra pars compacta* (Lees et al., 2009). As a consequence of cell death, dopamine content is reduced in the basal ganglia, leading to the motor symptoms observed in patients. The cardinal symptoms of PD are resting tremor, muscle rigidity (stiffness of limbs), bradykinesia (slowness of movements) and postural instability (gait or balance problems) (reviewed in Jankovic, 2008; Lees et al., 2009; Tolleson and Fang, 2013).

A second hallmark of PD is the accumulation of aggregated α-synuclein into Lewy bodies (LBs) (Polymeropoulos et al., 1997). Moreover, mutations in the *SNCA* (α-synuclein) gene were found to be associated with the familial cases of early-onset Parkinson's disease (Spillantini et al., 1997). α-synuclein is able to form amyloid fibrils, β-sheet structures prone to aggregation, which is its main pathogenic feature. α-synuclein overexpression results in endoplasmic reticulum (ER) stress, vesicle trafficking defects, impairment of the ubiquitin-proteasome system and mitochondrial dysfunction (reviewed in Auluck et al., 2010; Bendor et al., 2013).

α-synuclein is mainly found at the presynaptic terminals of neurons (Maroteaux et al., 1988). In presynaptic terminals, α-synuclein interacts with the membranes of synaptic vesicles and associated proteins, where it appears to be a critical regulator of vesicle dynamics at the synapse (reviewed in Auluck et al., 2010; Bendor et al., 2013). It acts as a trafficking partner of synaptobrevin II (sybII) (Gordon and Cousin, 2014). At this location, α-synuclein facilitates the entry of sybII into SNARE complexes, which is a key step in the exocytotic fusion of synaptic vesicles with the presynaptic terminal (Burre et al., 2010; Gordon and Cousin, 2014). The acidic C-terminal of α-synuclein interacts with sybII whereas its N-terminal membrane-associated region is an inducible amphipathic α-helix that obtains its structure only after contact with the membrane (Auluck et al., 2010; Bendor et al., 2013). The amphipathic helix does not enter the membrane bilayer, but aligns itself parallel to the bilayer axis. Amphipathic α-helices are found in several proteins that regulate membrane vesicle trafficking and it is becoming increasingly clear that they

function as membrane curvature sensors (Drin et al., 2007; Drin and Antonny, 2010; Jensen et al., 2011). Synucleins have been shown to both induce and sense membrane curvature (Middleton and Rhoades, 2010; Varkey et al., 2010; Pranke et al., 2011), which can have a significant impact on the basal fusogenic properties of synaptic vesicles.

α-synuclein aggregates accumulate in PD and are cleared via various routes, mainly including the ubiquitin-proteasome system, autophagy and lysosomal degradation pathways (Webb et al., 2003; Cuervo et al., 2004). Besides the accumulation of misfolded proteins, PD is further associated with mitochondrial dysfunction generating reactive oxygen species (ROS) and oxidative stress (Ayala et al., 2007; Auluck et al., 2010). These phenomena mutually affect each other as diseased mitochondria generate more ROS, which in turn exacerbates protein folding defects. Thus clearance of dysfunctional or damaged mitochondria and proper functioning of the protein quality control are essential for neuronal fitness and survival, but are impaired in PD (Devine et al., 2011; Jin and Youle, 2012; Tofaris, 2012; Dehay et al., 2012a). Protein quality control depends on both the proteasome and lysosome. The lysosome mediates end-stage degradation of obsolete or damaged cytoplasmic material, including protein aggregates and organelles such as mitochondria, through autophagy pathways (Webb et al., 2003; Cuervo et al., 2004; Mak et al., 2010; Jin and Youle, 2012; Tofaris, 2012; Dehay et al., 2012a).

ATP13A2 is a late endosomal/lysosomal P5-type transport ATPase that is emerging as a critical regulator of lysosomal functions (Ramirez et al., 2006; Usenovic et al., 2012a; Dehay et al., 2012b; Tsunemi and Krainc, 2014). Mutations in the ATP13A2 gene, belonging to the PARK9 PD susceptibility locus, lead to the Kufor-Rakeb syndrome (KRS), a severe early-onset autosomal recessive form of PD with dementia (Ramirez et al., 2006). Overexpression of ATP13A2 suppresses α-synuclein toxicity. This links two genetic risk factors of PD, *i.e.* ATP13A2 and α-synuclein, highlighting the central role of ATP13A2 in PD (Gitler et al., 2009). Loss of ATP13A2 function is also associated with neuronal ceroid lipofuscinosis, a lysosomal storage disorder (Farias et al., 2011; Bras et al., 2012; Schultheis et al., 2013).

This review will focus on ATP13A2 as an orphan member of the family of P-type transport ATPases. P-type ATPases are a large family of evolutionarily related primary transporters present in Archaea, Bacteria and Eukarya (reviewed in Kuhlbrandt, 2004; Palmgren and Nissen, 2011). They use the energy derived from ATP hydrolysis to transport various substrates, ranging from ions to lipids, across biological membranes against their concentration gradients. P-type ATPases are crucial for the generation of electrochemical gradients, which fuel vital cellular processes, such as secondary transport, excitability, vesicular transport and osmotic balance.

In the following sections we will give a short description of the architecture and transport mechanism of classical P-type transport ATPases. Then, we will provide an overview of those P-type ATPases that are implicated in neuronal disorders. Finally, we will review the recent literature of ATP13A2 and use available knowledge on P-type ATPase functions to discuss ATP13A2's putative cellular function and pathological role in PD.

THE FAMILY OF P-type ATPases

GENERAL FEATURES OF P-type ATPases

P-type ATPases are biological pumps omnipresent in all forms of life, which are recognized by several conserved signature motifs associated with their catalytic mechanism (Axelsen and Palmgren, 1998). The main characteristic of all P-type ATPases is the formation of an acid-stable aspartyl phosphate intermediate during the catalytic cycle (hence the name P-type). The phosphorylated Asp residue is located in a highly conserved DKTG sequence motif located in the cytoplasmic part of the proteins. The events of auto-phosphorylation and auto-dephosphorylation are tightly coupled to substrate binding, transport and release.

According to sequence comparison and phylogenetic analysis, the P-type transport ATPase family can be classified into five distinct subfamilies (P1–P5), which can be further divided into additional subgroups (A, B, etc.) (Axelsen and Palmgren, 1998) (reviewed in Kuhlbrandt, 2004; Palmgren and Nissen, 2011). Importantly, the phylogenetic division correlates well with differences in the preferred transport substrates. The P1-P3 ATPases are well-characterized ion pumps: the P1A are part of bacterial K^+ transport systems, the P1B transport heavy metals, the P2A and P2B are Ca^{2+} pumps, the P2C Na^+/K^+- and H^+/K^+-pumps are found in animals, the P2D are Na^+ pumps in fungi and mosses and the plasma membrane H^+ pumps of P3A are present in fungi and plants. The P3B corresponds to a small group of bacterial Mg^{2+} transporters. In contrast to inorganic ion transport, P4 ATPases participate in lipid flipping across membranes, generating membrane curvature and exposing or removing relevant signaling lipids. The substrate specificity of the last subfamily, the P5 ATPases, has yet to be revealed.

Based on the conserved P-type ATPase motifs, 36 human genes are recognized and annotated in databases to encode for P-type ATPases. These include 2 copper-ATPases, 4 Na^+/K^+-ATPases, 2 H^+/K^+-ATPases, 9 Ca^{2+}-ATPases, 14 putative lipid flippases and 5 P5-type ATPases with unknown substrate specificity (ATP13A1-5). **Figure 1** displays the phylogenetic relationship of these 36 human P-type ATPases and their orthologs in key animal model organisms. Many P-type ATPases display broad expression profiles and fulfill many housekeeping functions, while the expression of other P-type ATPases is restricted to specific tissues e.g., brain, heart, skeletal muscle, etc.

P-type ATPases use metabolic energy (ATP) to actively pump substrates against an electrochemical gradient. To prevent backflow of transported ligand(s), P-type ATPases use an alternating access mechanism (**Figure 2**). After substrate binding from one side, the access pathways from both sides of the membrane are transiently closed, effectively occluding the transported ion(s) in the membrane domain, before releasing them to the other side of the membrane. In addition, the affinity toward the substrate is different at both sides of the membrane. High affinity binding occurs at the side of the membrane with low substrate availability, whereas a drop in the affinity at the other side of the membrane leads to spontaneous release. These two features allow P-type ATPases to generate steep concentration

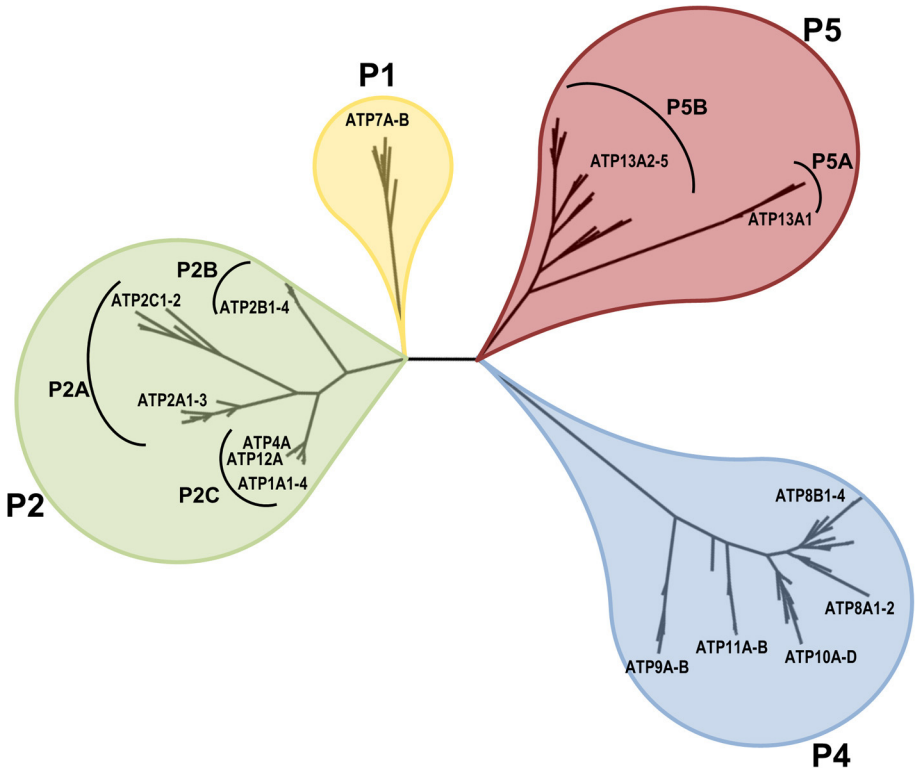

FIGURE 1 | Phyologenetic tree of the human P-type ATPases. Phylogenetic tree based on the core protein sequences of 137 animal homologues of the 36 human P-type ATPase isoforms. ATP13A2 homologues were obtained from the database Homologene http://www.ncbi.nlm.nih.gov/homologene. Core protein sequences were generated according to the methodology described in Axelsen and Palmgren (1998). The 36 human P-type ATPases are indicated. Of note, only animal isoforms are depicted, so the P3A-type ATPases, which are uniquely found in fungi and plants and the small class of bacterial Mg^{+}-ATPases of the P3B group and bacterial pumps belonging to P1A are not represented. The phylogenetic tree was rendered using www.phylogeny.fr (Dereeper et al., 2008, 2010).

and charge gradients (Kuhlbrandt, 2004; Palmgren and Nissen, 2011).

The transport mechanism of P-type ATPases can be described by the model of Post-Albers (Albers, 1967; reviewed in Kuhlbrandt, 2004; Palmgren and Nissen, 2011). During each catalytic cycle, the pumps oscillate between four major conformations (**Figure 2**): E1, E1P, E2P, and E2. The E1 state displays high-affinity ion-binding sites that are exposed to the cytosol. Upon ion binding, the protein reacts with an ATP nucleotide catalyzing auto-phosphorylation generating the E1P state in which ions are occluded. Then, the protein undergoes an often rate limiting transition to the E2P state where the transport binding sites are transformed into low-affinity sites facing the extra-cytosolic side of the membrane. This releases the ion(s) and allows the binding of specific counterion(s). This binding triggers E2P auto-dephosphorylation, returning to the E2 ground state in which counter-ions are occluded. All P-type ATPases are inhibited by orthovanadate, an inorganic phosphate mimic that locks the enzyme in the E2P conformation. Finally, the transition to E1 allows release of counter-ions at the cytosolic side to re-initiate the catalytical cycle.

The transport process can be overall electrogenic if translocation occurs of an unequal amount of charges at both sides of the membrane. Examples are the Na^{+}/K^{+}-ATPase, which transports two Na^{+} ions out of the cell in exchange for three K^{+} ions per hydrolyzed ATP, and the SERCA Ca^{2+}-ATPases, which transport two Ca^{2+} ions from the cytoplasm to the lumen of the ER/SR for two to three H^{+} in the other direction. Other P-type ATPases transport only in one direction using either the forward E1 to E1P step to bind the transported substrate (P1B copper-ATPase, P3 H^{+}-ATPase) while others use the E2P step for substrate binding (P4 lipid flippases) (Kuhlbrandt, 2004; Palmgren and Nissen, 2011).

THE P-type ATPase ARCHITECTURE

The two archetypical members of the P-type ATPase family are the sarco(endo)plasmic reticulum (SR/ER) Ca^{2+}-ATPase SERCA1a and the (α1 subunit of) Na^{+}/K^{+}-ATPase for which there is a wealth of structural and kinetic information. SERCA1a was the first P-type ATPase to have its structure solved at high resolution (Toyoshima et al., 2000), providing detailed insights into the overall domain organization of a P-type ATPase. Since then, several other conformational states of SERCA1a have been resolved using several inhibitors, transition analogs and nucleotides locking the protein in intermediate steps of the transport mechanism. Together with a strong biochemical characterization and extensive

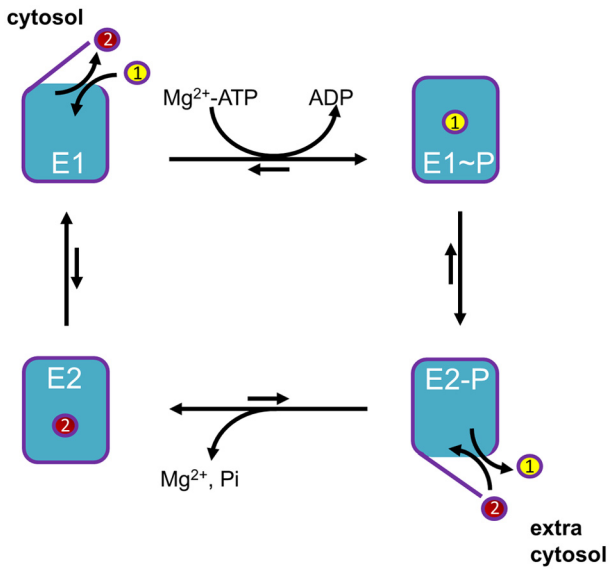

FIGURE 2 | General Post-Albers reaction scheme for P-type ATPases. A cytosolic ligand (yellow, transported ligand 1) is transported to the extracytosolic space, whereas an extracytosolic ligand (red, counter-transported ligand 2) is imported into the cytosol. Note that the number of ligands in each direction may vary between different P-type ATPase isoforms. In short, P-type ATPases switch between two major conformations E1, with ligand binding sites facing the cytosol, and E2, with ligand binding sites facing the extracytosolic side of the membrane. The induced fit of ligand 1 binding in E1 promotes phosphorylation by Mg^{+}-ATP. In this E1∼P state the ligand 1 becomes occluded. The rate-limiting E1∼P to E2-P transition is accompanied by major conformational changes, reorienting the ligand-binding sites toward the extracytosolic space. This decreases the affinity of the binding site for ligand 1, whereas the affinity for ligand 2 is increased. As a result, ligand 1 is released into the extracytosolic space via an open exit pathway for ligand 1 and the counter-transported ligand 2 can enter the binding cavity. The resulting conformational changes lead to dephosphorylation of E2P and the released inorganic phosphate is expelled. The ligand 2 becomes occluded, whereupon the pump is reset to the E1 state, reducing the affinity for ligand 2. The pump can now start a new cycle.

mutagenesis, this has culminated in a detailed description of the transport mechanism of the Ca^{2+}-ATPase at atomic resolution, which provides the scaffold to understand the transport process in other P-type ATPases. For a careful discussion of this topic, the reader is referred to excellent in depth reviews and references in (Toyoshima, 2009; Moller et al., 2010; Palmgren and Nissen, 2011).

Crystal structures have also been presented for other P-type ATPases including the H^{+}-ATPase of plants (Pedersen et al., 2007), the Na^{+}/K^{+}-ATPase (Morth et al., 2007; Shinoda et al., 2009; Kanai et al., 2013; Nyblom et al., 2013) and the copper-ATPase of *Legionella pneumophila* (Gourdon et al., 2011) (**Figure 3**). Comparison of these structures has revealed that P-type ATPases share a strikingly similar fold despite strong sequence divergence. Four principal domains are recognized, which are conserved throughout the family: three cytoplasmic domains (nucleotide-binding, N; phosphorylation, P; actuator, A) and a transmembrane (TM) domain (M domain) (**Figure 3**).

During the catalytic transport process the N-domain binds ATP and serves as a built-in protein kinase, which auto-phosphorylates the P-domain. The A-domain acts as an intrinsic protein phosphatase dephosphorylating the P-domain later in the catalytic cycle. The process of phosphorylation and dephosphorylation is tightly coupled to formation and deformation of high-affinity transport-binding sites in the M domain by an allosteric mechanism (Toyoshima, 2009; Moller et al., 2010; Palmgren and Nissen, 2011).

The N-domain is the least conserved cytoplasmic domain among P-type ATPases and forms the ATP binding pocket. It is situated as a large insert into the P-domain sequence stretch and is connected by a highly flexible hinge region linking the N- and P-domains (Toyoshima et al., 2000). The strongly conserved P-domain contains the P-type ATPase fingerprint with the critical Asp residue (DKTG). During each catalytic cycle, the Asp residue is alternately phosphorylated and dephosphorylated by the N-domain and the A-domain, respectively. A Lys in the nucleotide interaction site of the N-domain (KGAPE) interacts with the adenine ring of ATP delivering the γ-phosphate to the active-site residue of the P-domain. This reaction renders a high-energy aspartyl phosphate intermediate. Subsequently, the A-domain subjects the bond to hydrolysis, catalyzed by the Glu residue in the highly conserved signature motif TGE (Toyoshima, 2009; Moller et al., 2010; Palmgren and Nissen, 2011).

The M-domain, the largest of the four principal domains, comprises six to twelve α-helices (Bublitz et al., 2011) and plays a crucial role in substrate binding and transport. The cytoplasmic domains are connected to the M-domain by five flexible linker regions, four in P1-type ATPases (Palmgren and Nissen, 2011). The substrate translocation pathways are centered on the M1–M6 segments. M4 is critically important for substrate specificity and coordinating the substrate in the binding pocket. The sequence of the M4 region thus diverges between the five P-type subfamilies, corresponding to the difference in substrate specificities. M4 involves a highly conserved Pro residue, which induces unwinding of M4. This twist exposes backbone carbonyl oxygens that are used to coordinate the transported ligand (Palmgren and Nissen, 2011).

P-type ATPases also hold extended N- or C-terminal tails that regulate pump activity by intra-molecular interaction (Vandecaetsbeek et al., 2009) or via interaction of regulatory proteins (Vincenzi et al., 1980). The extensions may in addition control subcellular localization (Petris et al., 1998) or substrate delivery (Gourdon et al., 2011). Often, the N and C termini are auto-inhibitory, preventing the activation of the transporter and requiring additional stimuli for pump activation (Ekberg et al., 2010; Zhou et al., 2013).

THE P-type ATPase ION TRANSPORT CYCLE

To translocate substrates across the membrane, P-type pumps undergo extensive conformational changes, which are driven by ATP hydrolysis. In the next section, the general catalytic mechanism of P-type ATPases will be explained based on the Ca^{2+} transport cycle of the SERCA1a pump (based on references in Toyoshima, 2009; Moller et al., 2010; Palmgren and Nissen, 2011).

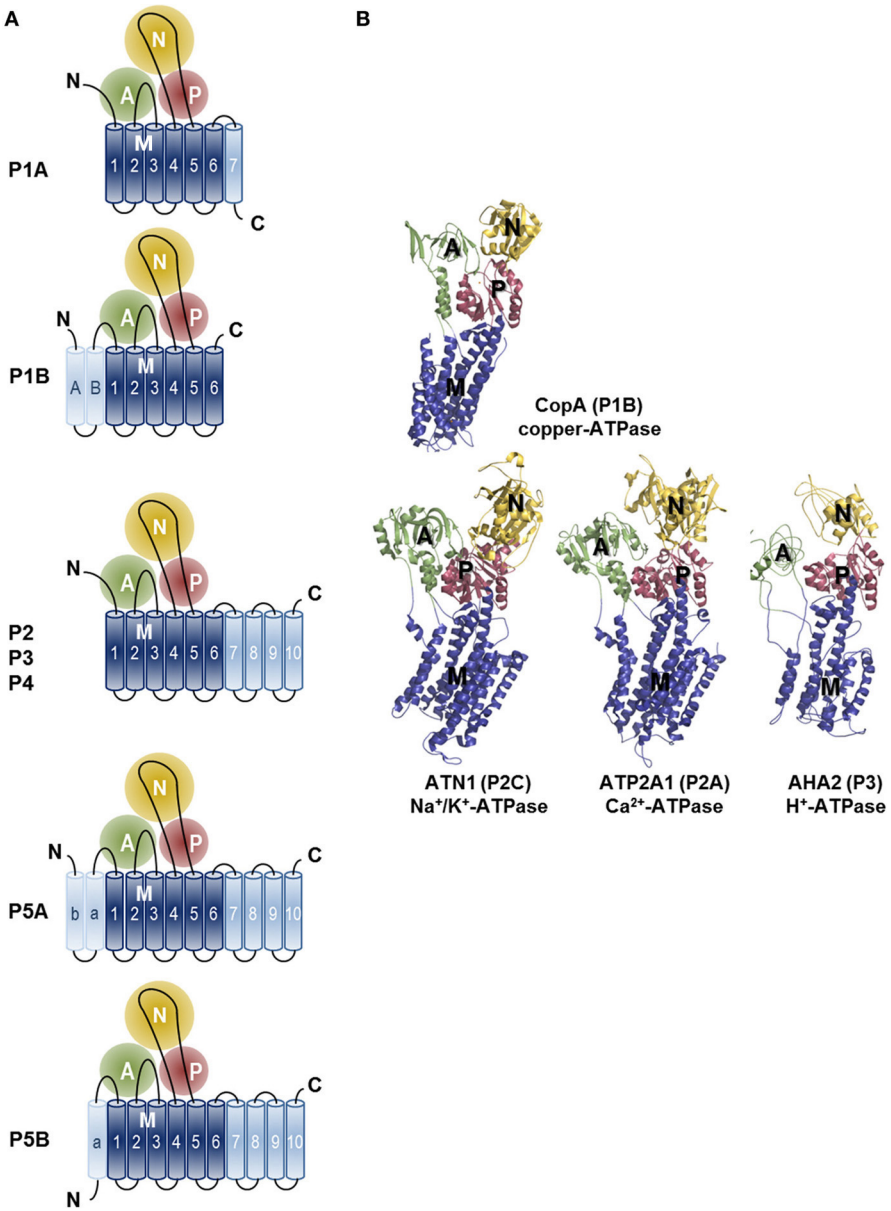

FIGURE 3 | Topology and architecture of the catalytic subunits of P-type ATPases. (A) Planar topology models of the five classes of P-type ATPases (P1-P5). Nucleotide-binding domains (N, yellow), actuator domains (A, green) and phosphorylation domains (P, red) are indicated. The 6 TM helices (Polymeropoulos et al., 1997; Spillantini et al., 1997; Jankovic, 2008; Lees et al., 2009; Auluck et al., 2010; Tolleson and Fang, 2013) form the core segment of the membrane (M) domain of all P-type ATPases, which is depicted in dark blue, whereas additional N- and C-terminal helices are shown in light blue. Of note, there is one exception for the P2 ATPases, a splice variant of ATP2A2, SERCA2b, harbors an 11th TM helix at the C-terminus (not shown, Vandecaetsbeek et al., 2009). **(B)** Resolved P-type ATPase crystal structures, known up until now. The *Legionella pneumophila* CopA copper-ATPase (PDB 3RFU), a P1B-type ATPase; the rabbit P2A-type ATPase ATP2A1 (SERCA1a, PDB 2AGV), the *Squalus acanthias* Na^+/K^+-ATPase α-subunit ATN1 (PDB 3A3Y), a member of the P2C group and the *Arabidopsis thaliana* proton pump AHA2 (PBD 3B8C) of the P3-type ATPases. N-, A-, P- and M-domains are indicated with similar colors as in the planar models. Note that the obligatory subunits of the P1A, P2C and P4 are not shown.

Ca^{2+} entry and binding: E2 → E1 · 2Ca^{2+}

At the start of the catalytic cycle, cytosolic Ca^{2+} ions interact with the high-affinity binding sites in the M-domain in the E1 conformation. The binding of the two Ca^{2+} ions is sequential and cooperative. The first Ca^{2+} proceeds to site I where its binding repositions a critical Asp residue on M6 (D800), which now forms the second Ca^{2+} binding site II. Upon binding of the second Ca^{2+}, the gating residue E309 on M4 will capture the second Ca^{2+} ion in site II.

Phosphorylation and occlusion: E1 · 2Ca^{2+} → E1∼P · 2Ca^{2+}

Via the induced fit mechanism of Ca^{2+} binding, the rearrangement of the TM helices is transmitted to the P-domain creating a Mg^{2+}-binding site near the critical Asp residue (Asp^{351} in

SERCA1a). Presence of Mg^{2+} is essential as this cofactor decreases the electrostatic repulsion of the γ-phosphate of ATP by the negatively charged Asp and hence, allows phosphate transfer. In this way, ATPase activity of P-type transporters in the cytosolic domains is tightly coupled to the ion binding in the M-domain, preventing unnecessary ATP hydrolysis. The transition toward the intermediate E1∼P phosphorylated state bends the P-domain and tilts the A-domain that rests on the P-domain. This exerts strain on the linkers between the A-domain and M1, M2, and M3 of the M-domain. As a result, M1-M2 is partially lifted out of the membrane forcing E309 in a fixed position, which closes the Ca^{2+} entry path (occlusion).

Ca^{2+} release: E1∼P → E2-P

Following complete γ-phosphate transfer, the ATP-mediated connection between the N- and P-domains is lost. As a consequence, the pump relaxes and the N-domain moves away from the catalytic site and stretches the linker region between the M3 helix and the A-domain. The generated tension triggers rotation of the A-domain and results in transition to the low-energy E2P state. The significant conformational changes associated with the E1P to E2P transition is the rate-limiting step in the catalytical cycle. The rearrangement of the pump opens the luminal exit pathway for Ca^{2+} by spreading out M1/M2 and M3/M4 away from M5/M6. In addition, this reduces the affinity of the Ca^{2+}-binding sites promoting the luminal release of Ca^{2+}. In exchange, two to three H^+ ions bind with high affinity to the E2P state leading to occlusion of the luminal gate and further rotation of the A-domain. The A-domain rotation also brings the TGE loop closer to the phosphorylation site, shielding the aspartyl-phosphate by restricting the access of ADP or water.

Dephosphorylation and occlusion: E2-P → E2

The Ca^{2+}-ATPase pump is reset to E1 by a series of reversal reactions leading to E2P dephosphorylation and proton countertransport. Entry of a water molecule induces a new rotation of the A-domain, which now precisely positions the Glu of the TGE-loop and the water molecule to catalyze an attack on the aspartyl phosphate. The rotation of the A-domain also repositions M1/M2 and the cation-binding site with the protons becomes occluded. Thereafter, the A-domain disengages from the phosphorylation site resulting in transition of the E2 state to the relaxed E1 conformation associated with release of the counterions.

DOMAIN ORGANIZATION AND SIGNATURE MOTIFS IN P5B-type ATPases

In comparison with the well-studied SERCA1a Ca^{2+} pump, little is known about the P5-type ATPases to which ATP13A2 belongs. P5-type ATPases are found in all eukaryotic genomes, but are absent in bacterial genomes (Moller et al., 2008; Sorensen et al., 2010). Based on the conservation of residues in the putative transport binding sites (Moller et al., 2008) and on their predicted TM topology (Sorensen et al., 2010), the P5 subfamily can be divided into two groups, P5A and P5B (**Figures 1**, **3**). Exactly one P5A (ATP13A1 in humans) and at least one P5B isoform is found in all eukaryotic genomes, except for land plants, which have lost the P5B genes (Moller et al., 2008). Multiple P5B isoforms exist in higher vertebrates (four in humans, ATP13A2-5) and some invertebrate lineages (three in *Caenorhabditis elegans*, CATP-5 to 7).

The P5-ATPase membrane topology is unusual (Sorensen et al., 2010). In addition to the 10 classical TM helices, two extra TM spanning helices are predicted in the N terminus of the P5A ATPases (Ma and Mb), whereas the P5B group is marked by a single predicted N-terminal TM helix (Ma). Therefore, the P5A would consist of 12 and the P5B of 11 TM helices. The poor sequence conservation of the extra N-terminal helices suggest that they are not critical for substrate coordination during transport (Sorensen et al., 2010). Instead, as in other P-type ATPases, the N-terminal region may directly function as a regulator of catalytic function or serve as the docking site for other regulatory proteins (Gourdon et al., 2011). Of all P-type ATPases, only the P5 and the P1B heavy metal pumps contain additional N-terminal membrane-spanning segments (**Figure 3**). The structure of a P1B pump, the CopA copper transporter, was recently solved (Gourdon et al., 2011). The structure clearly depicts that the Mb forms an N-terminal docking platform for the binding of a copper chaperone, which delivers copper to the pump directly, or via the N-terminal heavy metal binding domains (Gourdon et al., 2011). Interestingly, also the P5 ATPases contain conserved Pro or Gly residues at a similar position in the additional N-terminal membrane helices (Mb for P5A ATPases Sorensen et al., 2010 and Ma for P5B ATPases). In analogy herewith, the extended N terminus of the P5 may follow a similar fold as in the P1B, representing a docking platform for substrate delivery.

Like other P-type ATPases, P5 isoforms, including ATP13A2, contain the key signature motifs KGAPE for ATP coordination (N-domain), DKTG for auto-phosphorylation (P-domain) and TGE for dephosphorylation (A-domain) (Kwasnicka-Crawford et al., 2005; Ramirez et al., 2006). This indicates that P5-ATPases like other P-type ATPases catalyze the hydrolysis of ATP to autophosphorylate the enzyme on a conserved Asp residue in the P-domain.

The presence of a highly conserved M4 region within P5 ATPases indicates that in P5-type ATPases the hydrolysis of ATP may be coupled to the transport of a ligand close to the M4 region (Moller et al., 2008; Sorensen et al., 2010). Spontaneous auto-phosphorylation has already been observed for the yeast P5A ATPase Spf1p and the plant P5A ATPase HvP5A1 (Corradi et al., 2012; Sorensen et al., 2012), but so far it remains unclear whether P5B-ATPases such as ATP13A2 also form a phosphointermediate.

The M4 segment of the P5-type ATPase corresponds to the putative substrate binding site and contains a double Pro in (PPxxP) (Moller et al., 2008; Sorensen et al., 2010). This may exacerbate the twist of the M4 segment imposed by the initial Pro, which might have a significant impact on the mechanism of substrate coordination and transport. P5A sequences contain a PP(E/D)xPx(E/D) motif, whereas P5B sequences are characterized by a PP(A/V)xP(A/V)x motif (Moller et al., 2008; Sorensen et al., 2010) (**Figure 4**). The negative charges in the unwound M4 helix of P5A compared to the corresponding hydrophobic residues in P5B may suggest that both subgroups display different substrate specificities (Moller et al., 2008; Sorensen et al., 2010).

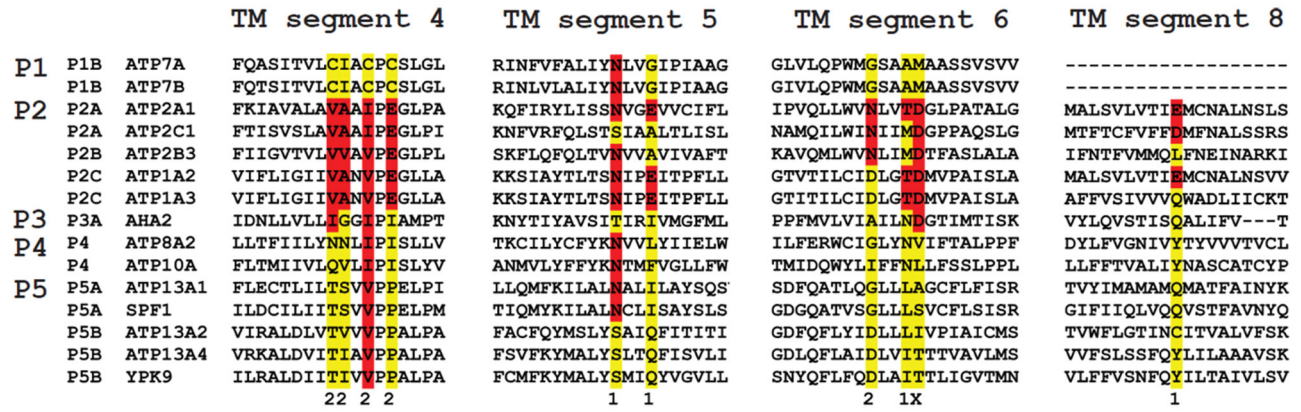

FIGURE 4 | Sequence comparison of the TM helices in P-type ATPases of various subfamilies. The residues involved in Ca^{2+} binding in the two Ca^{2+} binding sites (site 1 and 2) in the SERCA1a Ca^{2+} pump (ATP2A1) are distributed over four TM helices: M4, 5, 6 and 8. The colored residues are part of the Ca^{2+} binding sites in ATP2A1 and numbers 1 and 2 refer to the number of the Ca^{2+}-binding site to which the residue contributes (x is contributing to both site 1 and site 2). The sequence of the M4, M5, M6 and M8 helices is compared with those of the P-type ATPases that are involved in neurological disorders. Also the yeast P5 ATPases Spf1p and Ypk9p, the Ca^{2+}/Mn^{2+}-ATPase SPCA as well as the proton pump AHA2 are included for comparison. M4 shows the highest degree of conservation. Highlighted in red are conserved residues as compared to the ATP2A1 Ca^{2+} binding site sequence, whereas in yellow the non-conserved residues are indicated. For each subfamily, a signature motif can be recognized in M4, which corresponds well with the substrate specificity. The PPELP and PPALP sequences of P5A- and P5B-type ATPases have little in common with other P-type ATPase signature motifs, which might indicate that the transported ligand is significantly different.

For this reason, we will focus only on the P5B ATPases in this review.

P-type ATPases AND NEURODEGENERATION

P-type ATPases play important roles in the nervous system, ranging from regulation of Ca^{2+} homeostasis, osmotic balance, electrical excitability, uptake of trace elements to vesicular transport processes. It is therefore not surprising to see that loss-of-function mutations in many P-type ATPase isoforms are detrimental for neuronal functions. In the following sections we will provide a short overview of those P-type ATPases that according to genetic information are implicated in neurological disorders (**Table 1**).

P1B-type ATPases IN NEUROLOGICAL DISORDERS

The P1B-type ATPase subfamily consists of the two copper-transporting isoforms in human, ATP7A and ATP7B. ATP7A is ubiquitously expressed in all tissues, including the brain, but except the liver, regulating homeostatic maintenance of cell copper levels. ATP7B is highly expressed in the liver. Mutations in ATP7A are associated with Menkes disease (MD) (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993), while ATP7B mutations cause Wilson's disease (WD) (Bull et al., 1993) (reviewed in Gupta and Lutsenko, 2009; Kaler, 2011; Telianidis et al., 2013).

ATP7A

Mutations in *ATP7A* are associated with MD, an X-linked recessive disorder characterized by progressive neurodegeneration and connective tissue dysfunction (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993). The clinical manifestations of MD include severe seizures associated with cerebral atrophy, vascular abnormalities, kinky hair structure, hypopigmentation, growth retardation and death in early childhood. These features originate from a generalized copper deficiency that triggers dysfunction of several cuproenzymes (Kaler, 2011). *ATP7A* mutations also underlie occipital horn syndrome (OHS), a milder disease with moderate neurologic symptoms and prominent connective tissue disturbances (Kaler, 2011). Recently, a novel ATP7A-related disease phenotype was discovered, spinal muscular atrophy, distal, X-linked 3 (SMAX3), which is characterized by atrophy of the lower limb muscles (Kennerson et al., 2010).

ATP7A is targeted to the trans-Golgi network from where it supplies copper to the copper-dependent enzymes as they migrate through the secretory pathway. Under conditions of elevated copper, ATP7A relocalizes to the plasma membrane where it promotes the efflux of copper from cells (Petris et al., 1998). To date, over 500 disease-causing variations of *ATP7A* have been reported, mostly substitutions and deletions (ATP7A database, www.LOVD.nl/ATP7A) which lead to misfolding (Kim et al., 2002), impaired copper-induced trafficking (Kim et al., 2003) or reduced copper-ATPase activity (Paulsen et al., 2006).

ATP7B

WD is an autosomal recessive disorder caused by mutations in *ATP7B* (Bull et al., 1993), a copper-ATPase which is mainly expressed in liver and implicated in biliary copper excretion (Telianidis et al., 2013). ATP7B dysfunction results in the toxic buildup of copper in liver. Brain copper accumulation develops secondary to liver disease and leads to degeneration of the basal ganglia. As a consequence, WD patients present movement disorders such as tremor, dystonia and parkinsonism (Machado et al., 2006).

A number of single nucleotide polymorphisms in ATP7B are associated with an increased risk of AD (Squitti et al., 2013). Furthermore, it has been suggested that a single mutated ATP7B allele may confer susceptibility for (late-onset) parkinsonism (Sechi et al., 2007).

Table 1 | P-type ATPases involved in neuronal disorders.

P-type	Gene	Substrate	Disorder	Ref.
P1B	ATP7A	Cu^+	Menkes disease (MD) Occipital horn syndrome (OHS) Spinal muscular atrophy, distal, X-linked 3 (SMAX3)	[OMIM:309400] [OMIM:304150] [OMIM:300489]
P1B	ATP7B	Cu^+	Wilson disease (WD) Possible genetic risk factor for Alzheimer's disease (AD) and parkinsonism	[OMIM:277900] Bull et al., 1993; Telianidis et al., 2013
P2B	ATP2B3	Ca^{2+}	Early onset X-linked spinocerebellar ataxia 1	[OMIM:300014]
P2C	ATP1A2	Na^+/K^+	Familial hemiplegic migraine type 2 (FHM2) Alternating hemiplegia of childhood 1 (AHC1)	[OMIM:602481] [OMIM:104290]
P2C	ATP1A3	Na^+/K^+	Rapid-onset dystonia parkinsonism (DYT12, RDP) Alternating hemiplegia of childhood 2 (AHC2)	[OMIM:128235] [OMIM:614820]
P4	ATP8A2	PS	Cerebellar ataxia, mental retardation and disequilibrium syndrome 4 (CAMRQ4)	[OMIM:615268]
P4	ATP10A	?	Angelman syndrome (AS)	Blanco-Arias et al., 2009
P5B	ATP13A2	?	Kufor-Rakeb syndrome (KRS) Neuronal ceroid lipofuscinosis (NCL)	[OMIM:606693]
P5B	ATP13A4	?	Specific language impairment (SLI) autism spectrum disorders (ASD)	Gourdon et al., 2011; Lohmann and Klein, 2013 Ugolino et al., 2011

OMIM entries are shown for the most well established genetic disorders. References are shown for disorders that have been linked genetically to mutations in the indicated gene(s).

P2-type ATPases IN NEUROLOGICAL DISORDERS

The P2-type ATPases constitute the best characterized subfamily of P-type ATPases. The human P2 isoforms can be subdivided into three groups, P2A, P2B and P2C (P2D is not represented in humans, but consists of eukaryotic Na^+-ATPases). The P2A group contains the well-known SERCA and Secretory Pathway/Golgi (SPCA) Ca^{2+}-ATPases, whereas the plasma membrane Ca^{2+}-ATPases (PMCAs) belong to the P2B-ATPases. The P2C-subgroup encompasses the Na^+/K^+-ATPases and the gastric H^+/K^+-pumps (Kuhlbrandt, 2004; Palmgren and Nissen, 2011).

Na^+/K^+-ATPase

The Na^+/K^+-ATPase generates vital Na^+ and K^+ gradients over the plasma membrane by expelling three Na^+ ions in exchange for two K^+ ions. This is essential for many physiological functions in the nervous system such as cell volume control, the drive of secondary active transport systems and the support of electrical excitability (reviewed in Benarroch, 2011). The α-subunit is the catalytical subunit of the Na^+/K^+-ATPase that exists in four isoforms (ATP1A1-4 or α1-4), which display tissue specific and developmental dependent expression. Only the *ATP1A1-3* genes are expressed in the nervous system. The α-subunit forms a hetero-oligomer with a β- and γ-subunit. β is critical for proper targeting and affects the K^+ affinity (Hasler et al., 2001), whereas γ (belonging to the FXYD family) mainly regulates the Na^+ affinity of the pump (Geering, 2005).

The neurological disorders familial hemiplegic migraine type 2 (FHM2), alternating hemiplegia of childhood (AHC), and rapid-onset dystonia parkinsonism (RDP) are autosomal dominant disorders caused by mutations of the Na^+/K^+-ATPase α2 (FHM2 and AHC1) and α3 (AHC2 and RDP) isoforms. α2 (ATP1A2) is primarily expressed in astrocytes and drives Na^+-dependent Glu uptake and removes excess K^+ from the extracellular space during neuronal excitation. α3 (ATP1A3) is predominantly expressed in neurons and is involved in post-stimulus recovery (reviewed in Brashear et al., 2014).

ATP1A2. *ATP1A2* mutations lead to FHM2, a severe subtype of migraine with aura and temporary hemiparesis. More than 20 mutations in the α2-subunit are known to cause FHM2 (De Fusco et al., 2003; Jurkat-Rott et al., 2004; Schack et al., 2012). A disturbed clearance of extracellular K^+ by glial cells underlies FHM2, which is related to an impaired pumping rate (Schack et al., 2012). *ATP1A2* mutations may also underlie basilar migraine (BM), which is a subtype of migraine with aura originating from the brainstem or involvement of both hemispheres (Ambrosini et al., 2005). In addition, a mutation in the *ATP1A2* gene was also identified in affected members of a family with AHC1 (Swoboda et al., 2004). FHM2, BM, and AHC1 are allelic disorders with overlapping phenotypes.

ATP1A3. Mutations in *ATP1A3* (also known as dystonia-12, DYT12) lead to RDP, which is a rare autosomal dominant movement disorder with variable penetrance, characterized by the abrupt start of dystonia with signs of parkinsonism (de Carvalho Aguiar et al., 2004). The onset of RDP (at the age of 4–55) is often triggered by physical or emotional stress, fever, childbirth, or alcohol consumption. RDP-associated mutations are predominantly located in highly conserved residues in the TM domain of ATP1A3, which mainly affect the Na^+ affinity (Rodacker et al., 2006; Blanco-Arias et al., 2009). The resulting intracellular Na^+ increase may possibly affect the Na^+/Ca^{2+} exchange system and subsequently lead to increased intracellular Ca^{2+} impacting on Ca^{2+}-dependent signaling pathways, such as neurotransmitter

release (Rodacker et al., 2006). Mutations in *ATP1A3* are also implicated in AHC2 (Heinzen et al., 2012), which generally has earlier onset than RDP and is characterized by transient episodes of hemiplegia often shifting from one side of the body to the other. AHC2 and RDP present overlapping clinical features such as dystonia with a bulbar preference (Heinzen et al., 2012; Brashear et al., 2014).

According to the crystal structure of Na^+/K^+-ATPase, the C-terminal tail is inserted within a binding pocket between TM helices (Morth et al., 2007). This tail controls Na^+ and proton binding at the third Na^+ site. At least eight disease mutations occur in this C-terminal ion pathway (Poulsen et al., 2010). Two disease mutations have been reported in which the C terminus is extended by one Tyr residue in a patient with RDP (Blanco-Arias et al., 2009) and by a 28-residue long segment in a patient with FHM2 (Jurkat-Rott et al., 2004).

ATP2B3

PMCA isoforms (ATP2B1-4) remove Ca^{2+} from the cytosol to the extracellular environment. *ATP2B3* mutations cause early onset X-linked spinocerebellar ataxia-1, a disorder characterized by degeneration of the cerebellum (Zanni et al., 2012). Clinical manifestations include hypotonia at birth, dysarthria, gait ataxia difficulty standing, slow eye movements and delayed motor development. PMCA3 is highly expressed in the cerebellum, a critical region for motor coordination (Zanni et al., 2012).

P4-type ATPases IN NEUROLOGICAL DISORDERS

The human genome encodes 14 P4-type ATPases, which are putative lipid flippases involved in aminophospholipid transport across membrane bilayers. P4-type ATPases are the first class of P-type transporters that do not transport inorganic ions (reviewed in Graham, 2004; Poulsen et al., 2008; van der Mark et al., 2013).

ATP8A2

The P4-type ATPase ATP8A2 is a phosphatidylserine (PS) translocase, which is localized to the plasma membrane and highly expressed in retina and brain, particularly in the cerebellum. The P4 lipid flippase ATP8A2 is involved in localization of PS to the inner leaflet of the plasma membrane (Zhu et al., 2012). A missense mutation located in a M domain of ATP8A2 is associated with cerebellar ataxia, mental retardation and dysequilibrium syndrome (CAMRQ), an autosomal recessive disorder characterized by dysarthric speech and cerebellar atrophy with or without quadrupedal gait (Onat et al., 2013). Mice carrying loss-of-function mutations in the *Atp8a2* gene develop axonal degeneration resulting in progressive ataxia and neurodegeneration (Zhu et al., 2012). Loss of ATP8A2 disrupts PS asymmetry which might lead to fragile neuronal membranes that are more prone to degeneration. Defective vesicular trafficking may provide an alternative explanation (Zhu et al., 2012).

ATP10A

ATP10A is a putative aminophospholipid translocase. *ATP10A* maps within the most common interval of deletion (15q11-q13) leading to Angelman syndrome (AS). This syndrome is marked by neurobehavioral anomalies that include severe mental retardation, ataxia and epilepsy. AS patients with imprinting mutations or with maternal deletions of 15q11-q13 display little or no ATP10A expression (Meguro et al., 2001).

P5-type ATPases IN NEUROLOGICAL DISORDERS

Two members of the P5-type ATPases are implicated in neurological disorders. Little is known about the substrate specificity and cellular function of P5-type ATPases, but their putative function is extensively discussed in section 5 of this review.

ATP13A2

Loss-of-function mutations in ATP13A2 (PARK9) are a known cause of Kufor-Rakeb syndrome (KRS), an autosomal recessive disorder characterized by juvenile-onset Parkinsonism associated with dementia (Ramirez et al., 2006). This syndrome was first described in 1994 in a consanguineous family originating from Kufor-Rakeb, Jordan (Najim al-Din et al., 1994). Today, several homozygous and compound heterozygous mutations have been described that result in truncation of the ATP13A2 protein leading to loss-of-function (Ramirez et al., 2006; Schneider et al., 2010; Crosiers et al., 2011; Park et al., 2011). The clinical phenotype of KRS comprises pyramidal degeneration, supranuclear gaze palsy and severe cognitive decline (Williams et al., 2005). Brain MRI of KRS patients revealed generalized atrophy and putaminal and caudate iron accumulation, classifying KRS amongst neurodegeneration with brain iron accumulation (Bruggemann et al., 2010; Schneider et al., 2010), although others reported KRS patients without iron accumulation (Chien et al., 2011). KRS can be classified as a complex dystonia, i.e., a form of dystonia that occurs in conjunction with other neurological or non-neurological symptoms (Lohmann and Klein, 2013; Klein, 2014).

Whereas wild-type ATP13A2 is localized to late endosomal and lysosomal membranes (Ramirez et al., 2006), the truncating KRS mutations lead to retention of the protein in the ER resulting in ER stress and proteasomal degradation via the ER-associated degradation pathway (Ugolino et al., 2011). Other missense mutations in ATP13A2 have been identified that are associated with early-onset Parkinsonism (Di Fonzo et al., 2007; Lin et al., 2008; Ning et al., 2008; Santoro et al., 2011). Similar to KRS mutations, homozygous missense mutations disrupt normal localization and function of ATP13A2 while heterozygous missense mutations may impair ATPase activity (Podhajska et al., 2012). Moreover, ATP13A2 protein levels are increased in surviving neurons of humans with PD/dementia with LBs indicative of a putative protective function of high levels of ATP13A2. In LBs positive neurons ATP13A2 did not co-localize directly, but rather surrounds the LBs (Ramonet et al., 2012).

In dogs (Farias et al., 2011; Wohlke et al., 2011) and mice (Schultheis et al., 2013), loss of ATP13A2 elicits neuronal ceroid lipofuscinosis (NCL), a lysosomal storage disorder characterized by the accumulation of autofluorescent lipopigment. The phenotype of NCL partially overlaps with that of KRS and involves dysarthria, cerebellar ataxia, rigidity, bradykinesia and cognitive impairment. *Atp13a2* deficient mice exhibit age-dependent sensorimotor deficits that resemble motor symptoms observed in KRS, NCL and PD patients. Moreover, loss of Atp13a2 leads to lipofuscin accumulation and α-synuclein aggregation in the

hippocampus, critical features of NCL and PD, respectively (Schultheis et al., 2013). Also a homozygous ATP13A2 missense mutation was reported that is associated with juvenile NCL in humans (Bras et al., 2012). Extensive lipofuscinosis was demonstrated in neuronal and glial cells of cortex, basal ganglia and cerebellum (Bras et al., 2012). These findings underline the importance of ATP13A2 in the lysosomal pathway for α-synuclein degradation and suggest that lysosomal dysfunction might represent a link between lipofuscinosis and α-synuclein accumulation.

ATP13A4

ATP13A4 has been linked to language delay (Kwasnicka-Crawford et al., 2005; Worthey et al., 2013) and autism spectrum disorders (ASD) (Vallipuram et al., 2010). In two patients, disruption of ATP13A4 led to specific language impairment characterized by delayed expressive and receptive language, without further cognitive deficiencies (Kwasnicka-Crawford et al., 2005). Moreover, in a Finnish genome-wide screen for ASD, an autism susceptibility locus was identified on chromosome 3q25-27, nearby *ATP13A4* (Auranen et al., 2002). In six study participants, a Glu646Asp sequence variant was found, which is located between the fourth and fifth TM region of ATP13A4, near the conserved Asp residue and the N-domain (Kwasnicka-Crawford et al., 2005).

There is very little knowledge concerning the biological role of ATP13A4. In mice, Atp13a4 is mainly expressed in stomach and brain (Schultheis et al., 2004). Atp13a4 expression varies throughout all regions of the adult mouse brain, with the highest relative expression in cerebellum. Atp13a4 expression is also developmentally regulated, peaking at late neurogenesis, suggesting a function in neuronal development (Vallipuram et al., 2010; Weingarten et al., 2012). In humans, ATP13A4 mRNA has been detected in multiple organs, with relatively low expression in the brain, where expression was observed in the lateral inferior frontal cortex (Broca's area) and the temporoparietal cortex (Wernicke's area) (Kwasnicka-Crawford et al., 2005), areas of importance for language output and input, respectively. ATP13A4 is observed in the ER membrane and increases intracellular Ca^{2+} levels when overexpressed in COS-7 cells (Vallipuram et al., 2010). The intracellular Ca^{2+} increase is not observed in cells overexpressing the Glu646Asp variant, implying that the substitution might impair the ability of ATP13A4 to regulate Ca^{2+} transport (Vallipuram et al., 2010).

CELLULAR FUNCTION OF P5B ATPases IN MODEL ORGANISMS

A major bottleneck in unraveling the role of ATP13A2 in neurological disorders is the fact that virtually nothing is known concerning the molecular function and substrate specificity of the P5B-type ATPases. In this section, we will discuss the cellular role of ATP13A2 orthologs in different model organisms.

YPK9 IN THE YEAST *SACCHAROMYCES CEREVISIAE*

Ypk9p (yeast PARK9), is the single P5B-type ATPase in the yeast *Saccharomyces cerevisiae* and resides in the vacuolar membrane, the yeast equivalent of the mammalian lysosome (Gitler et al., 2009). Studies in yeast show that Ypk9p is involved in protecting cells against Mn^{2+} toxicity (Gitler et al., 2009) and, more broadly, heavy metals (Schmidt et al., 2009; Chesi et al., 2012; Kong et al., 2014), but the ion specificity might be strain dependent (Schmidt et al., 2009). Little or no other phenotypes have been reported. According to the *S. cerevisiae* genome database (http://yeastgenome.org) 74 genes or gene products have been reported as interactors of *YPK9*. Most interactions are genetic (72 in total) highlighting genes that when deleted in the absence of *YPK9* result in synthetic growth effects or phenotypic suppression or enhancement (**Figure 5**). **Supplemental Table 1** integrates data on interactions from high- and low-throughput studies in yeast available at BioGrid (http://thebiogrid.org). The interactors vary both with respect to cellular location and predicted function. Surprisingly, only a few of the interactors are located in the endosome and vacuolar systems (6 out of 74 interactors). The most prevalent interactions participate in vesicular trafficking and protein sorting (12 out of 74 interactors) and transcriptional flux (11 out of 74) (**Figure 5**).

Only one gene, TATA-binding protein-associated factor-1, *TAF1*, has so far been reported to be synthetic lethal in combination with deletion of *YPK9*. *TAF1* encodes the largest transcription factor TFIID subunit involved in RNA pol II transcription initiation, promoter binding and G1/S progression (Walker et al., 1997). Taf1p relocalizes to the cytosol in response to hypoxia, whereas subsequent oxygen exposure restores the nuclear localization (Dastidar et al., 2012). Also the expression of ATP13A2 in mammalian cells is upregulated in conditions of hypoxia (Xu et al., 2012). In humans, *TAF1* is associated with complex dystonia (X-linked parkinsonism, unconfirmed genetic evidence) (Makino et al., 2007) (reviewed in Lohmann and Klein, 2013; Klein, 2014). This might suggest a putative link between ATP13A2 and TAF1 in humans. Human TAF1 possesses protein kinase, ubiquitin-activating and -conjugating activities and histone acetyltransferase activities. These activities control transcription of genes involved in the G1 progression in mammalian cells such as cyclin D1 and cyclin A (Kloet et al., 2012).

OPI3 and *YME1* are so far the only two positive genetic interactors that have been identified. Thus, deletion of any of these genes in combination with a deletion of *YPK9* alleviates growth effects caused by loss of either. *OPI3* encodes the methylene-fatty-acyl-phospholipid synthase, which catalyzes the last two steps in phosphatidylcholine biosynthesis at the ER membrane in contact zones with the plasma membrane (Tavassoli et al., 2013). *YME1* encodes the catalytic subunit of the i-AAA protease complex that is responsible for degradation of unfolded mitochondrial proteins in the intermembrane space. Yme1p mediates Atg32p processing, which is required for mitophagy (Wang et al., 2013). It also contributes substantially to the proteolytic turnover of phosphatidylserine decarboxylase-1 (PSD1) located at the mitochondrial inner membrane (Nebauer et al., 2007).

The link between *YPK9* and the mitochondria is further underscored by negative genetic interactions with six other genes (*ATP5*, *MDL2*, *MMM1*, *FMT1*, *GEP3*, and *OXR1*) that encode proteins related to mitochondrial function. Negative interactions are defined as genes that upon deletion, in combination with a *YPK9* deletion, display aggravated growth effects as compared to the loss of either individual gene. The six genes are related

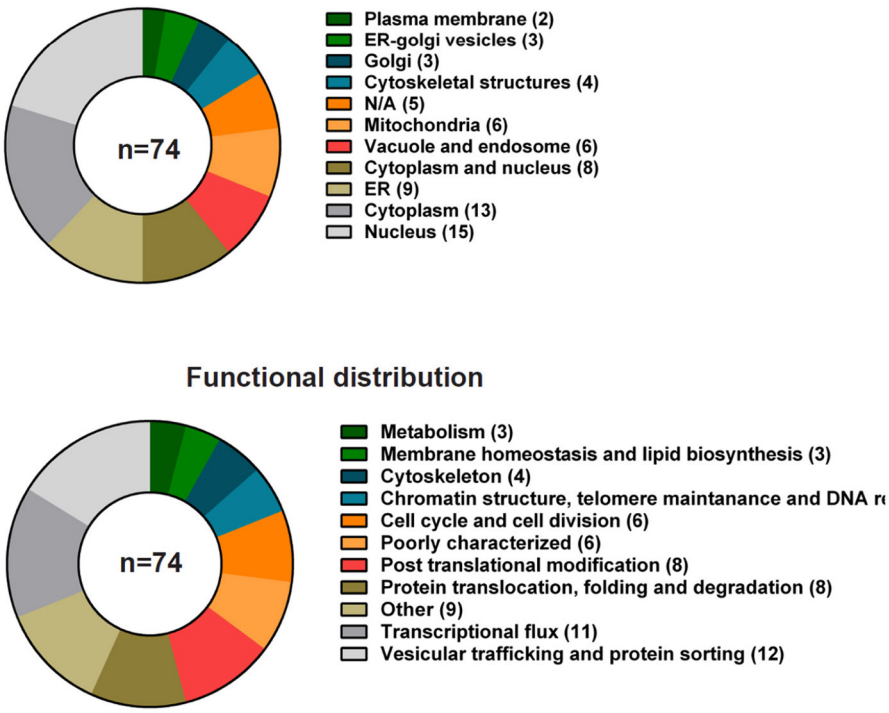

FIGURE 5 | Genetic interactions of *YPK9* in *S. cerevisiae* grouped according to cellular localization and function. Summary of the known genetic interactions of the yeast ATP13A2 ortholog *YPK9*. The data were collected from the yeast genome database (http://yeastgenome.org, see **Supplemental Table 1**) and were classified using BioGrid (http://thebiogrid.org) according to organellar distribution and function.

to maintenance of mitochondrial homeostasis (*ATP5*, *MDL2*, *MMM1*), mitochondrial biogenesis (*FMT1*, *GEP3*) and resistance to oxidative damage (*OXR1*). Several interactors of *YPK9* can thus be related to mitochondrial function, which substantiates a link between *YPK9* and protection from oxidative stress.

The remaining majority of interactors related to *YPK9* have been identified in a genome-wide screen aimed at defining mechanisms by which *YPK9* protects cells from Mn^{2+} toxicity in yeast (Chesi et al., 2012). At physiological conditions, *YPK9* genetically interacts with essential genes involved in the cell cycle (*APC5*, *CDC28*, *CDC53*, *SFH1*, *POL3*, *CDC11* and *CDC12*), cellular transport and vesicular trafficking (*ALG1*, *GAB1*, *BET2* and *MYO2*) or RNA processing (*DIM1*). Cdc11p and Cdc12p belong to the septin family, which includes highly conserved GTP-binding proteins found in eukaryotes. Septins provide a scaffold to support cell division, polarity and compartmentalization and have been implicated in diverse neurodegenerative disorders in humans, including in PD and α-synuclein mediated toxicity (Hall and Russell, 2004). *BET2* encodes the beta subunit of type II geranylgeranyl transferase (Rossi et al., 1991) that is required for vesicular transport between the ER and the Golgi (Newman et al., 1990). Bet2p provides a membrane anchor to the Rab-like protein Ypt1p, which like Ypk9p, protects yeast cells from α-synuclein toxicity (Cooper et al., 2006).

Yeast genes involved in Mn^{2+} protection mainly belong to categories of vesicle-mediated transport, vacuolar organization and chromatin remodeling (Chesi et al., 2012). *YPK9* deletion modifies Mn^{2+} tolerance of a subset of these genes. Interactors that increase Mn^{2+} sensitivity of *YPK9* are implicated in vacuolar and vesicle organization and membrane fusion (such as *VAM3*, *VAM6*, *SWF1*, and *GLO3*), whereas interactors that increase Mn^{2+} tolerance (such as *SIF2*, *HMO1*, *LEO1*, *APQ12*, and *MOG1*) seem to be involved in chromatin organization, histone modification and nuclear transport (Chesi et al., 2012). However, it is unclear why none of the established Mn^{2+} or heavy metal transport systems have been reported to be genetically linked to *YPK9*. This could be because *YPK9* is located upstream of established transport systems that take care of Mn^{2+} clearance. Ypk9p might for instance be implicated in the regulation of vesicular transport routes that control Mn^{2+} homeostasis. As Mn^{2+} ions are redox active Ypk9p may also regulate the removal pathway of damaged mitochondria via autophagy.

Taken together, the genetic interaction data from yeast suggest a potential role for *YPK9* in the cell cycle and vesicular trafficking in combination with proper mitochondrial function.

ANIMAL MODELS OF ATP13A2

C. elegans

Caenorhabditis elegans contains three P5B genes, *catp-5* to *7*. In contrast to the vacuolar/lysosomal localization of other P5B ATPases, CATP-5 locates to the plasma membrane at the apical side of intestinal cells (Heinick et al., 2010). *catp-5* mutant

strains are impaired in polyamine uptake (Heinick et al., 2010). Polyamines are ubiquitous cellular components that affect numerous biological processes such as cell cycle progression. Polyamines interact with anionic binding sites of macromolecules such as nucleic acids and phospholipids. *catp-5* might either encode a polyamine transporter or the gene might positively regulate polyamine uptake (Heinick et al., 2010). In mammalian CHO cells, ATP13A2 overexpression leads to a two-fold higher accumulation of the polyamine spermidine. It was shown that the ATP-dependent spermidine uptake was increased in a lysosomal and late endosomal fraction further supporting the notion that the ATP13A2 protein mediates polyamine uptake (De La Hera et al., 2013). The higher polyamine uptake rate might further explain the increased cytotoxic effects of paraquat, a toxic polyamine analogue that is an environmental risk factor for PD (Pinto Fde et al., 2012).

CATP-6 locates depending on the tissue type to either cytoplasmic punctae likely corresponding to vesicles associated with the lysosome or to the plasma membrane (Lambie et al., 2013). *catp-6*, was identified in a RNAi screen for genes stabilizing synthetic α-synuclein, representing a putative functional homologue of ATP13A2 (Hamamichi et al., 2008). The *catp-6* locus genetically interacts with *gon-2* and *gem-1* (Lambie et al., 2013). *gon-2* encodes a TRPM cation channel protein that is required for Mg^{2+} uptake, whereas *gem-1* encodes for the SLC16A transporter, which might be a putative monocarboxylate transporter. It was suggested that the *catp-6* gene product governs Mg^{2+} uptake by regulating the trafficking of transporters or other regulatory proteins to the plasma membrane (Lambie et al., 2013).

Mouse

The phenotype of a genetic knock-out mouse model of *Atp13a2* was recently described (Schultheis et al., 2013). The insertion site of the *Neo* gene in the genome of the *Atp13a2*$^{-/-}$ mice would still allow the formation of a truncated and mutated N-terminal Atp13a2 fragment consisting of the first 341 amino acids of Atp13a2 followed by 168 unrelated amino acids. Although the mutated transcript is clearly formed, no traces of the mutated protein were detected, suggesting that it may either be unstable or inefficiently translated (Schultheis et al., 2013).

As mentioned above, the *Atp13a2*$^{-/-}$ mice show α-synuclein accumulation as occurs in PD and related synucleinopathies, and accumulation of lipofuscin deposits, characteristic of NCL (Schultheis et al., 2013). The α-synuclein aggregation occurred predominantly in the hippocampus, but not in the cortex or cerebellum. Also the expression of some genes involved in PD is altered between the *striatum* and *substantia nigra* in *Atp13a2*$^{-/-}$ vs. *Atp13a2*$^{+/+}$ mice (Schultheis et al., 2013).

Twenty to twenty nine months old *Atp13a2*$^{-/-}$ mice perform more poorly on several sensorimotor tests. More specifically, the aged *Atp13a2*$^{-/-}$ mice display impaired aspects of motor learning. The gait analysis revealed a shortened stride length. Also reduced hindlimb stepping, impairments in fine motor skills and orofacial movements involved in nest building were reported (Schultheis et al., 2013). These deficits are similar to those observed in other genetic mouse models of PD and ataxia and also resemble aspects of motor dysfunction observed in KRS, NCL, and PD (Schultheis et al., 2013). Cognitive function and emotional reactivity were also changed in *Atp13a2*$^{-/-}$ mice. Mutant mice demonstrated greater exploratory behavior without changes in general locomotor activity or anxiety. Because the behavioral phenotype was not detectable until old age, some compensatory mechanisms might take place, which might be related to other members of the P5-ATPase family. But so far, no evidence was found for a compensatory upregulation of other P5-type ATPases (Schultheis et al., 2013).

Zebra fish

In contrast, knocking out *ATP13A2* in the zebra fish results in severe retardation already at the embryonic stage of development (Lopes da Fonseca et al., 2013). As the mouse genome contains four P5B genes (*Atp13a2-5*), while the zebra fish only one (*ATP13A2*), the late-onset mouse phenotype associated with loss of *ATP13A2* would suggest that some of the P5B homologues in mice may be functionally redundant, possibly compensating for each other's loss (Schultheis et al., 2013). Further studies will be required to shed light on the possible redundancy of P5B function and whether compensatory effects from other P5 alleles can take place.

CELLULAR ROLES OF HUMAN ATP13A2

ATP13A2 EXPRESSION PROFILE

ATP13A2 is mapped to the PARK9 PD susceptibility locus on chromosome 1p36. ATP13A2 is predominantly expressed in the brain, particularly in the dopaminergic neurons of the *substantia nigra* (Ramirez et al., 2006). Studies in mouse have shown that Atp13a1 (P5A) and Atp13a2 (P5B) are broadly expressed in many tissues with the highest expression of Atp13a2 observed in the brain. Atp13a4 and Atp13a5 (both P5B) are only expressed in brain and stomach while Atp13a3 (P5B) has a wider expression pattern that includes brain and other internal organs like colon, kidney and liver (Schultheis et al., 2004). It thus seems that all P5-type ATPase isoforms are expressed in the brain although individual members express at different levels during various developmental stages. Expression of Atp13a2 peaks during neurogenesis while Atp13a5 peaks at the adult stage (Weingarten et al., 2012). These observations are in line with a significant, yet undescribed role for P5B ATPases in brain development and function.

At least three ATP13A2 splice variants are reported (Ugolino et al., 2011). Variant 1 is the longest and counts 1180 amino acids. Variant 2 contains a five amino acid in-frame deletion in the N-terminus (1175 amino acids), whereas variant 3 is 1158 amino acids long and appears to be an anomalous protein. Here, the last two TM helices are replaced by an unusual sequence stretch and variant 3 also lacks an important part of the connection of the TM region with the cytosolic domains. Based on comparison with other P-type ATPases these alterations will probably have a significant impact on enzymatic activity. Variant 3 is retained in the ER and is rapidly degraded, questioning whether it serves a cellular role (Ugolino et al., 2011).

The promoter region of the human ATP13A2 gene contains hypoxia response elements, which can bind to the transcription factor hypoxia inducible factor 1a (HIF-1a). Hypoxic conditions

up-regulate transcription of the *ATP13A2* gene in both HEK293 and dopaminergic MN9D cells (Xu et al., 2012). Also Mn^{2+} and Zn^{2+} elevate ATP13A2 expression in several cell lines (see further details below) (Tan et al., 2011; Tsunemi and Krainc, 2014).

INTRACELLULAR LOCALIZATION OF ATP13A2

The general accepted view is that ATP13A2 is targeted to acidic compartments, *i.e.* the late endosomes and lysosomes, because of a co-localization with LAMP1/2a, Rab7, and Lysotracker. Also the loss of ATP13A2 leads to lysosomal dysfunction and an increase in the size and number of the lysosomes (Dehay et al., 2012b; Usenovic et al., 2012a). Originally, it was suggested that ATP13A2 is localized to late endosomes/lysosomes based on overexpression studies of tagged ATP13A2 (Ramirez et al., 2006). However, this view was recently challenged (Kong et al., 2014). In both differentiated SHSY5Y cells and rat primary neurons, the endogenous ATP13A2 associates closely together with LC3, a marker of the autophagosomes. More specifically, the authors concluded that the endogenous ATP13A2 occupies the outer limiting membrane of multivesicular bodies (MVBs), a morphologically distinctive late endosome compartment. MVBs undergo dynamic rearrangements and sorting of lipids and proteins via inward budding of the membrane generating multiple intra-luminal vesicles (ILVs). By fusing with the plasma membrane, MVBs can release the ILVs in the extracellular space as exosomes. MVBs can also fuse with autophagosomes from autophagy pathways producing hybrid structures referred to as amphisomes, which then can fuse with lysosomes for cargo degradation. ATP13A2 is also observed in the outer membrane of amphisomes (Kong et al., 2014).

ATP13A2 IS INVOLVED IN AUTOPHAGY AND MITOCHONDRIAL CLEARANCE

Mitochondrial dysfunction is tightly linked to the pathogenesis of PD (Auluck et al., 2010; Jin and Youle, 2012; Gautier et al., 2014). Strong support comes from the observations that 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), a potent mitochondrial complex I inhibitor, triggers a PD-like syndrome (Burns et al., 1983; Langston and Ballard, 1983). Several PD-associated genes, namely parkin, PINK1 and DJ-1 play a role in mitochondrial dynamics and clearance strengthening the concept that mitochondrial dysfunction and the production of ROS are consistent features of PD (Auluck et al., 2010; Jin and Youle, 2012). Thus, clearance of dysfunctional or damaged mitochondria and misfolded proteins is essential for neuronal fitness and survival (Jin and Youle, 2012; Gautier et al., 2014) and the lysosome is a vital organelle for this quality control (Jin and Youle, 2012; Tofaris, 2012; Dehay et al., 2013). Organelles, cytoplasmic material and protein aggregates are delivered to the lysosome via various autophagy pathways. Macroautophagy (or just autophagy) involves the formation of double-layered membrane autophagosomes, which encapsulate cytoplasmic materials for delivery to the lysosomes for degradation. Mitochondria are removed through a specific autophagy pathway called mitophagy (Jin and Youle, 2012). Soluble proteins can also be selectively degraded in the lysosome through uptake by the lysosomal receptor LAMP2a, via a process known as chaperone-mediated autophagy (CMA) (Mak et al., 2010). Micro-autophagy involves the direct engulfment of cytoplasmic material by invagination of the late endosomal/lysosomal membrane. Whereas the function of micro-autophagy in mammalian cells is unknown, both macro-autophagy and CMA are key processes in neurodegeneration and α-synuclein removal (Webb et al., 2003; Mak et al., 2010) (reviewed in Xilouri and Stefanis, 2011).

Studies in KRS patient-derived fibroblasts and ATP13A2-deficient cell lines have revealed that mutations of ATP13A2 or knockdown of the gene transcript lead to several lysosomal alterations. First the number and size of lysosomes is increased (Usenovic et al., 2012a). Also lysosomal dysfunction is reported involving impaired lysosomal acidification, decreased proteolytic processing of lysosomal enzymes, reduced degradation of lysosomal substrates (e.g., α-synuclein) and impaired lysosomal-mediated clearance of autophagosomes (Dehay et al., 2012b; Usenovic et al., 2012a; Tsunemi and Krainc, 2014).

In parallel, a strong link between ATP13A2 and mitochondrial dysfunction is emerging. Loss of ATP13A2 function impairs mitochondrial maintenance and leads to oxidative stress. ATP13A2 expression protects mammalian cells toward mitochondrial and oxidative stress (Covy et al., 2012). In fibroblasts of patients with non-functional ATP13A2 ATP production rates are decreased. Also a higher frequency of mitochondrial DNA lesions, increased oxygen consumption rates and increased fragmentation of the mitochondrial network have been observed (Grunewald et al., 2012).

A role for ATP13A2 in autophagy and mitochondrial clearance has been suggested, but mechanistic details are lacking (Grunewald et al., 2012; Gusdon et al., 2012; Park et al., 2014). ATP13A2 regulates mitochondrial bioenergetics through macroautophagy. ATP13A2 knockdown reduces the autophagic flux in SHSY5Y cells (Gusdon et al., 2012) and lysosomal-mediated clearance of autophagosomes was impaired in patient-derived $ATP13A2^{-/-}$ fibroblasts (Dehay et al., 2012b) and in ATP13A2 knockdown neurons (Usenovic et al., 2012a). Several physical interactors of ATP13A2 are involved in mitophagy whereas others are involved in protein quality, protein sorting, vesicular transport and membrane fusion (Usenovic et al., 2012b). Evidence from genetic interaction studies in yeast also provides a link between *YPK9* and mitochondrial clearance (see above).

The fact that ATP13A2 expression is upregulated under oxidative stress (Xu et al., 2012) seems to indicate that ATP13A2 is particularly important in conditions of oxidative stress, for instance arising from mitochondrial dysfunction. Defective mitochondrial clearance may also account for the increased sensitivity toward Zn^{2+} toxicity in $ATP13A2^{-/-}$ cells (Park et al., 2014). Indeed, Zn^{2+} induces mitochondrial ROS production, which results in mitochondrial dysfunction and fragmentation (Park et al., 2014). Importantly, treatment with an antioxidant completely abolishes Zn^{2+}-induced cell death in $ATP13A2^{-/-}$ cells (Park et al., 2014). The link between Zn^{2+} and mitochondrial stress is further emphasized by the observation that Zn^{2+} potentiates, whereas Zn^{2+} chelation protects against MPTP-induced PD (Sheline et al., 2013). Along the same lines, loss of ATP13A2 and impaired mitochondrial clearance may explain the observed intolerance toward Mn^{2+} (Gitler et al., 2009; Schmidt et al.,

2009) and paraquat (Pinto Fde et al., 2012). Since polyamines can function as ROS scavengers, the increased uptake of polyamines (De La Hera et al., 2013) may protect ATP13A2$^{-/-}$ cells toward oxidative stress, although high levels of polyamines are also toxic.

Together, these observations suggest that ATP13A2 controls mitochondrial maintenance, which would lend further support to converging lysosomal and mitochondrial pathways in PD pathogenesis (Jin and Youle, 2012; Tofaris, 2012; Dehay et al., 2013). By controlling the autophagy-lysosomal activity ATP13A2 may serve an essential function by removing damaged or dysfunctional proteins and organelles.

ATP13A2 IS INVOLVED IN VESICULAR TRANSPORT

The long list of putative physical interactors (Usenovic et al., 2012b) points into the direction of ATP13A2 as a scaffolding protein in the regulation of vesicular processes. Like other P-type ATPases, such as the Na^+/K^+-ATPase (Xie and Xie, 2005) and the SERCA2 Ca^{2+}-ATPase (Vangheluwe et al., 2005), ATP13A2 might be acting as a scaffold and exert a transporting function at the same time. Regulation of vesicular processes may include *de novo* vesicle formation, vesicular transport, vesicular sorting mechanisms and vesicle fusion. *E.g.* ATP13A2 directly interacts with several components of the SNARE complex that is involved in vesicle docking and fusion (Usenovic et al., 2012b). A function in vesicular transport can be easily reconciled with the established roles of ATP13A2 in mitochondrial clearance (Gusdon et al., 2012; Park et al., 2014) and α-synuclein removal (Gitler et al., 2009; Kong et al., 2014), which depend on autophagy pathways. In addition, ATP13A2 controls vesicle-dependent Zn^{2+} and α-synuclein removal mechanisms through exosomes, which are ILVs formed in the MVBs that fuse with the plasma membrane (Kong et al., 2014). Genetic evidence in yeast also provides a link between *YPK9* and vesicular transport (see above).

THE CONNECTION BETWEEN ATP13A2 AND α-SYNUCLEIN

ATP13A2 protects cells toward α-synuclein toxicity. This has been observed in several model systems including yeast, *C. elegans* and mammalian cells (Gitler et al., 2009). Like α-synuclein, ATP13A2 might be implicated in vesicle trafficking and mitochondrial dysfunction. Several hypotheses may explain the protective effect of ATP13A2.

(a) By regulating lysosomal functions ATP13A2 might control lysosomal α-synuclein degradation and prevent the build-up of α-synuclein aggregates (Dehay et al., 2012b; Usenovic et al., 2012a; Tsunemi and Krainc, 2014).
(b) Alternatively, ATP13A2 may control the delivery of α-synuclein to the lysosomes by regulating different autophagy pathways such as macro-autophagy (Dehay et al., 2012b; Usenovic et al., 2012a) and chaperone-mediated autophagy, two autophagic routes that control α-synuclein turnover (Webb et al., 2003; Mak et al., 2010).
(c) α-synuclein may prevent the membrane fusion of the mitochondria resulting in increased mitochondrial fragmentation (Kamp et al., 2010). α-synuclein also impairs mitochondrial function (Auluck et al., 2010). The protective effect of ATP13A2 toward α-synuclein toxicity might therefore be related to the positive effect of ATP13A2 on mitochondrial appearance (Grunewald et al., 2012; Gusdon et al., 2012; Park et al., 2014), which might compensate excessive mitochondrial fragmentation. One can speculate that via promotion of mitochondrial clearance, ATP13A2 might provide protection toward α-synuclein-induced mitochondrial fragmentation.
(d) ATP13A2 promotes the removal of α-synuclein out of the cell via exosomes reducing the α-synuclein stress in cells (Kong et al., 2014).
(e) As α-synuclein interacts with membranes and the amount of α-synuclein interaction with the membrane seems to correlate with the degree of toxicity (Auluck et al., 2010; Kuwahara et al., 2012), it is a tempting hypothesis that ATP13A2 might affect α-synuclein membrane interactions.

ATP13A2 CONTROLS CELLULAR ION HOMEOSTASIS

ATP13A2 causes protection toward several heavy metals (Gitler et al., 2009; Schmidt et al., 2009; Kong et al., 2014) and in KRS patients iron deposits in the brain are observed (Bruggemann et al., 2010; Schneider et al., 2010). The prevailing hypothesis is therefore that ATP13A2 is a lysosomal cation pump (Gitler et al., 2009). This hypothesis is furthermore based on sequence similarity with other P-type ATPases (Ramirez et al., 2006), among which most transport cations, e.g., to generate vital electrochemical ion gradients, to relocalize essential elements or dispose toxic metal ions.

WHAT IS THE TRANSPORTED LIGAND—IF ANY—OF ATP13A2?

Although P5B ATPases have essentially all sequence requirements to act as transporters, their ligands so far remain unidentified. ATP13A2 may regulate lysosomal function by transporting ions (Gitler et al., 2009) or an essential co-factor required for lysosomal enzyme activity (Covy et al., 2012). ATP13A2 might also take up metal ions in lysosomes or MVBs/exosomes to remove excess ions (Kong et al., 2014). The possibility that ATP13A2 works closely together with the V-type ATPases to pump protons to contribute to the low pH in the late endosome/lysosome can also not be excluded (Dehay et al., 2012b). In this section we will critically review the prevalent concept that ATP13A2 is a cation transporter by comparing sequence characteristics of ATP13A2 with better described P-type ion pumps and discussing the available physiological and biochemical evidence for transport of proposed ligands. Finally, we discuss the possibility that ATP13A2 might pump organic ions, such as lipids or peptides from one membrane leaflet to the other.

CATION(S) POSSIBLY TRANSPORTED BY ATP13A2

ATP13A2 has been linked to Mn^{2+}, Zn^{2+}, Mg^{2+}, and H^+ homeostasis suggesting that ATP13A2 might be implicated in the transport of these cations.

Is ATP13A2 a Mn^{2+} transporter?

ATP13A2 was first suggested to be a lysosomal Mn^{2+} transporter (Gitler et al., 2009). Mn^{2+} is a biologically relevant metal that functions as a cofactor of many enzymes, such as carboxylases and phosphatases in the cytosol, sugar transferases and sulfatases

in the Golgi and the mitochondrial superoxide dismutase SOD2 (Vangheluwe et al., 2009; Tuschl et al., 2013). Little is known about Mn^{2+} requirements in lysosomes or MVBs, but Mn^{2+} uptake in the MVBs and lysosome could constitute a Mn^{2+} detoxification pathway to remove excessive Mn^{2+}. This would prevent Mn^{2+} toxicity, which evokes extrapyramidal syndromes resembling PD and dystonia (Vangheluwe et al., 2009; Tuschl et al., 2013). Mn^{2+}-induced cell death involves oxidative stress, interference with Ca^{2+} and iron homeostasis, DNA damage and mitochondrial dysfunction (Tan et al., 2011). It is thus clear that Mn^{2+} levels need to be properly controlled, but the responsible pathways remain incompletely understood.

A role of ATP13A2 in Mn^{2+} homeostasis and Mn^{2+} toxicity has been proposed based on the following observations. Loss of *YPK9* in yeast leads to an increased sensitivity toward Mn^{2+} (Gitler et al., 2009; Schmidt et al., 2009), whereas mammalian cells (HEK293, Neuro2a and NLF neuroblastoma cells) that overexpress ATP13A2 showed resistance to $MnCl_2$-induced cytotoxicity (Tan et al., 2011; Covy et al., 2012). Atomic absorption spectrophotometry further revealed that HEK293 cells overexpressing ATP13A2 accumulate less Mn^{2+} when cells were pre-exposed to $MnCl_2$. Also the endogenous ATP13A2 expression levels increase when HEK293 cells are exposed to $MnCl_2$ (Tan et al., 2011). Thus, ATP13A2 regulates and is controlled by the intracellular Mn^{2+}-concentration providing a strong link between ATP13A2 and Mn^{2+} homeostasis. Although these observations indicate that ATP13A2 is implicated in the removal pathway for Mn^{2+}, the available evidence that ATP13A2 would be a Mn^{2+} transporter remains circumstantial since no direct Mn^{2+} transport or lysosomal Mn^{2+} uptake has been demonstrated.

For instance, instead of possibly transporting Mn^{2+} directly, ATP13A2 might influence other Mn^{2+} removal pathways which can depend on vesicular transport and/or other Mn^{2+} carriers. It is clear that efficient Mn^{2+} resistance in yeast depends on all steps in the secretory pathway involving proteins of vesicle-mediated transport, vacuolar organization and chromatin remodeling (Chesi et al., 2012). This suggests that Mn^{2+} removal may occur mainly via vesicular transport routes. In the brain, Mn^{2+} detoxification also depends on several members of the solute carrier (SLC) family including the proton coupled transporters SLC11A2/DMT1/NRAMP2 and SLC40A1/ferroportin, the putative Zn^{2+}/Mn^{2+} transporter SLC30A10/ZnT10 and SLC39A14/ZIP14 (DeWitt et al., 2013; Tuschl et al., 2013). These proteins are involved in Mn^{2+} transport and typically carry several metal species. SLC members are involved in the transport of several divalent metal cations, such as Zn^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, and Cd^{2+}. This is also the case for the transferrin receptor (TfR) carrying Fe^{2+} and trivalent Mn^{3+} (DeWitt et al., 2013) and for the Secretory Pathway Ca^{2+}/Mn^{2+}-transport ATPase SPCA1/PMR1 (Vangheluwe et al., 2009). The relative importance of all these Mn^{2+} transporters or removal pathways in the brain or how ATP13A2 affects these pathways remains to be determined.

Several Mn^{2+} transport routes were studied in neuronal cells. Mn^{2+} is sequestered into the Golgi/secretory pathway compartments by SPCA1/PMR1, which also belongs to the P-type ATPase family (P2-type) (Vangheluwe et al., 2009). So far, the two SPCA isoforms SPCA1 (ubiquitous) and SPCA2 (secretory cells) are the only known P-type ATPases in animals that transport Mn^{2+} in intracellular stores with a high affinity. As such, SPCA1 might be implicated in the removal of toxic Mn^{2+} from neurons through the secretory pathway (Vangheluwe et al., 2009). However, SPCA expression levels decrease with Mn^{2+}-exposure and SPCA activity is inhibited by high concentrations of Mn^{2+}, suggesting that other Mn^{2+}-removal mechanisms may prevail in neurons (Sepulveda et al., 2012).

The ubiquitous ZIP14 is present in the plasma membrane and promotes Mn^{2+} uptake in SHSY5Y neuroblastoma cells. Conversely, SLC30A10 controls Mn^{2+} secretion in SHSY5Y cells, which is thought to be a Mn^{2+} transporter (Quadri et al., 2012). Mutations in SLC30A10 cause extreme neurotoxic accumulation of Mn^{2+} in liver and brain triggering dystonia and parkinsonism (Quadri et al., 2012). The subcellular localization of SLC30A10 matches with different compartments, such as the Golgi system, endosomes, and the plasma membrane (Quadri et al., 2012). The human SLC30A10 complements defective Mn^{2+} uptake in yeast cells lacking the Ca^{2+}/Mn^{2+} ATPase PMR1 (Tuschl et al., 2012) and the endogenous SLC30A10 expression increases with Mn^{2+} exposure in HepG2 hepatocellular carcinoma cells. These observations provide a strong link between SLC30A10 and maintaining Mn^{2+} homeostasis. Finally, neurons also take up Fe^{2+} and trivalent Mn^{3+} via DMT1 and TfR (DeWitt et al., 2013).

Is ATP13A2 a Zn^{2+} transporter?

Besides Mn^{2+}, Ypk9p protects yeast against other heavy metals, including Zn^{2+}, Cd^{2+}, Ni^{2+}, and Se^{2+} (Gitler et al., 2009; Schmidt et al., 2009; Kong et al., 2014). In mammalian cell systems, a link between ATP13A2 and Mn^{2+}, Ca^{2+}, Cd^{2+}, Zn^{2+}, or Ni^{2+} homeostasis has been reported. A protective effect of ATP13A2 overexpression toward Ni^{2+} and Mn^{2+} in mammalian NLF cells was described (Covy et al., 2012), whereas the knockdown of ATP13A2 increases the sensitivity of SHSY5Y cells toward Zn^{2+}, but strangely not Mn^{2+}. This challenges the view that ATP13A2 would be a Mn^{2+} transporter. Peptide fragments of ATP13A2 bind Mn^{2+}, Zn^{2+}, and copper (Remelli et al., 2013). ATP13A2 also regulates basal and Cd^{2+}-induced intracellular Ca^{2+} levels in neurons (Ramonet et al., 2012).

Several recent studies underscored a strong link between ATP13A2 and Zn^{2+} homeostasis (Kong et al., 2014; Park et al., 2014; Tsunemi and Krainc, 2014). Thus, Zn^{2+} is another strong candidate ligand for ATP13A2-mediated transport. Neurons are sensitive to both Zn^{2+} deficiency and excess (Sensi et al., 2009) and Zn^{2+} levels are increased in PD (Hozumi et al., 2011). Fibroblasts carrying homozygous or compound heterozygous ATP13A2 disease mutations and also patient-derived olfactory neurospheres (hONs), mouse primary embryonic cortical neurons and SHSY5Y cells with ATP13A2 knock down are highly sensitive to Zn^{2+} exposure, whereas sensitivity to Mn^{2+} is less pronounced (Kong et al., 2014; Park et al., 2014; Tsunemi and Krainc, 2014). In addition, the expression of endogenous ATP13A2 in primary neurons was elevated in the presence of Zn^{2+} (Tsunemi and Krainc, 2014). In conditions of Zn^{2+} overload, the acidic or LC3-positive vesicles of *ATP13A2*$^{-/-}$ cells accumulate less Zn^{2+} (Park et al., 2014; Tsunemi and Krainc, 2014). Conversely, via

X-ray fluorescence microscopy total intracellular Zn^{2+} levels were estimated to be 60% higher in *ATP13A2*$^{-/-}$ hONs than in control cells (Kong et al., 2014). This would suggest that ATP13A2 contributes to Zn^{2+} efflux from the cell.

Whether ATP13A2 pumps Zn^{2+} directly or rather affects other Zn^{2+} transporters or vesicular transport remains to be clarified. In the hONs, the majority of known secondary Zn^{2+} transporters were upregulated in *ATP13A2*$^{-/-}$ cells, pointing to a severe Zn^{2+} dyshomeostasis. These include 9 members of the SLC30 family of Zn^{2+} transporters (ZnTs) that mediate Zn^{2+} efflux and 14 members of the SLC39 family of ZRT/IRT-related proteins (Zn^{2+} importing proteins, ZIP) that facilitate influx of Zn^{2+} (Park et al., 2014). It is unclear how much of the altered expression pattern of these Zn^{2+} transporters explains the mislocalization of Zn^{2+} in *ATP13A2* deficient cells.

Nevertheless, the result of impaired ATP13A2 activity is a rise in cytosolic Zn^{2+} concentrations. Zn^{2+} dyshomeostasis has been associated with a variety of neurological disorders (Sensi et al., 2009). This affects multiple cellular functions including the mitochondria (Park et al., 2014) and lysosomes (Tsunemi and Krainc, 2014), making it difficult to assess the underlying mechanism of ATP13A2-mediated Zn^{2+} protection. Zn^{2+} induces lysosomal dysfunction, which negatively impacts on lysosomal pH, lysosomal proteolysis and accumulation of α-synuclein (Tsunemi and Krainc, 2014). Loss of ATP13A2 also leads to lysosomal dysfunction, which potentiates the Zn^{2+}-related effects (Dehay et al., 2012b; Tsunemi and Krainc, 2014).

Zn^{2+} is not involved in redox reactions and therefore does not generate oxidative stress by itself. However, excessive mitochondrial Zn^{2+} uptake in conditions of high Zn^{2+} exposure inhibits several enzymes and complexes of the mitochondria leading to the production of ROS. This imposes oxidative stress, which can induce cell death (Park et al., 2014). As mentioned above, the loss of ATP13A2 is associated with mitochondrial dysfunction, which is related to impaired mitochondrial clearance, presumably due to insufficient lysosomal degradation (Grunewald et al., 2012; Gusdon et al., 2012; Ramonet et al., 2012). The improper cytosolic removal of Zn^{2+} in ATP13A2$^{-/-}$ cells might further impose mitochondrial stress, triggering severe ROS production (Park et al., 2014). Moreover, an increased accumulation of failing mitochondria in ATP13A2$^{-/-}$ cells in conditions of Zn^{2+} exposure may contribute to the increased sensitivity of Zn^{2+}. The extensive mitochondrial dysfunction and fragmentation may lead to ATP depletion and/or ROS production resulting in cellular degeneration. Importantly, treatment with an antioxidant completely abolishes Zn^{2+}-induced cell death in ATP13A2$^{-/-}$ cells, indicating that the ROS production during Zn^{2+}-induced mitochondrial failure largely accounts for the toxic effects of Zn^{2+} (Park et al., 2014).

In conclusion, it appears that the Zn^{2+} phenotype of *ATP13A2*$^{-/-}$ cells can largely be explained without imposing that ATP13A2 is a Zn^{2+} transporter.

Is ATP13A2 a Mg^{2+} transporter?

The ATP13A2 homologue Kil2 in *Dicytostelium discoideum*, a phagocytic bacterial predator, is required for Mg^{2+}-dependent killing of ingested *Klebsiella* (Lelong et al., 2011). Also the ATP13A2 homologue CATP-6 in *C. elegans* has been implicated in Mg^{2+} uptake (Lambie et al., 2013). In a purified system, the ATPase activity of the P5A ATPase Spf1p in *S. cerevisiae* is stimulated by Mg^{2+} ions (Cronin et al., 2002). These observations might indicate that Mg^{2+} plays a role in both P5A and P5B ATPases. However, all P-type ATPases require Mg^{2+} for proper ATP coordination and phosphorylation of the conserved Asp in the P-domain (Moller et al., 2010). So for any P-type ATPase it is difficult to discriminate between transport or non-transport related effects of Mg^{2+} on the ATP-hydrolytic activity.

Is ATP13A2 a H^+ transporter?

ATP13A2 is present in acidic compartments. Because loss of ATP13A2 leads to an elevated lysosomal pH, ATP13A2 might be involved in organellar acidification (Dehay et al., 2012b; Tsunemi and Krainc, 2014). However, whether the lysosome would require additional H^+ pumps is questionable, as lysosome acidification primarily depends on the activity of V-type ATPases, which are highly efficient and abundant lysosomal H^+ pumps. V-type ATPases also work together with Cl^- channels for generating the steep H^+ gradient in the lysosome (Marshansky and Futai, 2008).

Can the ATP13A2 TM sequence support ion transport?

Establishing conclusively that ATP13A2 is a cation transporter will depend on a biochemical characterization of the purified pumps reconstituted in lipid vesicles that allow for measurements of transport. So far, we can only question whether the ATP13A2 protein supports Zn^{2+}, Mn^{2+}, Mg^{2+}, or H^+ transport. As explained before, the M4 region in P-type ATPases is critically involved in substrate coordination and specific M4 sequence motifs correlate well with substrate specificity. We first compared the sequence of ATP13A2 with SPCA, an established P-type Mn^{2+} transport ATPase (**Figure 4**). M4 of ATP13A2 (PPALP) strikingly differs from the SPCA M4 region (PEGLP). The Glu residue that coordinates Mn^{2+} and serves as a gating residue is absent in ATP13A2 and is replaced by a hydrophobic residue that is difficult to reconcile with ion binding. However, ion-coordination in SPCA not only depends on M4, but also involves oxygen atoms of the peptide backbone and polar residues positioned on M5 and M6 (Vangheluwe et al., 2009). In ATP13A2, at least one conserved negatively charged residue is found in M6 and conserved polar residues are found on M4, M5 and M6, which in theory could support ion coordination (**Figure 4**) (Sorensen et al., 2010). Notably, critically conserved Asp residues in the M6 segment of the P3 plasma membrane H^+-ATPase (Buch-Pedersen et al., 2009) and the P2 Ca^{2+}-ATPases (Toyoshima et al., 2000) play a role in respectively H^+ coordination and Ca^{2+} coordination.

Coordination of Zn^{2+} typically involves His and/or Cys residues (Simonson and Calimet, 2002). Also in the P-type ATPase heavy metal transporters, conserved Cys residues are found in the M4 region (a CPC motif in the CopA copper transporter) (Gourdon et al., 2011). No obvious conserved His, Cys or even a Met are found in the TM region of ATP13A2, questioning whether coordination of Zn^{2+} may occur. However, a striking similarity is observed between ATP13A2 and the P1B heavy metal P-type ATPases, which both have extra N-terminal helices in common (Sorensen et al., 2010; Gourdon et al., 2011).

In the CopA copper transporter, the N-terminal platform recruits metal-chaperones for copper delivery (Gourdon et al., 2011). Whether metal chaperones would bind ATP13A2 remains to be tested. Several putative ATP13A2 interactors were identified with a split-ubiquitin yeast two hybrid screening (Usenovic et al., 2012b) and several genetic interactions for *YPK9* were identified in yeast (Chesi et al., 2012), but no obvious metal chaperones or proteins involved in metal transport were found.

In conclusion, at least some residues in the M-region of ATP13A2 might support ion binding, but the overall composition significantly differs from other P-type Mn^{2+} and Zn^{2+} transporters. So until strong biochemical evidence becomes available, we may need to consider other possibilities.

IS ATP13A2 A LATE ENDOSOMAL/LYSOSOMAL FLIPPASE?

Among the P-type ATPases the 14 members of the P4 ATPases transport other ligands than inorganic cations. Members of this subfamily flip phospholipids from one membrane leaflet to the other. Because P5 ATPases phylogenetically are more related to P4 ATPases than to any other P-type ATPase subfamily (**Figure 1**) (Axelsen and Palmgren, 1998), it may be interesting to consider the possibility that P5 ATPases likewise might be involved in lipid or organic ion transport. The structural requirements of P4-type flippases are only just emerging and candidate residues have been identified that are important for lipid transport (Coleman et al., 2012; Baldridge and Graham, 2013; Baldridge et al., 2013). An Ile residue in the conserved P4 sequence motif on M4 (PISL) appears important for the binding and translocation of the phospholipid and shows functional analogy to a conserved Glu in M4 of P2-type ATPases that in these pumps serve as a gating residue (Vestergaard et al., 2014). At this point it remains hard to say whether P5B-type ATPases fulfill the sequence requirements of a typical flippase. Remarkably, a Pro in the M4 motif of the P5 is found at the position of the conserved Glu in P2 and Ile in P4.

Amongst other possible hypotheses, we should consider the possibility that ATP13A2 might be a flippase that transports a lipid or another organic molecule from one membrane leaflet to the other. Such an activity might in turn control vesicular dependent processes that regulate ion homeostasis, exosome formation, mitophagy/autophagy and α-synuclein clearance.

As a putative (lipid) flippase, ATP13A2 might alter membrane curvature, alter lipid dynamics, organize lipid microdomains or expose/remove important signaling molecules at one or the other membrane leaflet (Graham, 2004; Palmgren and Nissen, 2011), which might for instance regulate α-synuclein membrane interactions. Moreover, ATP13A2 is implicated in mitochondrial clearance and exosome formation at the site of the late endosome, MVB and lysosome. A putative (lipid) flippase might here be strategically important as these organelles undergo continuous vesicle forming and vesicular fusion events to deliver, sort or remove cargo. This might require a tight regulation of membrane dynamics. At the end station for autophagsome delivery, ATP13A2 might control the fusion process of the autophagosomes. ATP13A2 might for instance compose a fusion-compatible lipid microdomain or expose important signaling molecules required for fusion. Alternatively, changes in the lysosomal lipid distribution may regulate autophagy pathways, which are known to be sensitive to changes in the lipid environment (Ferguson et al., 2009; Rodriguez-Navarro et al., 2012). ATP13A2 might regulate micro-autophagy, a process depending on membrane invagination to take up cargo for degradation. It might also be involved in the formation of intraluminal vesicles of the MVBs which impacts on α-synuclein removal (Kong et al., 2014).

CONCLUSION

Although the cell biological context in which ATP13A2 is involved is gradually emerging, studying the molecular function and substrate specificity of ATP13A2 using biochemical methods and isolated systems will be required to unravel the substrate specificity and transport properties of ATP13A2. Understanding ATP13A2 at the molecular level will reveal its link to KRS, NCL, dystonia, and PD. This might open new therapeutic possibilities to treat this spectrum of disorders.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnmol.2014.00048/abstract

Supplemental Table 1 | Genetic interactions of *YPK9* in *S. cerevisiae* obtained from the yeast genome database (http://yeastgenome.org). The data were were classified using BioGrid (http://thebiogrid.org) according to organellar distribution and function. A summary is provided in **Figure 5**.

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Upstream deregulation of calcium signaling in Parkinson's disease

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Parkinson's disease (PD) is a major health problem affecting millions of people worldwide. Recent studies provide compelling evidence that altered Ca^{2+} homeostasis may underlie disease pathomechanism and be an inherent feature of all vulnerable neurons. The downstream effects of altered Ca^{2+} handling in the distinct subcellular organelles for proper cellular function are beginning to be elucidated. Here, we summarize the evidence that vulnerable neurons may be exposed to homeostatic Ca^{2+} stress which may determine their selective vulnerability, and suggest how abnormal Ca^{2+} handling in the distinct intracellular compartments may compromise neuronal health in the context of aging, environmental, and genetic stress. Gaining a better understanding of the varied effects of Ca^{2+} dyshomeostasis may allow novel combinatorial therapeutic strategies to slow PD progression.

Keywords: Parkinson's disease, dopamine, calcium, mitochondria, endoplasmic reticulum, lysosomes, Golgi

INTRODUCTION – WHICH NEURONS DIE IN PD?

Parkinson's disease (PD) is an incurable late-onset neurodegenerative disorder which is strongly associated with aging, as evidenced by the exponential increase in incidence above the age of 65 (de Rijk et al., 1997; de Lau et al., 2004). Due to extended life expectancy, the prevalence of PD is estimated to double by 2030. Therefore, deciphering the molecular mechanisms underlying the disease, with the aim of developing novel disease-modifying therapies, has become an urgent and crucial task in PD-related research. Whilst PD is a disease of neurons, not all neurons are affected. The motor symptoms of PD, such as resting tremor, bradykinesia, and rigidity are clearly linked to the death of dopamine (DA) neurons in the substantia nigra pars compacta (SNc). Similarly, the clinical gold-standard treatment of L-DOPA (3,4-dihydroxy-L-phenylalanine), a DA precursor, indicates that DA neurons are crucial to the disease. However, the neuropathological hallmarks of PD, which are the presence of proteinaceous intracellular deposits called Lewy bodies or Lewy neurites in surviving neurons, are more distributed and not exclusive to DA neurons. Non-DA neurons which show pathology in PD include cholinergic neurons in the dorsal motor nucleus of the vagus (DMV) and basal forebrain (BF), noradrenergic neurons in the locus ceruleus (LC), and serotonergic neurons in the raphe nuclei (RN; Braak et al., 2004). Neurodegeneration is also not evident in all dopaminergic neuronal populations. For example, DA neurons in the ventral tegmental area (VTA) are relatively unaffected (Matzuk and Saper, 1985; Kish et al., 1988; Ito et al., 1992; Damier et al., 1999). Thus, elucidating why the diverse neurons are at risk for degeneration is essential if we want to formulate testable hypotheses as to the cause(s) underlying PD.

WHY DO NEURONS DIE IN PD – FROM DOPAMINE TO MITOCHONDRIA

Distinct mechanisms have been proposed to account for the preferential loss of DA neurons in PD. One hypothesis proposed that DA itself may be the culprit, as oxidation of cytosolic DA and its metabolites can lead to the production of cytotoxic free radicals and oxidative stress (Greenamyre and Hastings, 2004). However, since not all dopaminergic neurons are at risk in PD, and since elevating DA levels in PD patients by L-DOPA administration does not accelerate the progression of PD (Fahn, 2005), DA unlikely is the principal culprit, even though its effects may further worsen the cellular deficits related to oxidant stress and/or protein aggregation triggered by other means (see below).

Another hypothesis has linked PD to mitochondrial dysfunction (Henchcliffe and Beal, 2008; Schapira, 2008; Vila et al., 2008). Mitochondria are crucial organelles for cellular energy production. The transport of electrons down the electron transport chain (ETC) releases energy which is used by complex I, III, and IV to pump protons from the mitochondrial matrix to the mitochondrial intermembrane space, creating a proton gradient and an electrochemical gradient across the mitochondrial inner membrane, the latter of which is being used by ATP synthase to convert ADP to ATP. Mitochondria comprise one of the major cellular producers of reactive oxygen species (ROS), as electrons in the ETC are occasionally captured by oxygen to produce superoxide anion radicals, with complex I and III being the major culprits for production of these radicals (Cali et al., 2011).

There is extensive evidence for mitochondrial involvement in both sporadic and genetic PD. Toxins such as MPTP, rotenone, and paraquat, which inhibit complex I, can cause a Parkinsonian

phenotype (Betarbet et al., 2000; Przedborski et al., 2004). In addition, postmortem tissue samples derived from the SNc from sporadic PD patients display a drastic decrease in the activity of complex I (Mann et al., 1994). A deficit in ETC can cause mitochondria-derived oxidative stress in the form of ROS and other radicals. Indeed, the decreased activity of complex I in PD patients seems due to oxidative damage (Keeney et al., 2006) and also affects other cellular components such as lipids and DNA (Zhang et al., 1999). Oxidative damage may also be responsible for the high levels of somatic mitochondrial DNA (mtDNA) deletions in SNc DA neurons (Bender et al., 2006; Kraytsberg et al., 2006), and the physical proximity of mtDNA to the site of ROS generation may indeed make them a vulnerable target. Since seven proteins involved in the formation of complex I are encoded by the mitochondrial genome, this may give rise to further ETC dysfunction and oxidative stress, leading to accelerated loss of SNc DA neurons.

However, the observed decrease in complex I deficiency in homogenates from nigral tissue from PD patients is too big to be restricted to SNc DA neurons, and only a proportion of PD patients show complex I inhibition in the SNc (Jenner, 2001). In addition, whilst toxins such as the herbicide rotenone cause ubiquitous complex I inhibition, dopaminergic degeneration is observed in the SNc, but not in the VTA area (Betarbet et al., 2000). Thus, inhibition of mitochondrial complex I activity *per se* cannot explain the selective vulnerability of neurons which die in PD.

WHY DO NEURONS DIE IN PD – PACEMAKING, Ca^{2+} DYSHOMEOSTASIS, AND OXIDANT STRESS

A hypothesis, put forward by Surmeier's group, suggests that specific and shared physiological features are responsible for the risk of a subset of neurons to degenerate in PD (Guzman et al., 2010; Surmeier et al., 2011; Goldberg et al., 2012), and comprises probably the best working model to explain disease pathomechanism to date (**Figure 1**).

Neurons are electrically excitable, using steep electrochemical gradients (mainly Na^+ and K^+ gradients) across their plasma membrane to integrate incoming chemical signals, and pass them on to other neurons. Voltage-dependent Ca^{2+} channels in most neurons are only opened by strong depolarization during an action potential. These channels close relatively slowly during membrane repolarization, such that the total Ca^{2+} influx during a spike is very sensitive to spike duration. To minimize global increases in Ca^{2+}, neurons which need to spike at high frequencies tend to restrict Ca^{2+} entry by keeping spikes very brief, and tend to express Ca^{2+} buffering proteins to help manage intracellular Ca^{2+} levels (Augustine et al., 2003).

In contrast to many other neurons, SNc DA neurons are autonomously active in the absence of synaptic input (Grace and Bunney, 1983). Such pacemaking activity is necessary to maintain a basal DA tone in the striatum; without it, movement ceases (Surmeier and Schumacker, 2013). Whilst most neurons rely on Na^+ to drive this pacemaking activity, SNc DA neurons also engage L-type Ca^{2+} channels with a Cav1.3 pore-forming subunit (Bonci et al., 1998; Puopolo et al., 2007). Although not strictly necessary for pacemaking, L-type Ca^{2+} channels help support pacemaking (Guzman et al., 2009). SNc DA neurons exhibit slow, broad spikes, causing a significant increase in intracellular Ca^{2+} levels, and they lack relevant intrinsic Ca^{2+} buffering capacity (Foehring et al., 2009; Guzman et al., 2009). The combination of these features, namely spontaneous activity that can be intrinsically generated, broad action potentials, prominent Ca^{2+} currents and low intrinsic Ca^{2+} buffering capacities are common to all neurons at risk for neurodegeneration in PD, irrespective of their neurotransmitter content (Surmeier and Schumacker, 2013). In contrast, relatively non-affected VTA DA neurons, whilst also slow pacemaking neurons, have low L-type Ca^{2+} channel densities and express high levels of the Ca^{2+} buffering protein calbindin (German et al., 1992; Khaliq and Bean, 2010).

GETTING RID OF Ca^{2+} – AN ENERGETICALLY COSTLY PROCESS

The shared physiological phenotype of at-risk neurons means that they will have a larger burden to handle increased intracellular Ca^{2+} levels. As Ca^{2+} is a universal second messenger, controlling a wide variety of cellular events ranging from regulation of enzyme activity to programmed cell death, it is under tight homeostatic control (Petersen et al., 2005). Pumping Ca^{2+} out of the cytosol is an energy-consuming process. Cytosolic Ca^{2+} levels are set to around 100 nM, which is 20,000-fold lower than the Ca^{2+} concentration in the extracellular space. This contrasts with the concentration differences of Na^+ and K^+ ions across the plasma membrane, which is in the range of 10–30-fold. Thus, thermodynamic considerations dictate that it will be energetically much more expensive to move Ca^{2+} ions across the plasma membrane as compared to Na^+ or K^+ ions (Surmeier and Schumacker, 2013).

Ca^{2+} ions are removed from the cytosol by either exchangers or pumps. Exchangers, such as the Na^+/Ca^{2+} exchanger use the Na^+ gradient to move Ca^{2+} ions out of the cytosol. Pumps, such as the plasma membrane Ca^{2+}-ATPase, use ATP to drive the movement of ions against a concentration gradient. Ca^{2+} buffering proteins further help to decrease the free Ca^{2+} concentration. Importantly, Ca^{2+} which is not rapidly pumped out of the neuron is sequestered into intracellular organelles including the endoplasmic reticulum (ER), mitochondria, Golgi, and lysosomes (**Figure 1**; Berridge et al., 2000; Rizzuto, 2001; Pinton et al., 2008; Lloyd-Evans and Platt, 2011; Kaufman and Malhotra, 2014).

How the increased demand for Ca^{2+} handling causes increased risk for degeneration of the vulnerable neuronal populations remains to be fully elucidated. One hypothesis proposes that due to their high basal ATP consumption rates related to Ca^{2+} handling, vulnerable neurons will have a lesser bioenergetic or respiratory reserve, which is defined as the difference between the maximum capacity for ATP generation by oxidative phosphorylation and the basal ATP consumption rate (Nicholls, 2008). A smaller respiratory reserve may put these neurons at risk when their metabolic demands increase, such as during bursts of spiking or upon toxin exposure. Indeed, when ATP levels are not sufficient to meet demands, a deterioration of the membrane potential would be followed by massive Ca^{2+} influx and cell death.

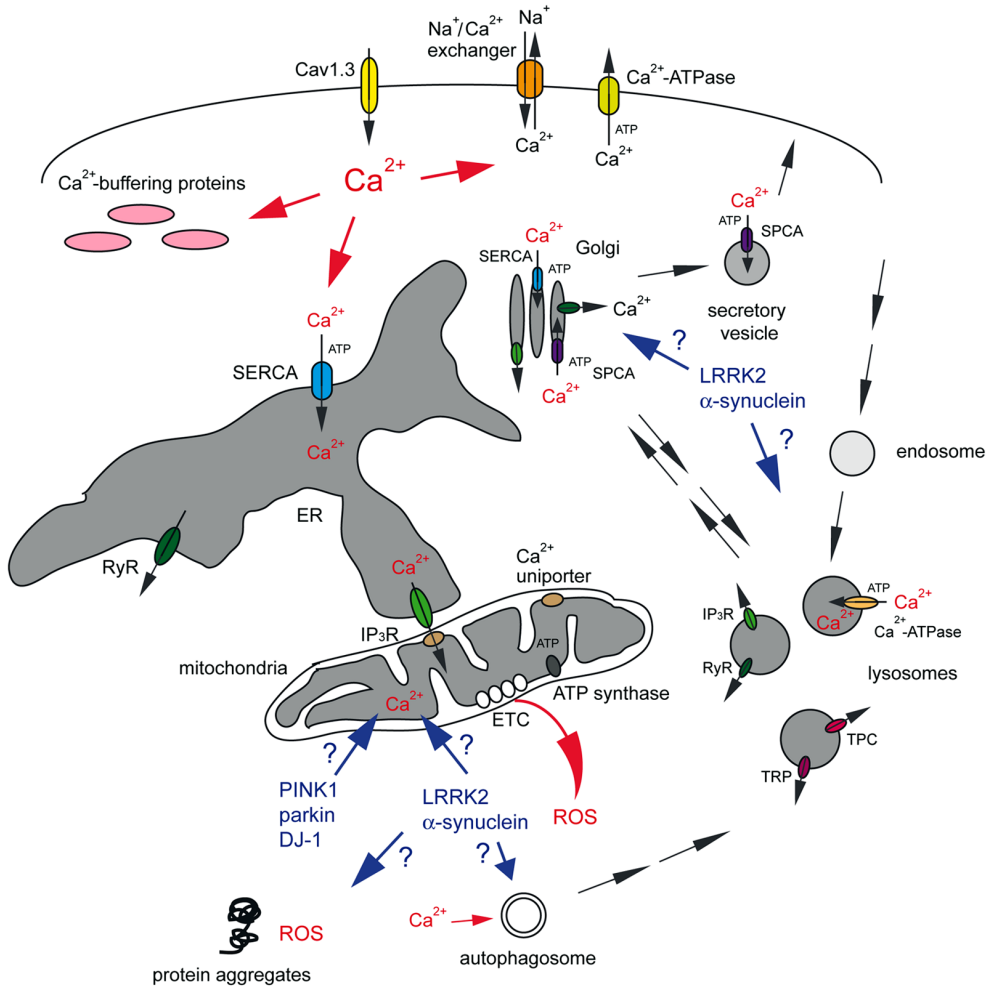

FIGURE 1 | Abnormal Ca^{2+} signaling in SNc DA neurons may cause mitochondrial oxidant stress, proteostasis deficits and eventual cell death. Vulnerable neuronal populations display spontaneous slow pacemaking activity employing Cav1.3 L-type Ca^{2+} channels, prominent Ca^{2+} currents and low intrinsic Ca^{2+} buffering capacities. Ca^{2+} inside the neuron can be transported back across the plasma membrane either via plasma membrane Ca^{2+}-ATPase at the cost of ATP consumption, or through the Na^{+}/Ca^{2+} exchanger which uses the Na^{+} gradient across the plasma membrane. Ca^{2+} is rapidly sequestered by interactions with Ca^{2+} buffering proteins or taken up into a variety of intracellular organelles. The ER uses a high-affinity Ca^{2+}-ATPase [the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA)] to pump Ca^{2+} into the ER lumen at the cost of ATP consumption. This pump is also present on cis and medial Golgi membranes, whilst secretory vesicles employ a secretory pathway Ca^{2+}-ATPase (SPCA) which is also be present on the trans Golgi complex. Ca^{2+} uptake into acidic organelles is mediated by a molecularly unidentified Ca^{2+}-ATPase. Ca^{2+} flows back into the cytosol from the ER lumen through IP_3 receptors (IP_3R) or ryanodine receptors (RyR). IP_3R are also present on cis and medial Golgi membranes, RyR on trans Golgi membranes, and RyR, TRP and TPC channels are present on acidic organelles. Mitochondria, often in close apposition to the ER or plasma membrane, can take up Ca^{2+} into the matrix through a mitochondrial Ca^{2+} uniporter. Ca^{2+} transfer between ER and mitochondria involves the IP_3R on the ER membrane. Ca^{2+} within mitochondria is necessary for proper ETC function to generate ATP by ATP synthase, but mitochondrial Ca^{2+} overload can cause mitochondrial oxidant stress (ROS). Toxins as well as familial mutations in PINK1, parkin and DJ-1 affect mitochondrial ATP production and Ca^{2+} handling, even though the molecular details remain to be determined. The effects of familial mutations in LRRK2 and α-synuclein on mitochondrial functioning are even less clear, but those mutant proteins may cause additional deficits in proteostasis through mechanisms involving Ca^{2+}-regulated events such as autophagy. This may also include alterations in the trafficking of Golgi-derived vesicles to the plasma membrane, resulting in changes in vesicle secretion and in the steady-state levels of surface receptors. Golgi deficits may cause altered trafficking of enzymes destined to lysosomes, with concomitant deficits in lysosomal degradative capacity, or alterations in retromer-mediated retrieval from endolysosomes back to the Golgi. Finally, changes in acidic store Ca^{2+} levels may affect various endo-lysosomal trafficking steps or the degradative capacity of acidic organelles *per se*. For further details see text.

The increased metabolic demand of SNc neurons may also give rise to an increase in the basal level of mitochondrial oxidant stress, as high rates of metabolic activity cause increased ROS production (**Figure 1**; Lee et al., 2001). In support of this, pacemaking in SNc neurons was shown to generate mitochondrial oxidant stress, which was not apparent in neighboring VTA DA neurons (Guzman et al., 2010). Such oxidant stress was largely prevented in the presence of L-type Ca^{2+} channel antagonists, clearly implicating those channels and the resultant increase in intracellular Ca^{2+} as culprits for downstream oxidant stress

generated by high demands for mitochondrial ATP production.

Mitochondrial oxidant stress causes mild mitochondrial depolarization or uncoupling (Guzman et al., 2010), which leads to a decline in energy production and generation of ROS, causing damage to proteins, lipid, and DNA. In accordance with this, mtDNA deletions are significantly greater in SNc DA neurons from older as compared to younger subjects, and from neurons from PD patients as compared to unaffected individuals (Bender et al., 2006; Kraytsberg et al., 2006), with no changes observed in other brain areas. The accumulation of mtDNA deletions, with effects on mitochondrial respiratory chain function, will thus lead to further bioenergetic deficiency that manifests over time.

GETTING RID OF Ca^{2+} – NOT JUST A PROBLEM OF ENERGY

It is clear that cytosolic Ca^{2+} levels have to be maintained within a small range of concentrations for optimal survival of SNc DA neurons (Michel et al., 2013). However, apart from the extra bioenergetic burden to control intracellular Ca^{2+} levels, altered Ca^{2+} handling by various intracellular organelles may threaten neuronal viability as well. Indeed, mitochondrial oxidant stress in SNc DA neurons can be diminished when limiting mitochondrial Ca^{2+} uptake, without affecting pacemaking (Guzman et al., 2010). This is important, as it suggests that mitochondrial oxidant stress may be the consequence of increased mitochondrial Ca^{2+} load, rather than a mere reflection of the need for increased ATP production.

Ca^{2+} is well-known to modulate mitochondrial function. The Ca^{2+} uniporter uses the mitochondrial membrane potential to take Ca^{2+} up into the mitochondrial matrix (Kirichok et al., 2004; Santo-Domingo and Demaurex, 2010), where it increases ATP production by stimulating enzymes of the tricarboxylic acid (TCA) cycle, and thus helps to maintain increased metabolic demands associated with electrical activity and influx of Ca^{2+} (McCormack and Denton, 1990). However, too much Ca^{2+} in mitochondria compromises mitochondrial function by causing a transient collapse of the mitochondrial membrane potential (McCormack and Denton, 1990), which thus transiently halts the production of ATP.

The mitochondrial Ca^{2+} uniporter drives rapid and massive Ca^{2+} entry at high cytosolic Ca^{2+} concentrations only thought to be reached in microdomains near plasma membrane Ca^{2+} channels and Ca^{2+} release channels on the ER. Indeed, the primary intracellular organelle dealing with Ca^{2+} homeostasis is thought to be the ER (Berridge, 2002; Verkhratsky, 2005). The ER is responsible for the coordinated production, delivery, and degradation of proteins in a process called proteostasis. It forms a continuous intracellular network which extends throughout the somatodendritic tree (Choi et al., 2006), and contains high-affinity ATP-dependent transporters [(sarco-ER Ca^{2+}-ATPase (SERCA)] to move Ca^{2+} from the cytoplasm into the ER lumen. Ca^{2+} sequestered in the ER can be released at sites where it can be pumped back across the plasma membrane, or can be used locally to modulate cellular function (Verkhratsky, 2005). The Ca^{2+} store in the ER is highly interconnected with other intracellular Ca^{2+} stores, such that ER Ca^{2+} dyshomeostasis will affect Ca^{2+} handling in other organelles as well. For example, inositol 1,4,5-trisphosphate (IP_3) receptors which reside at direct ER-mitochondrial contacts termed MAMs (mitochondria-associated ER membranes) allow for direct flux of Ca^{2+} from ER into mitochondria (Csordas et al., 2006; Rizzuto and Pozzan, 2006; Kaufman and Malhotra, 2014), which may then lead to the mitochondrial Ca^{2+} overload described above (**Figure 1**). Indeed, stimulation of Ca^{2+} release from the ER by ryanodine, accompanied by an increase in cytosolic Ca^{2+} levels, was found to protect DA neurons from spontaneous or induced neurodegeneration (Guerreiro et al., 2008). Thus, relieving the Ca^{2+} load in the ER, without significantly causing Ca^{2+} transfer from ER to mitochondria through IP_3 receptors, may prove beneficial to the survival of DA neurons, possibly via preventing ER-mediated mitochondrial Ca^{2+} overload. Altered ER Ca^{2+} concentrations are also associated with altered changes in cytosolic Ca^{2+} concentration upon ER release, and thus can affect the downstream signaling functions of this organelle (Morikawa et al., 2000; LaFerla, 2002).

Apart from its signaling function, Ca^{2+} plays an inherently important role for the functioning of the ER by acting as an allosteric regulator of protein processing and folding. Depletion of ER Ca^{2+} stores induces ER stress and the unfolded protein response (Paschen and Mengesdorf, 2005). Too much intraluminal ER Ca^{2+} may compromise proteostasis as well. For example, L-type Ca^{2+} channel blockers have been shown to restore folding and lysosomal delivery of mutant lysosomal enzymes responsible for a variety of lysosomal storage diseases (Mu et al., 2008). Similarly, decreasing ER Ca^{2+} levels by SERCA inhibitors seems to enhance the folding and plasma membrane trafficking of mutant cystic fibrosis transmembrane conductance regulator (CFTR; Egan et al., 2002, 2004). Precise Ca^{2+} imaging experiments will be required to determine the intraluminal ER Ca^{2+} levels upon such treatments. Nevertheless, these data indicate that altering ER Ca^{2+} homeostasis can have profound effects on folding and trafficking of proteins destined to other subcellular locations including lysosomes and the plasma membrane (**Figure 1**), with obvious downstream effects both on plasma membrane functioning/signaling and lysosomal degradative capacity.

INTRACELLULAR Ca^{2+} STORES AND Ca^{2+} HANDLING: THE NEGLECTED PLAYERS

In addition to the ER and mitochondria, two other compartments deserve attention as significant intracellular Ca^{2+} store. The first is the Golgi apparatus, which shares some functions and biochemical markers with the ER. The Golgi complex is a highly dynamic intracellular organelle which processes and sorts membrane proteins derived from the ER to the cell surface, secretory vesicles or lysosomes, and which also receives retrograde transport input. Thus, damage to neuronal Golgi structure can have important functional consequences for protein and vesicular trafficking (Fan et al., 2008). Interestingly, Golgi fragmentation has been observed in nigral neurons from PD patients (Fujita et al., 2006), and recent studies indicate that increased neuronal activity causes reversible Golgi fragmentation in a manner dependent on Ca^{2+}-calmodulin-dependent protein kinase (Thayer et al.,

2013). It will be interesting to determine whether Golgi fragmentation is a shared phenotype of vulnerable neurons in PD, and if it can be modulated by L-type Ca^{2+} channel antagonists. In addition, it remains to be seen whether neuronal activity-dependent Golgi fragmentation causes Golgi-derived Ca^{2+} release which may alter the spatio-temporal complexity of cellular Ca^{2+} signaling.

The Golgi complex serves as a bona fide Ca^{2+} store, containing Ca^{2+}-ATPases, Ca^{2+} release channels and Ca^{2+}-binding proteins (**Figure 1**; Scherer et al., 1996; Pinton et al., 1998; Lin et al., 1999). The Golgi seems to handle Ca^{2+} differently dependent on its sub-compartments. Whilst *cis*- and medial Golgi compartments contain the SERCA ATPase and IP_3 receptors, the trans Golgi takes Ca^{2+} up exclusively via SPCA1 (secretory pathway Ca^{2+}-ATPase isoform 1), and at least in some cells contains ryanodine receptors (Lissandron et al., 2010). Thus, the Golgi can serve as a Ca^{2+} store responding to local Ca^{2+}-induced Ca^{2+} release or to second messengers such as cyclic ADP ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) which have been shown to activate ryanodine receptors (Fliegert et al., 2007). Decreasing Ca^{2+} in the trans-Golgi complex alters the structure of the entire Golgi apparatus, with effects on sorting of proteins to the plasma membrane through the secretory pathway (Lissandron et al., 2010; Micaroni, 2012). For example, depletion of SPCA1 has been shown to disrupt polarized trafficking, impairing neuronal differentiation, and the generation of functional neurites (Sepúlveda et al., 2009). The mechanism by which intraluminal Ca^{2+} in the Golgi may regulate sorting is starting to emerge. For example, sorting of some secretory proteins has been shown to require actin remodeling by ADF/cofilin, SPCA1, and a soluble Golgi-resident Ca^{2+}-binding protein (von Blume et al., 2011, 2012). Sorting may depend on a transient influx of Ca^{2+} into the trans Golgi induced by the binding of ADF/cofilin to SPCA1, which may facilitate the association of secretory proteins with the Golgi-resident Ca^{2+}-binding protein, acting as a soluble receptor to segregate a subset of secretory proteins (Kienzle and von Blume, 2014). In sum, alterations in intraluminal Ca^{2+} concentrations can impact both on cellular Ca^{2+} signaling as well as on Golgi structure and secreted protein cargo sorting (Micaroni, 2012), and it will be interesting to determine whether this may cause cell-autonomous deficits for example by altering the formation and trafficking of small dense-core DA-containing vesicles (Bauerfeind et al., 1995), or non-cell-autonomous events such as altering the secretion of neurotrophic factors with downstream effects on dopaminergic cell survival (Kordower and Bjorklund, 2013).

Ca^{2+} is also stored in a variety of acidic organelles (Patel and Docampo, 2010). Acidic organelles containing Ca^{2+} include endosomes, lysosomes, lysosome-related organelles and secretory granules. Amongst acidic organelles, lysosomes probably comprise the most prominent Ca^{2+} stores, and may contain an average free Ca^{2+} concentration in the range of 500 μM, similar to the Ca^{2+} concentration within the ER (Lloyd-Evans et al., 2008). Ca^{2+} uptake into lysosomes is thought to be mediated by pumps. Indeed, purified lysosomes from neutrophils, fibroblasts, and rat liver have been shown to take up Ca^{2+} in an ATP-dependent manner (Klempner, 1985; Lemons and Thoene, 1991; Ezaki et al., 1992; Adachi et al., 1996). The molecular nature of the lysosomal Ca^{2+}-ATPase remains to be determined, even though some data indicate that it may be driven by SERCA3 (López et al., 2005). Alternatively, Ca^{2+} loading into lysosomes has been suggested to involve ER Ca^{2+} leak, such that small fluctuations in ER Ca^{2+} levels may cause large effects on lysosomal Ca^{2+} load (Bezprozvanny, 2012). Acidic stores also possess Ca^{2+}-permeable channels such as IP_3/ryanodine receptors, TRP channels (transient receptor potential channel superfamily), and TPCs (two-pore channels), which are members of the TRP channel superfamily as well (**Figure 1**). TPC channels located on endosomes and lysosomes have been reported to be targets for NAADP, the most potent Ca^{2+} mobilizing messenger (Churchill et al., 2002; Guse and Lee, 2008). However, they do not directly bind to NAADP (Lin-Moshier et al., 2012; Walseth et al., 2012), and their gating properties and ion selectivity have recently been questioned (Wang et al., 2012; Cang et al., 2013). This may be due to the fact that they can heterodimerize in-between themselves as well as with a subset of TRP channels, which are gated by NAADP as well (Patel and Docampo, 2010), and further work will be necessary to elucidate how second messengers such as NAADP may trigger Ca^{2+} release from acidic organelles, and the precise channels involved.

Lysosomal impairments seem intricately linked to PD pathogenesis. Lysosomes are the primary degradative organelle in all cell types, and their function is particularly important in non-dividing cells such as neurons. Several diseases associated with lysosomal dysfunction (lysosomal storage diseases) have been identified, and many of them affect brain function. Conversely, many neurodegenerative diseases also exhibit lysosomal dysfunction (Schultz et al., 2011). Lysosomal impairments are observed in sporadic PD brain and toxic as well as genetic rodent models of PD-related neurodegeneration (Dehay et al., 2013). The mechanisms involved may be varied, including defects in the lysosomal delivery of enzymes required for degradation, defects in lysosomal acidification or altered intralysosomal Ca^{2+} handling. Importantly, the lysosomal degradative system is characterized by many vesicular fusion events along the endocytic pathway which depend on intraluminal Ca^{2+}, and lysosomal Ca^{2+} is also required for luminal content condensation (Pryor et al., 2000; Luzio et al., 2007). Whilst precise Ca^{2+} imaging experiments will be required to determine whether SNc neurons display alterations in intralysosomal Ca^{2+} levels, such lysosomal Ca^{2+} dyshomeostasis is expected to cause impaired turnover of dysfunctional mitochondria, which would further aggravate mitochondria-derived oxidant stress in vulnerable neurons.

In the context of proteostasis, it is also worthy considering effects of altered intracellular Ca^{2+} levels on autophagy, a process employed by cells to get rid of protein aggregates and defunct organelles, and deficits of which are also clearly implicated in PD (Lynch-Day et al., 2012). There is some controversy as to whether increases in Ca^{2+} promote or inhibit autophagy. This may be due to the subcellular localization of the source of the Ca^{2+} signal and may also depend on cellular state (Decuypere et al., 2011). Under normal conditions, the IP_3 receptor-mediated Ca^{2+} transfer from the ER to mitochondria, which maintains

mitochondrial ATP production, seems to inhibit autophagy. In contrast, an increase in cytosolic Ca^{2+} concentrations can stimulate autophagy (**Figure 1**; Decuypere et al., 2011). In both cases, this may involve the activity of AMPK, which is activated when cellular ATP levels drop and/or when cytosolic Ca^{2+} levels increase. Activation of autophagy, combined with a decrease in lysosomal degradative capacity, may then lead to the observed accumulation of autolysosomal structures observed in PD brains (Anglade et al., 1997).

PD, AGING, RISK FACTORS, AND GENETICS

Age is clearly the single strongest risk factor for PD. The physiological properties of SNc DA neurons indicate that they will be at a higher risk of age-related cell death due to their enhanced burden of Ca^{2+} handling. Indeed, these neurons seem to be lost at a higher rate (5–10% every 10 years) than many other neurons in the brain, some of which do not display significant loss over 60–70 years (Stark and Pakkenberg, 2004). This means that we may all develop PD if we live long enough. Environmental and genetic factors may then dictate which people become symptomatic (Sulzer, 2007). Environmental factors may further alter intracellular Ca^{2+} handling, or may impact upon downstream cellular events triggered by Ca^{2+} dyshomeostasis, playing either protective or damaging roles. As mentioned above, for example toxins known to cause PD increase mitochondrial oxidant stress, thus impacting upon the same pathway already affected in vulnerable neurons.

Similarly, genetic forms of PD would be expected to converge on pathways affected by altered intracellular Ca^{2+} handling. Familial mutations in a variety of genes, with either autosomal-recessive (parkin, PINK1, DJ-1) or autosomal-dominant [(α-synuclein, leucine-rich repeat kinase (2LRRK2)] inheritance account for approximately 10% of PD cases (Trinh and Farrer, 2013). Of those, parkin, PINK1, and DJ-1 are clearly implicated in mitochondrial homeostasis and Ca^{2+} handling (Scarffe et al., 2014). For example, DJ-1 seems to protect against mitochondrial oxidant stress evoked by pacemaking in dopaminergic neurons by interfering with mitochondrial uncoupling in response to calcium-induced stress (Guzman et al., 2010). Depletion of DJ-1 seems to decrease expression of certain mitochondrial uncoupling proteins, even though the underlying mechanism(s) remain to be determined. PINK1 has been proposed to contribute to maintaining bioenergetic function of mitochondria by regulating Ca^{2+} efflux via the Na^{+}/Ca^{2+} exchanger, and PINK1 deficiency was reported to cause mitochondrial Ca^{2+} overload, resulting in mitochondrial oxidant stress (Gandhi et al., 2009). Other studies indicate that PINK1 deficiency is associated with mitochondrial fragmentation, decreased membrane potential and decreased agonist-stimulated Ca^{2+} entry, thus pinpointing to a role for PINK1 in mitochondrial Ca^{2+} uptake rather than Ca^{2+} extrusion, and concomitant decreased ATP production (Heeman et al., 2011). Similarly, parkin deficiency has been reported to cause mitochondrial fragmentation and ER-mitochondria Ca^{2+} crosstalk, thus affecting cellular bioenergetics (Cali et al., 2013). Both parkin and PINK1 cooperate to regulate mitochondrial quality control events such as fission and fusion, degradation of defunct mitochondria by autophagy (mitophagy), mitochondrial transport, and biogenesis (Scarffe et al., 2014). Whilst the molecular mechanism(s) at present remain sketchy, these three proteins seem to be implicated in the same Ca^{2+}-mediated pathway which is already compromised in sporadic PD (**Figure 1**).

Other proteins implicated in familial PD such as α-synuclein and LRRK2 have been consistently shown to cause dysfunction of the autophagy/lysosomal degradation system (**Figure 1**; Manzoni and Lewis, 2013), but how they may impact upon ER-mitochondrial Ca^{2+} handling and mitochondrial oxidant stress is less clear. Autosomal-dominant mutations in LRRK2 have been shown to cause deficits in Ca^{2+} homeostasis, leading to mitochondrial depolarization and enhanced mitophagy, which can be prevented by L-type Ca^{2+} channel inhibitors (Papkovskaia et al., 2012; Cherra et al., 2013). Greater levels of mtDNA damage can be observed in LRRK2 mutant patient cells as compared to healthy subjects (Sanders et al., 2014), but whether this is due to altered mitochondrial Ca^{2+} handling remains to be determined.

Apart from directly affecting mitochondrial Ca^{2+} handling, gene products involved in familial PD may also affect Ca^{2+} homeostasis in other intracellular organelles such as ER, Golgi, or lysosomes, with downstream effects on proteostasis and protein aggregation. Precise Ca^{2+} imaging experiments in the context of both sporadic and familial PD models will be required to reveal possible alterations in intracellular Ca^{2+} handling by these distinct organelles. For example, altered lysosomal Ca^{2+} levels may be responsible for the observed changes in lysosomal morphology, clustering, and degradative capacity described for mutant LRRK2-expressing cells (MacLeod et al., 2006; Tong et al., 2010; Dodson et al., 2012; Gómez-Suaga et al., 2012; Orenstein et al., 2013). Such changes, concomitant with an increase in cytosolic Ca^{2+} levels (Gómez-Suaga et al., 2012), may lead to aberrations in autophagic clearance, followed by a deficit in proteostasis. Impaired proteostasis in the presence of mutant α-synuclein has recently been shown to indirectly increase mitochondrial oxidant stress, suggesting that proteostatic extra-mitochondrial stress may be additive with mitochondrial oxidant stress observed in SNc DA neurons (**Figure 1**; Dryanovski et al., 2013). Whilst the mechanism(s) by which this occurs requires further investigation, it seems to involve NADPH oxidase activity. These data indicate that extramitochondrial oxidant stress may significantly contribute to PD, such that reverting proteostasis deficits may also be therapeutically beneficial in slowing down PD progression. In this context, Golgi-derived proteostasis effects may be worth considering as well, and may underlie altered risk for sporadic (Beilina et al., 2014) as well as familial PD, where Golgi phenotypes have been observed upon mutant α-synuclein and LRRK2 expression (Lin et al., 2009), even though whether this is related to altered Ca^{2+} handling in the Golgi remains to be determined. In sum, Ca^{2+} dyshomeostasis seems to be central towards our understanding of both sporadic and familial PD, and can affect a plethora of cellular events related to mitochondrial bioenergetics and oxidant stress as well as proteostasis (at the level of the ER, Golgi, and lysosomes) which may in turn increase extramitochondrial-derived oxidant stress to further threaten the viability of affected neurons.

NOVEL HOPES FOR TREATMENT OPTIONS?

The above-mentioned findings indicate that L-type Ca^{2+} channel antagonists may be viable therapeutic targets in the early stages of PD. There are oral antagonists [dihydropyridines (DHP)] available, with good blood–brain barrier permeability and a long record of safe use in humans. Adult SNc DA neurons can compensate for L-type Ca^{2+} channel antagonism and continue pacemaking (Chan et al., 2007), and mice do not show obvious motor, learning, or cognitive deficits when treated with L-type Ca^{2+} channel antagonists (Bonci et al., 1998), suggesting that these compounds do not alter the functional activity of SNc DA neurons. Indeed, several studies in humans indicate that these compounds diminish the risk of developing PD (Becker et al., 2008; Ritz et al., 2010; Pasternak et al., 2012). However, they do not seem to slow progression of PD (Marras et al., 2012), maybe because of their relatively poor potency against Cav1.3 L-type Ca^{2+} channels, or because other factors may become more prominent during disease manifestation. Such factors may in part derive from alterations in intracellular Ca^{2+} stores, with the resultant varied downstream effects on cellular proteostasis.

Much work remains to be done before gaining a clearer understanding of the role of Ca^{2+} dysregulation in the pathogenesis of PD. It is becoming increasingly clear that abnormal Ca^{2+} handling may have pleiotropic effects on a variety of intracellular events resulting in mitochondrial oxidant stress, deficits in ER proteostasis, endolysosomal/autophagic trafficking and alterations in Golgi function which require further investigation. Thus, whilst L-type Ca^{2+} channel antagonists may attack the source of the problem, improving the deteriorated cellular functions of mitochondria, ER, lysosomes, or Golgi may be an efficient complementary strategy to attack the varied downstream effects of the increased burden of handling intracellular Ca^{2+} in vulnerable neurons. Maybe a feasible future therapeutic strategy should not involve a "hit-hard" principle employed for example to treat cancer patients, but rather a "hit-softly, continue hitting, and hit at multiple places at a time" principle aimed at correcting a combination of cellular deficits derived from improper Ca^{2+} handling employing combination-type therapies.

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Protein phosphorylation in neurodegeneration: friend or foe?

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Protein misfolding and aggregation is a common hallmark in neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), and fronto-temporal dementia (FTD). In these disorders, the misfolding and aggregation of specific proteins occurs alongside neuronal degeneration in somewhat specific brain areas, depending on the disorder and the stage of the disease. However, we still do not fully understand the mechanisms governing protein aggregation, and whether this constitutes a protective or detrimental process. In PD, alpha-synuclein (aSyn) forms protein aggregates, known as Lewy bodies, and is phosphorylated at serine 129. Other residues have also been shown to be phosphorylated, but the significance of phosphorylation in the biology and pathophysiology of the protein is still controversial. In AD and in FTD, hyperphosphorylation of tau protein causes its misfolding and aggregation. Again, our understanding of the precise consequences of tau phosphorylation in the biology and pathophysiology of the protein is still limited. Through the use of a variety of model organisms and technical approaches, we are now gaining stronger insight into the effects of phosphorylation in the behavior of these proteins. In this review, we cover recent findings in the field and discuss how targeting phosphorylation events might be used for therapeutic intervention in these devastating diseases of the nervous system.

Keywords: alpha-synuclein, tau, Parkinson's disease, Alzheimer's disease, phosphorylation, neurodegeneration

NEURODEGENERATIVE DISORDERS AS PROTEIN AGGREGOPATHIES

Neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD) and frontotemporal dementia (FTD), result from the progressive loss of specific neuronal populations leading to the progressive appearance of the clinical symptoms that are characteristic of each disorder. Current treatments for these disorders are palliative rather than curative and their effectiveness is still far from satisfactory. Thus, tremendous efforts are underway to elucidate the causes underlying these disorders and to find a cure. From a molecular perspective, the common hallmark of neurodegenerative disorders is the misfolding and aberrant aggregation of proteins in amyloid-like beta-sheet filaments. This feature is not only characteristic of classic neurodegenerative disorders but also of prion disorders and other amyloidosis inside and outside the central nervous system, suggesting that neurodegenerative disorders are part of a much greater superfamily of protein misfolding disorders, or aggregopathies (Frost and Diamond, 2010).

While it is clear that protein misfolding and aggregation are pathological hallmarks of neurodegenerative disorders, the precise mechanisms linking protein aggregation and neurotoxicity are largely unknown. Protein aggregates are dynamic structures, allowing small soluble species to detach or attach from or to larger protein inclusions relatively easily (Kim et al., 2002). As a result of this dynamism, protein inclusions have variable solubility, stability and size. Big, insoluble protein inclusions inside or outside neurons were initially thought to be neurotoxic. However, current evidence indicates that they might be rather neuroprotective (Arrasate et al., 2004; Bodner et al., 2006), and that the smaller, more soluble oligomers are the ones that exert neurotoxicity. A consensus in this matter remains to be reached but, regardless of the nature of the toxic and non-toxic species, unraveling the mechanisms determining protein aggregation is absolutely necessary for the understanding, diagnosis and treatment of neurodegenerative disorders.

POSTTRANSLATIONAL MODIFICATIONS AS MODULATORS OF PROTEIN FATE

Protein aggregation can be regulated by various cellular events including different types of stress, molecular crowding, or the local micro-environment. In addition, diverse posttranslational modifications (PTMs), such as phosphorylation, ubiquitination or sumoylation, which alter the conformation and/or biological function of proteins, can also affect protein folding and aggregation, and thereby play a critical role in neurodegenerative disorders. For example, ubiquitination can direct proteins for either degradation by the proteasome or to certain subcellular compartments, and glycosylation is related to the secretion of proteins to the extracellular medium. Both processes could

therefore influence protein concentration, folding, localization and, ultimately, aggregation. Phosphorylation can also affect protein conformation, function and fate in many different ways: it may be required for proper protein folding; it may induce conformational changes that can result in lower or higher catalytic activity; it may precede or function as a recognition signal for further modifications, such as ubiquitination; it may alter the subcellular localization of the protein; and it may modify protein-protein interactions (Salazar and Hofer, 2009). In the particular case of neurodegenerative and protein misfolding diseases, phosphorylation has been shown to be involved in both protein aggregation and toxicity, as illustrated by the paradigmatic examples described below (**Figure 1**).

PHOSPHORYLATION IN PARKINSON'S DISEASE AND OTHER SYNUCLEINOPATHIES

Alpha-synuclein (aSyn) is the main protein component of Lewy bodies (LBs), the typical pathological hallmarks of PD and other disorders collectively known as synucleinopathies. The vast majority of PD cases are sporadic, multiplications and missense mutations in the gene encoding for aSyn have been associated with familial forms of PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Singleton et al., 2003; Chartier-Harlin et al., 2004; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Kiely et al., 2013; Lesage et al., 2013).

Several PTMs have already been identified in aSyn. These include ubiquitination (Shimura et al., 2001), phosphorylation at S129 (Fujiwara et al., 2002), a C-terminal truncation (Li et al., 2005), nitration on tyrosine residues (Giasson et al., 2000), glycosylation, and SUMO modification (Dorval and Fraser, 2006). However, most attention was devoted to phosphorylation at S129 (pS129). While only 4% of the soluble, monomeric aSyn appears phosphorylated under physiological conditions *in vivo*, approximately 90% is phosphorylated in LB lesions (Fujiwara et al., 2002; Anderson et al., 2006), suggesting a close relationship between aSyn phosphorylation at S129 and its aggregation. In particular, pS129 aSyn is found in LBs occurring and other pathogenic inclusions found in substantia nigra of PD patients (Fujiwara et al., 2002; Saito et al., 2003; Anderson et al., 2006) as well as in different brain regions of patients suffering from other synucleinopathies, such as dementia with LBs (DLB), multiple system atrophy (Fujiwara et al., 2002; Kahle et al., 2002; Saito et al., 2003; Nishie et al., 2004; Waxman and Giasson, 2008), Hallervorden-Spatz disease (Fujiwara et al., 2002), pure autonomic failure (Arai et al., 2000b), and LB variant of AD (LBVAD) (Waxman and Giasson, 2008). In addition, aSyn was found to be phosphorylated on S129 in transgenic mice expressing human mutant A30P, A53T, or WT aSyn (Kahle et al., 2002; Freichel et al., 2007; Wakamatsu et al., 2007).

In addition to S129, other three serine, four tyrosine and ten threonine residues are putative sites of phosphorylation (**Figure 2**). These residues are mostly localized in the C-terminal region of the protein, with the exception of Y39 and S87. Increased levels of phosphorylated S87 (pS87) were also reported in synucleinopathies (Paleologou et al., 2010). Phosphorylation aSyn on tyrosine 39 (pY39) and 125 (pY125) was also reported in human brains but no correlation was established between increased levels of phosphorylation in these residues and the pathological condition (Chen et al., 2009; Mahul-Mellier et al., 2014). Other residues were found to be phosphorylated *in vitro* but it is unknown if their phosphorylation also occurs *in vivo*, even if in small extension.

THE ROLE OF PHOSPHORYLATION ON aSyn CYTOTOXICITY AND AGGREGATION

The phosphorylation status of aSyn clearly influences its aggregation and toxicity, but it is still unclear whether phosphorylation promotes or prevents aggregation and toxicity. To better understand this trinomial relation, would also be important to clearly establish what are the toxic forms of aggregated aSyn, although recent studies suggest that the soluble oligomeric/protofibrillar species may be more toxic than larger aggregated forms of aSyn (Spillantini et al., 1997; Conway et al., 2001; El-Agnaf et al., 2003; Outeiro et al., 2007; Diogenes et al., 2012).

In vitro and *in vivo* studies, correlating phosphorylation of aSyn in several residues to its aggregation and/or toxicity, resulted in conflicting results (**Table 1**). Several of these studies employed S129A and S129D/E mutants, to block and mimic phosphorylation, respectively. Other studies modulated the levels of phosphorylation of aSyn by either co-expressing specific kinases or phosphatases, or by using kinase inhibitors (**Table 1**). Moreover, these studies included three types of assays: (i) *in vitro* biochemical studies; (ii) single cell models (yeast and mammalian cells); and (iii) animal models of PD (mice or rat models). In these studies the relation between toxicity and aggregation was not always explored (**Table 1**).

The genetic mutant that attempts to mimic pS129 (S129D) aSyn was initially associated with pathology in a transgenic *Drosophila* model (Chen and Feany, 2005; Chen et al., 2009) while aSyn hyperphosphorylation and insolubility were correlated with the disease in transgenic mouse models of PD (Kahle et al., 2002; Freichel et al., 2007). However, opposite results were obtained in yeast, rat and *Caenorhabditis elegans* models of PD. Namely, S129E had no effect while the mutation S129A increased aSyn toxicity in budding yeast (Fiske et al., 2011; Sancenon et al., 2012). Moreover, in rat models using retrovirus-mediated expression of aSyn in neurons of the *substantia nigra*, the S129A variant also showed toxicity while the results for S129D were variable, showing either protecting (Gorbatyuk et al., 2008) or no effect (Azeredo Da Silveira et al., 2009). In *C. elegans* models, S129D aSyn was also protective, reducing neuronal dysfunction, while S129A expression resulted in severe motor dysfunction, growth retardation, and synaptic abnormality by lowering its membrane interaction (Kuwahara et al., 2012).

Similarly, while some reports suggest that pS129 promotes inclusion formation (Fujiwara et al., 2002; Smith et al., 2005; Arawaka et al., 2006; Takahashi et al., 2007; Gorbatyuk et al., 2008; Kragh et al., 2009; Wu et al., 2011a), others suggest that phosphorylation prevents or has no effect on inclusion formation (Lee et al., 2004a; Chen and Feany, 2005; Paleologou et al., 2008; Waxman and Giasson, 2008; Azeredo Da Silveira et al., 2009; Chau et al., 2009; Chen et al., 2009; Fiske et al., 2011; Sancenon et al., 2012) (**Table 1**).

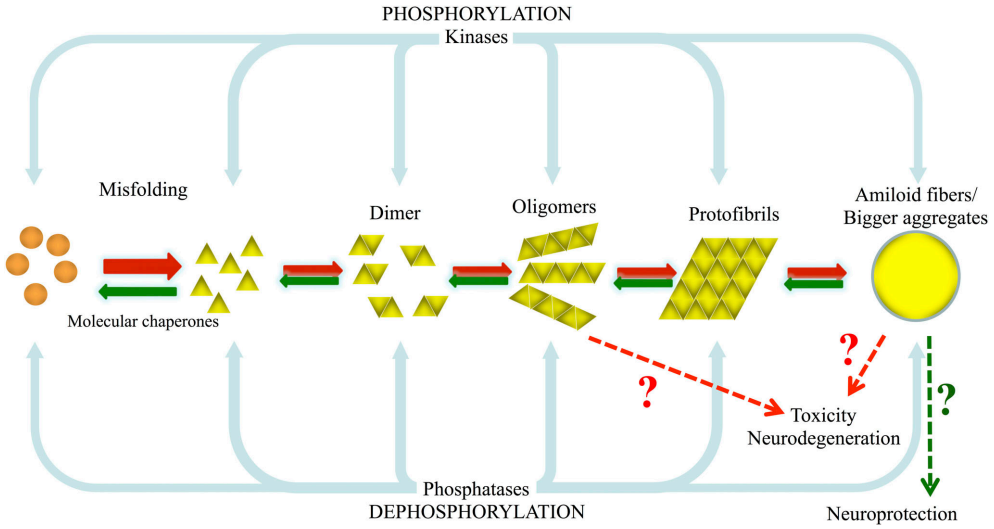

FIGURE 1 | Model of aSyn and tau misfolding and aggregation, and the involvement of kinases and phosphatases on their phosphorylation/dephosphorylation. Under pathological conditions, due to genetic or environmental factors such as exposure to pesticides, normal highly soluble aSyn and tau misfold and are converted into pathological oligomers and larger species that fibrillize and deposit into inclusion bodies as LBs and Lewy neurites and into PHFs and NFTs. In this situation the normal cellular quality-control systems (molecular chaperones, ubiquitin proteasome system (UPS), phagosome/lysosome system) are not able to counteract and prevent or reverse protein misfolding or eliminate proteins that have misfolded or assembled into pathological aggregates and amyloid fibrils.

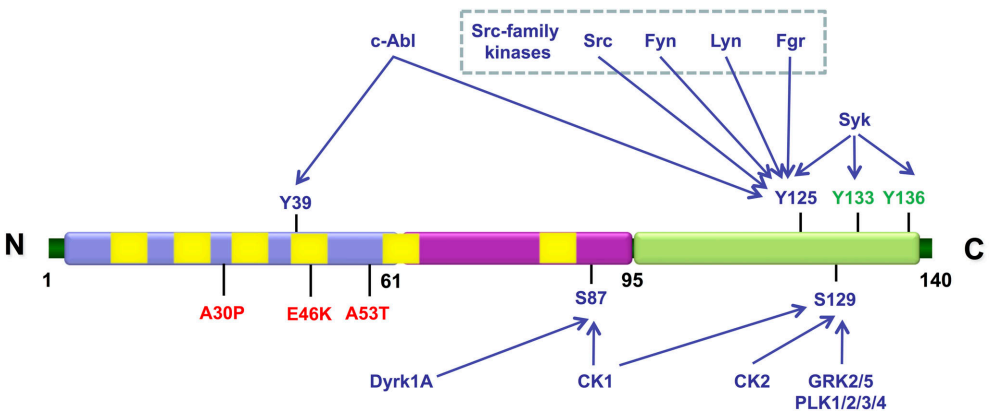

FIGURE 2 | Schematic representation illustrating the various residues in aSyn that can be phosphorylated *in vivo* (represented in blue) and *in vitro* (represented in green). The mutations associated with familial PD are shown in red. The N-terminal amphipathic region of the protein is represented in blue, the hydrophobic central region that contains the non-amyloid-β component (NAC) domain is represented in purple and the highly acidic C-terminal is represented in green. The imperfect KTKEGV repeats are represented in yellow. The kinases described as being able to phosphorylate each of the indicated residues are also indicated.

In vitro biochemical studies also lead to conflicting results regarding the correlation between pS129 and fibrillization of aSyn. While S129 aSyn phosphorylated by casein kinase (CK)2 was found to form fibrils more readily than unphosphorylated aSyn *in vitro* (Fujiwara et al., 2002), different studies observed that fibrillization of aSyn is inhibited in purified pS129 S87A aSyn (where phosphorylation at S87 is blocked) (Paleologou et al., 2008; Waxman and Giasson, 2008), while a more recent study reposted that pS129 aSyn by polo-like kinase (PLK) 2 displays comparable fibrillization kinetics to the WT protein *in vitro* (Schreurs et al., 2014).

Studies performed in different cell and animal models are also not consensual regarding the correlation between aSyn pS129 and aggregation. Most studies performed in cell lines associate aSyn pS129 with increased formation of soluble oligomers (Arawaka et al., 2006; Kragh et al., 2009), cytoplasmic and nuclei aggregates (Arawaka et al., 2006; Wu et al., 2011a), and cytoplasmic inclusions (Smith et al., 2005; Takahashi et al., 2007) (**Table 1**). Toxicity was evaluated only in some of these studies and was interrelated with increased aggregation in one study (Kragh et al., 2009) but was found to be protective in another (Wu et al., 2011a).

Table 1 | aSyn phosphorylation sites and effects.

aSyn residue	Kinase	Model	Cytotoxicity	Aggregation	References
Y39	c-Abl	*In vitro* biochemical assay/M17 neuroblastoma cell lines/ primary cultures of mouse cortical neurons / mThy1 aSyn transgenic mice / Rat model involving viral delivery	c-Abl inhibition increases aSyn degradation by proteasome and autophagy pathways	–	Mahul-Mellier et al., 2014
S87	CK1	*In vitro* biochemical assay/K293 and PC12 cells	–	–	Okochi et al., 2000
	Dyrk1A	*In vitro* biochemical assay/SH-SY5Y and H19-7 cells	Increased pS87 increases citotoxicity	Increased pS87 increases aSyn aggregation in cultured cells	Kim et al., 2006
	CK1	*In vitro* biochemical assay/SH-SY5Y cells/ transgenic mice M20 and M83	–	pS87 reduce recombinant aSyn fibril formation	Waxman and Giasson, 2008
	CK1	*In vitro* biochemical assay/ transgenic mouse models of PD/LBD and MSA	–	S87E or pS87 blocks aSyn fibrillization	Paleologou et al., 2010
	–	Rat model involving viral delivery of WT, S87A, and S87E aSyn	S87E protects against aSyn induced toxicity by reducing dystrophic fibers, and motor impairment	S87E inhibits aSyn aggregation	Oueslati et al., 2012
Y125	Fyn	*In vitro* biochemical assay/COS7 cells	–	–	Nakamura et al., 2001
	Src, Fyn	*In vitro* biochemical assay/HEK293T cells	–	–	Ellis et al., 2001
	Src-family kinases	COS7 cells	–	–	Nakamura et al., 2001
	Syk, Lyn, Fgr	*In vitro* biochemical assay/SH-N-BE and CHO cells	–	Syk-mediated aSyn phosphorylation decreases oligomerization	Negro et al., 2002
	kinase shark (Syk *Drosophila* homolog)	*Drosophila*	Increased pY125 is protective; Y125F is toxic	Increased pY125 decreases aSyn oligomerization while Y125F increases it	Chau et al., 2009
	Fyn	*In vitro* biochemical assay	–	*in vitro* pY125 fibrillate similarly to WT aSyn while Y125F or Y125E fibrillate significantly slower than WT aSyn	Schreurs et al., 2014
	c-Abl	*In vitro* biochemical assay/M17 neuroblastoma cell lines/ primary cultures of mouse cortical neurons / mThy1 aSyn transgenic mice / Rat model involving viral delivery	c-Abl inhibition increases aSyn degradation by proteasome and autophagy pathways	–	Mahul-Mellier et al., 2014
S129	CK1, CK2	*In vitro* biochemical assay/K293 cells	–	–	Okochi et al., 2000
	CK1, CK2, Grk2, Grk5	*In vitro* biochemical assay/COS-1 cells	–	–	Pronin et al., 2000
	CK2	*In vitro* biochemical assay	–	pS129 increases aSyn fibrillization *in vitro*	Fujiwara et al., 2002
	PLK2	*In vitro* biochemical assay/HEK293 cells/Mouse	–	–	Inglis et al., 2009

(Continued)

Table 1 | Continued

aSyn residue	Kinase	Model	Cytotoxicity	Aggregation	References
	CK1, CK2	*In vitro* biochemical assay	–	pSer129 inhibits rather than promotes aSyn fibrillization; S129A promotes aSyn aggregation	Paleologou et al., 2008
	PLK2	*In vitro* biochemical assay	–	*in vitro* pS129, S129A or S129D fibrillate similarly to WT aSyn	Schreurs et al., 2014
	Yck1 and Yck2 yeast CK1 kinases	*S. cerevisiae*	pS129 by plasma membrane CK1 kinases correlates with aSyn toxicity	pS129 by plasma membrane CK1 kinases correlates with aSyn inclusion formation	Zabrocki et al., 2008
	–	*Schizosaccharomyces pombe* and*S. cerevisiae*	Neither S129A nor S129D mutants altered WT aSyn toxicity	Both S129A and S129D increased endomembrane association in *S. pombe*, but only S129D decreased plasma membrane association in *S. cerevisiae*	Fischer et al., 2009
	CK1	*S. cerevisiae*	Yck1 CK1 yeast ortholog phosphorylate S129 aSyn and attenuate aSyn toxicity by an S129 phosphorylation-independent mechanism; S129A increases aSyn toxicity in a yeast genetic context-dependent manner	S129A increases aSyn inclusion formation a in a yeast genetic context-dependent manner;	Sancenon et al., 2012
	PLK1, PLK2, PLK3, PLK4	*S. cerevisiae,* mouse CAD cathecolaminergic cells and human H4 neuroglioma cells	PLK2 increased aSyn cytotoxicity in yeast but by an S129 phosphorylation-independent mechanism phosphorylation	PLK2 promotes aSyn inclusion formation in yeast and in mammalian cells by an S129 phosphorylation-independent mechanism	Basso et al., 2013
	–	*S. cerevisiae*	S129A increases aSyn toxicity	S129A aSyn forms more inclusions and oligomeric species with higher molecular weight than the WT form	Tenreiro et al., 2014
	GRK5	HEK293, SH-SY5Y cells and primary neurons from the cerebral cortex of fetal mice	–	Increased aggregation by co-expression with GRK5	Arawaka et al., 2006
	CK2	293T, PC-12 and NS20Y cells stably or transiently transfected with synphilin-1, aSyn and/or CKII	–	S129A mutation does not influence aSyn aggregation with synphilin-1	Lee et al., 2004a
	–	293T cells stably transfected with synphilin-1 co-expressing aSyn WT or S129A	S129A mutation does not influence aSyn toxicity	S129A mutation does not influence aSyn aggregation	Tanaka et al., 2004
	CK2	SH-SY5Y cells	No toxicity detected	S129A decreases inclusion formation while pS129 levels correlates with inclusion formation	Smith et al., 2005
	CK2 and other unidentified kinases	SH-SY5Y cells	S129D is toxic; S129A does not affect aSyn toxicity	increased pS129 was not attend with increased insoluble aggregates	Chau et al., 2009
	CK2	3D5 neuroblastoma cell line	–	pS129 promotes aSyn oligomerization and inclusion formation	Takahashi et al., 2007

(Continued)

Table 1 | Continued

aSyn residue	Kinase	Model	Cytotoxicity	Aggregation	References
	CK1, CK2	*In vitro* biochemical assay/SH-SY5Y cells/ transgenic mice M20 and M83	–	pS129 reduced recombinant aSyn fibril formation	Waxman and Giasson, 2008
	–	Mouse MN9D dopaminergic cells coexpressing human aSyn WT or S129D	S129D is protective	S129D promotes aSyn fibril or inclusion formation	Wu et al., 2011a
	GRK2, GRK5, PLK2, PLK3	Human brain neuroglioma H4 cell line	–	S129A increases inclusion formation	Gonçalves and Outeiro, 2013
	CK2 and PLKs	Rat oligodendroglial cell line OLN-93 coexpressing human p25aand aSyn WT or S129A/D	pS129 increases microtubule retraction followed by apoptosis and cell dead; S129A is protective while S129D behaves as WT, whoever with a smooth phenotype	pS129 promotes aSyn oligomers formation while S129A mutagenesis or CK2 and PLKs kinase inhibitors prevent it	Kragh et al., 2009
	PLK1, PLK2, PLK3	HEK293T/HeLa cells/ primary rat Neurons/ (Thy1)-h[A30P] aSyn transgenic mice	–	–	Mbefo et al., 2010
	Gprk2 (Grk2 *Drosophila* homolog)	*Drosophila*	Increased pS129 is toxic; S129D is toxic; S129A is protective	pS129 increases soluble oligomers formation but has no effect on inclusion formation	Chen and Feany, 2005; Chen et al., 2009
	–	SH-SY5Y cells/ transgenic *C. elegans*	S129D is protective while S129A is toxic	No insoluble oligomers or bigger aggregates were observed	Kuwahara et al., 2012
	–	Rat model involving viral delivery of WT or S129D/A aSyn	S129A is toxic while S129D is protective	S129D promotes inclusion formation while S129A reduce it	Gorbatyuk et al., 2008
	–	Rat model involving viral delivery of WT or A30P aSyn with S129D/A mutations	S129A is toxic while S129D has no effect	S129A increases aggregates formation while S129D forms fewer but larger aggregates	Azeredo Da Silveira et al., 2009
	CK1, CK2, PLK1, PLK2, PLK3	*In vitro* biochemical assay / QBI293 cells transfected with WT aSyn and treated with recombinant aSyn fibrils to induce the formation of aggregates, treated with kinases inhibitors or co-expressing kinases	–	Results obtained with different kinases suggest that phosphorylation of aSyn is independent of aSyn aggregate formation	Waxman and Giasson, 2011
	PLK2	HEK239T cells co transfected with aSyn and WT PLK2 or the kinase dead mutant (DM) PLK2; treated or not with PLK2 inhibitor / Rat model involving viral delivery of aSyn with either PLK2 WT or KDM	Increased pS129 aSyn by PLK2 reduces aSyn accumulation, suppresses dopaminergic neurodegeneration, and reverses hemiparkinsonian motor impairments by promoting aSyn autophagic clearence	–	Oueslati et al., 2013
Y133	Syk	*In vitro* biochemical assay/SH-N-BE and CHO cells	–	Syk-mediated aSyn phosphorylation decreases oligomerization	Negro et al., 2002
Y136	Syk	*In vitro* biochemical assay/SH-N-BE and CHO cells	–	Syk-mediated aSyn phosphorylation decreases oligomerization	Negro et al., 2002

In yeast cells, S129A aSyn is more toxic and forms more inclusions and oligomeric species of higher molecular weight than S129E or WT forms of aSyn (Sancenon et al., 2012; Tenreiro et al., 2014). Consistently, higher toxicity of the S129A variant is also associated with an increase in the generation of small, more soluble aggregates in rats (Azeredo Da Silveira et al., 2009).

To explain the discrepancies between the results obtained in different cell and animal models, several ideas have been put forward. One possibility is that the predominance of pS129 aSyn in LBs is not caused by its inherent propensity to aggregate but could be more related to the presence or absence of additional factors in the different models employed. Namely, the distinct results obtained in rat models could be eventually associated to dose-dependent interactions between rat aSyn and virally expressed mutant human aSyn, altering the aggregation properties of the protein, as has been demonstrated *in vitro* for mixtures of mouse and human aSyn (Rochet et al., 2000). On the other hand, in SH-SY5Y cells, co-expression of aSyn S129A with synphilin-1, an aSyn interacting protein that is also present in LBs, resulted in the formation of fewer inclusions than WT aSyn (Smith et al., 2005). In a *Drosophila* model, the obvious differences in the complexity of the nervous system and the absence of an aSyn homolog might explain the differences observed (Goedert, 2001; Hamilton, 2004).

Recently, it was also suggested that the discrepancies observed in the various studies might be due to different efficiencies of the different kinases in phosphorylating either S129 or other residues, as well as their differential pattern of expression in the different models (Oueslati et al., 2013; Schreurs et al., 2014). The conflicting results might also be due to differences in the dephosphorylation machinery involved in the dephosphorylation of aSyn, a process that is still understudied.

Another hypothesis is that phosphorylation could be an indirect cause of aSyn pathology, namely due to the impairment of the proteolytic machinery (Azeredo Da Silveira et al., 2009). There are several examples of proteins where phosphorylation works as a signal for protein degradation. If this is also the case for aSyn, then phosphorylated aSyn could accumulate in LBs due to proteasomal impairment (McNaught and Jenner, 2001; Shimura et al., 2001; Tanaka et al., 2001; Snyder et al., 2003; Grunblatt et al., 2004) leading to its accumulation and consequent aggregation.

Phosphorylation of aSyn in inclusions may be partially due to the intrinsic properties of aggregated aSyn to act as substrate for kinases but not phosphatases, as indicated by *in vitro* studies, suggesting that fibril and inclusion formation occur prior to phosphorylation and that this modification becomes more pronounced with disease progression (Waxman and Giasson, 2008; Mbefo et al., 2010; Paleologou et al., 2010; Waxman and Giasson, 2011).

In addition to S129, there are other phosphorylation sites in aSyn that may be relevant to aggregation and toxicity in synucleinopathies. This could either be due to a direct effect of the phosphorylation, or due to an effect on the cross-talk that likely occurs between phosphorylated states of these different residues. For example, phosphorylation of tyrosine residues Y125, Y133, and Y136 in the C-terminal segment of aSyn suppresses eosin-induced oligomerization (Negro et al., 2002). Phosphorylation at Y125 (pY125) has opposing effects to phosphorylation of S129 on aSyn neurotoxicity and soluble oligomer formation in a transgenic *Drosophila* model (Chen et al., 2009). Although pY125 does not directly affect the pS129 or vice versa, tyrosine phosphorylation is possibly acting downstream of pS129, as increasing pY125 levels rescued the neurotoxicity of a phospho-mutant S129D (Chen et al., 2009). This could be easily explained considering that different kinases are involved in the two phosphorylation events, which in turn can have behind completely different regulation pathways and physiological roles. Phosphorylation at Y125 diminishes with aging and is reduced in cortical tissue of DLB patients indicating a neuroprotective role (Chen et al., 2009). However, another recent study did not observe any significant differences in the levels of pY125 between PD brains and controls (Mahul-Mellier et al., 2014). In fact, phosphorylation at this residue was not detected in LBs of patients with DLB, in PD patients carrying the A53T mutation, nor in MSA cases (Anderson et al., 2006). This might be due to an increased sensitivity of Y125 to be dephosphorylated post mortem (Chen et al., 2009). Additionally, it could not be completely excluded that the observed effect on reduced oligomerization and concomitant toxicity was exclusively due to pY125, as the degree of Y133 and Y136 phosphorylation was not evaluated in this study performed in a *Drosophila* PD model (Chen et al., 2009). In fact, studies using recombinant aSyn demonstrated that the single phosphorylation of Y125 by Lyn and Fgr kinases does not affect oligomerization while the phosphorylation of all residues Y125, Y133 and Y136 by Syk prevents it (Negro et al., 2002). Recently, a new residue was detected as being phosphorylated in human brain tissues, the Y39, but without significant differences in the levels of pY39 between PD brains and controls (Mahul-Mellier et al., 2014). Importantly, in this same study, phosphorylation at Y39 and Y125 was found to play an important role in regulating aSyn clearance through proteasome and autophagy pathways (Mahul-Mellier et al., 2014).

S87 is, in addition to Y39, the only other residue outside the C-terminal region reported to undergo phosphorylation (pS87) *in vivo* (Paleologou et al., 2010). pS87 was found to be increased in brains of rat and mice models of synucleinopathies as well as in human brains from AD, LBD, and MSA patients (Paleologou et al., 2010) contradicting previous studies where phosphorylation of aSyn at this residue was not detected in either human brain samples or a transgenic mouse model of synucleinopathies (Fujiwara et al., 2002; Anderson et al., 2006; Waxman and Giasson, 2008). Again, results obtained using different systems were contradicting. pS87 may promote aSyn inclusion formation and decrease cell viability in SH-SY5Y and H19-7 cell lines (Kim et al., 2006). On the other hand, *in vitro* phosphorylation at this site inhibits aSyn fibril formation (Waxman and Giasson, 2008; Paleologou et al., 2010). Moreover, immunofluorescence staining of LBs isolated from fresh human brains using a specific anti-pS87 antibody allowed its detection and suggested that this phosphorylation occurs throughout the life span of LB development (Paleologou et al., 2010). More recently, S87E was found to inhibit aggregation and to protect against aSyn induced toxicity *in vivo*, namely by reducing aSyn aggregates, dystrophic fibers, and motor impairment in a rat model of PD where viral

delivery was used to overexpress WT, S87A, and S87E aSyn in the substantia nigra (Oueslati et al., 2012).

Another aspect that might explain the discrepancies observed in the different studies is the employment of aSyn phosphorylation mutants. While several studies reported consistent results using in parallel genetic or pharmacological methods to alter aSyn phosphorylation status (Chen and Feany, 2005; Smith et al., 2005; Chen et al., 2009), other studies indicate that phospho-mutants may not fully recapitulate the real phosphorylation/unphosphorylation states of aSyn (Paleologou et al., 2008; Schreurs et al., 2014). In particular, it was shown that S129A and S129D/E mutations themselves could have effects on aSyn aggregation properties independent of their effects on phosphorylation, with the S129A mutation stimulating fibril formation while S129D/E mutations do not reproduce the effect of phosphorylation on the structural and aggregation properties of aSyn *in vitro* (Paleologou et al., 2008). However, in yeast we observed that S129G and S129A mutations, both blocking aSyn phosphorylation, were more toxic and resulted in increased inclusion formation excluding that the observed phenotypes were due to specific structural consequences of S129A mutation on aSyn (Tenreiro et al., 2014). Moreover, in this yeast model, S129E aSyn exhibited the same phenotype of toxicity and inclusion formation as the WT protein that is strongly phosphorylated on S129 by endogenous kinases (Tenreiro et al., 2014). Despite the inherent caveats, the use of aSyn phospho-mutants still remains as a unique and powerful means to interrogate the effects of phosphorylation. Regarding the use of phosphomimic mutants of other residues, S87E was shown to behave as pS87, at least with respect to its effects on aSyn aggregation, while the S87A mutant exhibited similar secondary structure and similar membrane binding and aggregation properties as the WT protein (Waxman and Giasson, 2008; Paleologou et al., 2010; Oueslati et al., 2012).

Mutants that abolish phosphorylation at tyrosine residues of aSyn (by replacing tyrosine by phenylalanine residues) were used in several *in vitro* and *in vivo* studies (Chen et al., 2009; Mahul-Mellier et al., 2014). However, there are no mutants that mimic the phosphorylated state of a tyrosine residue, restricting the use of mutants that attempt to mimic tyrosine phosphorylation.

PHYSIOLOGICAL AND PATHOLOGICAL IMPLICATIONS OF aSyn PHOSPHORYLATION

Initial studies suggested aSyn might be predominantly unphosphorylated under physiological conditions (Okochi et al., 2000; Fujiwara et al., 2002). It was hypothesized that changes in aSyn phosphorylation could represent a response to biochemical events associated with PD pathogenesis. Among these, mitochondrial complex I dysfunction, oxidative stress and proteasome dysfunction are processes that are known to be involved in synucleinopathies (Lee and Trojanowski, 2006; Lashuel et al., 2013). Increased levels of pS129 aSyn were observed upon proteasome inhibition or oxidative stress in SH-SY5Y cells over-expressing aSyn. In the case of proteasomal impairment, this seems to result in pS129 aSyn accumulation through an increase in the activity of the kinase(s) involved, a decrease in protein turnover and, ultimately, in increased cell death (Waxman and Giasson, 2008; Chau et al., 2009). The kinases involved were not fully characterized but CK2 was found to be one of them. On the other hand, it is known that aggregation of aSyn itself leads to proteasome impairment (Tanaka et al., 2001; Snyder et al., 2003; Lindersson et al., 2004), which in turn could lead to CK2 activation and eventually to increased levels of pS129. It is important to note that phosphorylation of S129 appears not to be a general response to cellular stress, as inhibition of complex I had little effect on pS129 aSyn levels (Waxman and Giasson, 2008; Chau et al., 2009).

The low levels of pS129 aSyn under physiological conditions as well as the absence of other phosphorylated residues such as pY39, pS87 and pY125 (Okochi et al., 2000; Fujiwara et al., 2002; Anderson et al., 2006) could also be related to a faster degradation of this form under normal conditions. In fact, the phosphorylation status of aSyn was recently correlated with clearance mechanisms (Oueslati et al., 2013; Mahul-Mellier et al., 2014). Namely, blocking S129 phosphorylation in a yeast model lead to impaired aSyn clearance by autophagy (Tenreiro et al., 2014). In line with this observation increased levels of pS129 by overexpression of PLK2 suppress dopaminergic neurodegeneration, and reverse hemiparkinsonian motor impairments in a rat model of PD by promoting aSyn autophagic degradation (Oueslati et al., 2013). Moreover, phosphorylation at Y39 and Y125 by c-Abl kinase protects aSyn against its degradation via the autophagy and proteasome pathways in cortical neurons (Mahul-Mellier et al., 2014).

Phosphorylation also seems to alter the subcellular localization of aSyn. While pS129 aSyn was found to be preferentially localized in the nuclei of dopaminergic neurons in rat and mouse models of synucleinopathy (Yamada et al., 2004; Wakamatsu et al., 2007), in other studies using PD rat models the phosphoresistant S129A was found to be localized in the nucleus at higher levels than the S129D form, and was found to correlate with enhanced toxicity (Gorbatyuk et al., 2008; Azeredo Da Silveira et al., 2009). Our group demonstrated that S129 phosphorylation modulates the shuttling of aSyn between nucleus and cytoplasm in human neuroglioma cells, using photoactivatable green fluorescent protein as a reporter. Moreover, we also found that co-expression of aSyn with different kinases altered the translocation dynamics of the protein. While G protein-coupled receptor kinase 5 (GRK5) promotes the nuclear localization of aSyn, PLK2 and 3 modulate the shuttling of the protein between the nucleus and cytoplasm (Gonçalves and Outeiro, 2013). This difference might reflect different aSyn phosphorylation patterns in S129 and/or other residues, or phosphorylation of other targets besides aSyn. Very recently, G51D aSyn was found to exhibit enhanced nuclear localization and to be hyperphosphorylated on S129 in primary neurons (Fares et al., 2014). Although the function of aSyn in the nucleus is still unclear, it seems this is related with a pathological role that is independent of aSyn aggregation. In particular, nuclear localization of aSyn increases under oxidative stress conditions (Xu et al., 2006; Monti et al., 2010; Siddiqui et al., 2012). Nuclear aSyn interacts with histones, inhibits acetylation and promotes neurotoxicity (Goers et al., 2003; Kontopoulos et al., 2006). Moreover, aSyn might act as a transcriptional regulator, binding promoters such as PGC1-alpha, a master regulator of mitochondrial gene expression (Siddiqui et al., 2012).

Phosphorylation at S129 reduces the affinity of aSyn for lipids (Okochi et al., 2000; Pronin et al., 2000; Fujiwara et al., 2002; Yamada et al., 2004). Also pS87 was described to significantly reduce aSyn binding to lipid vesicles (Paleologou et al., 2010). Therefore, the phosphorylation status of aSyn might regulate its role in synaptic vesicle dynamics in physiological conditions and might contribute to its pathological role in abnormal dopamine neurotransmission (Lundblad et al., 2012; Scott and Roy, 2012).

It was also reported that aSyn inhibits tyrosine hydroxylase activity, a rate-limiting enzyme in dopamine biosynthesis, in dopaminergic MN9D cells, while the phosphomimic mutant of aSyn, S129D, relieves this inhibition and results in an increase of dopamine content in cells (Wu et al., 2011a). Recently, it was also observed that membrane-associated aSyn enhances dopamine uptake capacity in dopaminergic SH-SY5Y cells by the dopamine transporter through GRKs-mediated S129 phosphorylation (Hara et al., 2013).

The phosphorylation status of aSyn could also modulate its protein-protein interactions. The unphosphorylated form of S129 associates mainly with mitochondrial electron transport proteins while pS129 associates with cytoskeletal, vesicular trafficking proteins and enzymes involved in protein serine phosphorylation (McFarland et al., 2008). Phosphorylation also appears to have an important role in the regulation of aSyn axonal transport as the S129D mutation significantly reduces its rate of transport in neurons, likely due to the modulation of the interaction of aSyn with motor and/or accessory proteins involved in this process (Saha et al., 2004). Moreover, the interplay between the different phosphorylated residues could also contribute to increase the diversity in the possible protein interactors. In fact, several differences were observed in the set of proteins that were found to interact with S129 or Y125-phosphorylated forms of aSyn (McFarland et al., 2008). Both, S129 and Y125 residues are localized in the C-terminal region of aSyn which has been implicated in the majority of aSyn interactions with proteins (Jensen et al., 1999; Giasson et al., 2003a; Fernandez et al., 2004) reinforcing the relevance that phosphorylation in these residues could modulate the biological role of aSyn.

The C-terminus of the protein was also implicated in aSyn interactions with metal ions (Paik et al., 1999; Brown, 2007). These interactions influence the structure and propensity for aggregation of aSyn *in vitro* and in cell culture models of synucleinopathies (Paik et al., 1999; Brown, 2007; Wright et al., 2009). Interestingly, a recent study showed that pY125 and pS129 alter the binding sites of metal ions and increase the binding affinity of Cu(II), Pb(II), and Fe(II), but not Fe(III), a feature that could modulate aSyn function as well as aggregation (Lu et al., 2011).

Phosphorylation at S87 could also modulate protein-protein interactions, as some proteins were found to interact via the non-amyloid component (NAC) region, in which this residue is located. As an example, it is possible that S87 phosphorylation alters the interaction with phospholipase D (PLD) 2, an enzyme involved in lipid-mediated signaling cascades and vesicle trafficking (Outeiro and Lindquist, 2003; Payton et al., 2004).

Fyn and Src kinases are able to phosphorylate aSyn at Y125, suggesting phosphorylation in this residue might also modulate spatial learning and synaptic plasticity, due to the role these kinases play in these processes (Zhao et al., 2000). On the other hand, Fyn and Src are non-receptor-type PTKs activated by extracellular factors like neurotrophic factors and growth factors, suggesting that the phosphorylation state of aSyn can be regulated by extracellular signaling molecules, such as neurotrophins, cytokines, and cell adhesion molecules (Nakamura et al., 2001). Recently, a new connection between tyrosine phosphorylation of aSyn and synaptic plasticity was established with the identification of Y39 as the main target of phosphorylation by c-Abl protein tyrosine kinase (Mahul-Mellier et al., 2014), a kinase that plays an important role in the development of the central nervous system (CNS) and in neuronal plasticity (Moresco and Koleske, 2003; Moresco et al., 2003). Interestingly, c-Abl is upregulated in PD brains (Ko et al., 2010; Hebron et al., 2013a) as well as in other neurodegenerative diseases (Schlatterer et al., 2011). This tyrosine kinase is activated by increased levels of aSyn and, in turn, increased c-Abl activity leads to aSyn accumulation (Hebron et al., 2013a,b) by increasing pY39 and, to a lesser extent pY125, thereby affecting clearance pathways (Mahul-Mellier et al., 2014). Thus, phosphorylation of aSyn at tyrosine residues could be relevant in the context of the alterations of synaptic functions observed in PD and other synucleinopathies.

It remains unclear how familial mutations of aSyn might alter the phosphorylation of the protein, but it is likely that different mutations may influence phosphorylation in different residues. In transgenic mice expressing either E46K or A53T aSyn, inclusions were found to be strongly phosphorylated at S129 (Emmer et al., 2011). In HEK cells, S129 phosphorylation by GRK6 or PLK2 is equally efficient in WT or in G51D aSyn, although pS129 enhanced nuclear localization of G51D compared to WT aSyn (Fares et al., 2014). Moreover, while the A53T mutant shows similar phosphorylation levels to WT aSyn in SH-SY5Y cells (Smith et al., 2005), and slower *in vitro* phosphorylation kinetics by CK2 (Ishii et al., 2007), it was observed that detergent-insoluble aSyn from patients carrying the A53T mutation was hyper-phosphorylated at S129 (Anderson et al., 2006). In any case, additional studies on the interplay between aSyn mutations and phosphorylation are needed.

KINASES INVOLVED IN aSyn PHOSPHORYLATION

Several kinases have been implicated in aSyn phosphorylation (**Table 1**). S129 can be phosphorylated by G-protein coupled receptor kinases (GRK1, GRK2, GRK5 and GRK6) (Pronin et al., 2000; Arawaka et al., 2006; Sakamoto et al., 2009), casein kinases 1 and 2 (CK1, CK2) (Okochi et al., 2000; Smith et al., 2005; Ishii et al., 2007; Takahashi et al., 2007; Wakamatsu et al., 2007; Waxman and Giasson, 2008; Zabrocki et al., 2008), and the polo-like kinases (PLKs) (Inglis et al., 2009). The leucine-rich repeat kinase 2 (LRRK2) was also shown to phosphorylate aSyn at S129 (Qing et al., 2009), but this remains highly controversial as no other studies were able to confirm this, despite the existence of a clear interaction between the two proteins (Qing et al., 2009; Guerreiro et al., 2013).

Recent studies revealed that, in addition to phosphorylating agonist-occupied G protein-coupled receptors (GPCRs), GRKs may also phosphorylate non-receptor substrates, including the four members of the synuclein family (aSyn,

beta-, gamma-synuclein and synoretin) (Pronin et al., 2000). Overexpression of GRK2 or GRK5 in COS-1 cells showed that these kinases phosphorylate aSyn at S129 (Pronin et al., 2000). Phosphorylation of aSyn at S129 by endogenous GRKs was also demonstrated in HEK293 cells and it was observed that GRK3 and GRK6 play the main role in this modification (Sakamoto et al., 2009).

GRK5 was found to colocalize with aSyn in the LBs of the substantia nigra of PD patients, but was not detected in cortical LBs of DLB, or in the glial cytoplasmic inclusions of MSA (Arawaka et al., 2006). Overexpression of aSyn increased GRK5 protein expression in both, SH-SY5Y cells and in brain extracts of transgenic mice expressing human aSyn (Liu et al., 2010). A genetic association study performed in the Japanese population revealed a haplotypic association of the GRK5 gene with susceptibility to sporadic PD (Arawaka et al., 2006). However, another genetic association study performed in Southern Italy failed to correlate GRK5 polymorphisms with sporadic PD (Tarantino et al., 2011). The knockdown of endogenous GRK5 in SH-SY5Y cells fails to suppress phosphorylation of aSyn (Liu et al., 2010) confirming the involvement of other kinases in this phosphorylation.

CK1 and CK2 also phosphorylate aSyn at S129 in yeast (Zabrocki et al., 2008), in mammalian cells (Okochi et al., 2000; Waxman and Giasson, 2008) and in rat primary cortical neurons (Ishii et al., 2007).

CK1-mediated phosphorylation at S129 may counteract aSyn toxicity by attenuating vesicular trafficking defects and restoring synaptic transmission in some extension (Sancenon et al., 2012). However, higher levels of aSyn could result in protein mislocalization in other compartments, ultimately leading to defects in synaptic vesicle homeostasis and neurotransmission. In addition, excess aSyn may form inclusions that sequester CK1, depleting CK1 activity and exacerbating synaptic defects, generating a toxic vicious cycle. CK1 was also found to phosphorylate aSyn at S87 (Okochi et al., 2000), and to colocalize with pS87 in transgenic mice and in LB-like structures in LBD/PD diseased brains (Paleologou et al., 2010). β subunits of CK2 were also found to colocalize with LBs in PD brains (Ryu et al., 2008). Interestingly, oxidative stress imposed by iron overload causes upregulation of CK2 which, in turn, leads to increased pS129 aSyn with a concomitant increase in oligomerization and inclusion formation (Takahashi et al., 2007). In SH-SY5Y cells, the increase in aSyn phosphorylation under oxidative stress is mediated by CK2 and correlates with enhancement of inclusion formation (Smith et al., 2005).

In vitro studies, employing kinase assays, showed that PLK1, PLK2 and PLK3 are also capable of phosphorylating aSyn at S129 (Inglis et al., 2009; Mbefo et al., 2010). The PLKs comprise a family of conserved Ser/Thr protein kinases that are known to play critical roles on cell cycle regulation, cellular response to stress and carcinogenesis (Ng et al., 2006). PLK2 and PLK3 are expressed in response to synaptic activation and appear to be involved in synaptic plasticity, remodeling and homeostasis (Kauselmann et al., 1999; Seeburg et al., 2005, 2008), suggesting these kinases could be important actors in modulating the normal physiology of aSyn.

PLK2 and PLK3 partially colocalize with pS129 aSyn in primary hippocampal neurons as well as in cortical brain areas of aSyn transgenic mice, reinforcing the idea that S129 phosphorylation by the PLKs might also occur in human brain (Mbefo et al., 2010). Consistently, PLK2 levels are elevated in brains of patients with AD and DLB, and correlate with the increased levels of pS129 aSyn, further supporting a role for this kinase in disease (Mbefo et al., 2010). PLK2 is also involved in the phosphorylation of aggregated aSyn *in vitro* (Mbefo et al., 2010) and in cell culture (Waxman and Giasson, 2011). A prominent role of PLK2 as a regulator of aSyn turnover was recently described (Oueslati et al., 2013). Importantly, PLK2-mediated pphosphorylation at S129 of aSyn is protective in a rat model of PD, by promoting aSyn autophagic degradation (Oueslati et al., 2013). Recently, we also described that PLK2 mediates aSyn inclusion formation in yeast and in mammalian cells by a S129 phosphorylation-independent mechanism (Basso et al., 2013).

Only CK1 and the dual specificity tyrosine regulated kinase 1A (Dyrk1A) were found to phosphorylate aSyn at S87, and this was based on *in vitro* kinase assays and cells culture models (Okochi et al., 2000; Waxman and Giasson, 2008). CK1 colocalizes with pS87 in neuronal inclusions in a PD mouse model and in LB-like structures in LBD/PD diseased brains (Paleologou et al., 2010).

Several tyrosine kinases phosphorylate aSyn. The Y125 residue is target of phosphorylation by Fyn (Nakamura et al., 2001), Syk (Negro et al., 2002), Lyn (Negro et al., 2002), c-Frg (Negro et al., 2002), Src (Ellis et al., 2001) and c-Abl (Mahul-Mellier et al., 2014). Syk also phosphorylates Y133 and Y136 (Negro et al., 2002), and c-Abl also phosphorylates Y39 aSyn (Mahul-Mellier et al., 2014).

An emerging concept is that certain phosphorylation events might promote or prevent subsequent phosphorylation events in other residues (Negro et al., 2002; Mbefo et al., 2010). In fact, the double mutation of Y133 and Y136 to phenylalanines, designed to prevent phosphorylation in these residues, augments Y125 phosphorylation by Lyn (Negro et al., 2002). Phosphorylation or binding of c-Abl at Y125 was also found to decrease the propensity of this kinase to phosphorylate aSyn at Y39 (Mahul-Mellier et al., 2014).

TAU PHOSPHORYLATION IN ALZHEIMER'S DISEASE AND OTHER TAUOPATHIES

The "amyloid cascade hypothesis" was formulated after an amyloid precursor protein (APP) mutation was reported in a family with AD-typical histology and proposes that accumulation of an APP cleavage product, beta amyloid (Aβ), induces the biochemical, histologic, and clinical changes AD patients manifest (Hardy and Higgins, 1992). Later, Aβ oligomers were suggested to trigger neurotoxicity in AD probably via tau phosphorylation. Glycogen synthase kinase-3β (GSK-3β) activation was proposed as mediator of Aβ42 oligomer-induced effects on tau phosphorylation in P301L mice (Selenica et al., 2013).

THE ROLE OF PHOSPHORYLATION ON TAU CYTOTOXICITY AND AGGREGATION

Tau, in its longest isoform, contains 35 threonine, 45 serine, and 5 tyrosine residues meaning that nearly 20% of the tau protein

has the potential to be phosphorylated. Early studies revealed that tau is more efficient at promoting microtubules (MT) assembly in a more unphosphorylated state (Lindwall and Cole, 1984). A few years later, tau was demonstrated to make up the paired-helical filaments (PHFs) which form the neurofibrillary tangles (NFTs) found in AD brain and to be abnormally phosphorylated in these structures (Grundke-Iqbal et al., 1986; Goedert et al., 1988; Kosik et al., 1988; Wischik et al., 1988). Further analyses revealed that PHF-tau is phosphorylated at "pathological" sites, which was assumed to contribute to pathological processes in AD. Enhanced immunoreactivity in human AD tissue was observed with the phosphorylation-dependent antibodies AT8 (epitope pS199/pS202/pT205), PHF-1 (epitope pS396/pS404), and pS262 (Gu et al., 2013a; Mondragon-Rodriguez et al., 2014). Hyperphosphorylation of tau was shown to be involved in tau aggregation and cytotoxicity (**Table 2**) (Kosik and Shimura, 2005; Noble et al., 2013).

Abnormal high levels of intracellular tau are frequently observed in AD patients and may be directly implicated in tau aggregation, PHF formation, and neuron loss (Gomez-Isla et al., 1997). It was speculated that the hyperphosphorylation of tau precedes NFT pathology and, more important, is a key event for the integration of tau into fibrils (Bancher et al., 1991). The staging of AD-related neurofibrillary pathology using a silver stain technique was revised using immunostaining for hyperphosphorylated tau at the AT8 epitope (Braak et al., 2006). Several studies addressed the question whether the pattern of tau hyperphosphorylation correlates with the progression of neuronal cytopathology and the formation of higher order tau species in AD. Brain tissue was classified into pre-NFTs, intra-neuronal NFTs and extra-neuronal NFTs, and was examined regarding the most prominent staining of phosphorylation-dependent tau antibodies. Epitopes that were associated with pretangle, non-fibrillar tau include pS199, pS202, pT231, pS262, pT153, and S409. Intraneuronal fibrillar structures were stained with antibodies recognizing pS46, pT175/pT181, pT231, pS262/pS356 (12E8 epitope), pS396, pS422, and pS214. Epitopes associated with extracellular filamentous tau include AT8, AT100 (pT212/pS214), and PHF-1 (Morishima-Kawashima et al., 1995b; Kimura et al., 1996; Augustinack et al., 2002). Notably, with progression of the disease, tau is phosphorylated at pathological multiple-site epitopes (AT8, AT100, AT180, PHF-1, 12E8). Tau inclusions were observed in other neurodegenerative disorders such as MSA (Giasson et al., 2003b), familial and sporadic PD (Ishizawa et al., 2003; Rajput et al., 2006), and in Down syndrome (Flament et al., 1990; Mondragon-Rodriguez et al., 2014). Elevated levels of AT180 (pT231/pS235)-phosphorylated tau were detected in the cerebrospinal fluid (CSF) of patients with mild cognitive impairment who later went on to develop AD (Arai et al., 2000a).

Several animal models were generated to recapitulate hyperphosphorylation of tau and the formation of NFTs as key aspects of tauopathies (Ribeiro et al., 2013). Some studies showed that the overexpression of human mutant tau in transgenic mice led to increased phosphorylation of tau and the formation of tau inclusions, aggregates, and fibrils. Phosphorylation of tau was detected at the well-known disease-related epitopes S202, T205, S212, S216, T231, S262, S356, S422, AT100 (Kohler et al., 2013; Nilsen et al., 2013; Sahara et al., 2013). Likewise, overexpression of LRRK2 or p25/Cyclin-dependent kinase-5 (Cdk5) in mice resulted in hyperphosphorylation of tau, tau aggregation into NFT-like structures, and neuronal death (Cruz et al., 2003; Noble et al., 2003; Bailey et al., 2013). Other models took advantage of the co-expression of other disease-associated proteins such as APP and presenilin 1 (Oddo et al., 2003; Grueninger et al., 2010), or made use of the injection of Aβ fibrils (Gotz et al., 2001).

Almost all currently available animal models in AD are based on the over-expression of pathogenic mutant tau forms. Therefore, it debatable how well these models recapitulate AD cases where there are no mutations in either tau or APP. However, the first models of tauopathy, based on the overexpression of either 3-repeat or 4-repeat human WT tau, presented tau hyperphosphorylation but no NFT formation. Expression of tau-P301L, often in conjunction with other disease-associated proteins, is the most widely used and most successful approach to recapitulate key aspects of AD such as tau hyperphosphorylation, aggregation, and filament formation as well as neuron death. In these models, it is often not clear what drives tau hyperphosphorylation. *In vitro* studies may help to decipher the impact of specific pathogenic mutations on tau phosphorylation but existing data are not consistent. The well-known FTDP-17-associated missense tau mutations R406W, V337M, G272V, and P301L were shown to make tau a more favorable substrate for phosphorylation by rat brain kinases, in comparison to WT tau protein (Alonso Adel et al., 2004). In another study, the same mutations were shown to promote or inhibit phosphorylation at specific sites (Han et al., 2009). *In vitro* phosphorylation by recombinant GSK-3b exerted reduced phosphorylation of the R406W mutation, probably through long-range conformational changes. Conversely, P301L and V337M mutations had no effect (Connell et al., 2001). Similar results were obtained in cell culture (Dayanandan et al., 1999). In contrast, several other studies using cell culture models and human brain tissue indicate that the R406W mutation reduces tau phosphorylation, not only at the neighboring PHF1 epitope but at several positions (Miyasaka et al., 2001; Deture et al., 2002; Tackenberg and Brandt, 2009; Gauthier-Kemper et al., 2011). However, depending on the cellular context, R406W was also shown to increase phosphorylation, and other mutations, such as V337M, reduced phosphorylation of tau at specific sites (Deture et al., 2002; Krishnamurthy and Johnson, 2004). Alterations in the phosphorylation state can have tremendous effects on the structural properties, function, and pathology of tau as discussed below.

In vitro data imply that phosphorylation of tau at certain epitopes directly impacts on local structural properties or the global conformation of tau which in turn may affect its assembly into PHFs. Different sites were suggested to be important for the aggregation propensity and filament formation of tau including AT8, AT100, AT180, PHF-1, and S305 upstream of the PHF6-hexapeptide motif which is known to be important for tau fibrillization (Sun and Gamblin, 2009; Bibow et al., 2011; Inoue et al., 2012). Some studies suggested that the compaction of the paperclip conformation of tau becomes tighter or looser depending on phosphorylation at the AT8, PHF-1, and AT100 epitopes (Jeganathan et al., 2008; Bibow et al., 2011). Likewise,

Table 2 | Tau phosphorylation sites and effects.

Tau residue	Kinase	Model	Cytotoxicity	Aggregation	References
S199/S202/T205 (AT8, CP13 epitopes)	–	*In vitro* biochemical assay	–	S199E/S202E/T205E affects MT binding, MT polymerization and aggregation of tau	Sun and Gamblin, 2009; Bibow et al., 2011
	GSK-3β	*In vitro* biochemical assay	–	Pre-assembled pS199/pT205 tau filaments form large tangle-like structures	Rankin et al., 2008
	–	PC12 cells	S199E/S202E/T205E cause expansion of the space between MTs and inhibit mitochondrial movement in neurites and axons	–	Shahpasand et al., 2012
	GSK-3β	Rat hippocampal slices	NMDA receptor activation induces pS199/pS202 and facilitates LTD induction	–	Mondragon-Rodriguez et al., 2012
	–	rTg4510 tau transgenic mice	O-linked N-acetylglucosamine modification (O-GlcNAcylation) of tau lessens pS202/pT205, reduces the number of dystrophic neurons	O-GlcNAcylation of tau protects against tau aggregation	Graham et al., 2013
	–	TPR50 tau transgenic mice	pS202/pT205 increased with age, MT hyperdynamics, impaired axonal transport, cognitive deficits earlier than aggregates	Tau insolubility and intracellular accumulation	Onishi et al., 2014
	GSK-3β	pR5 tau transgenic mice	–	Increased pS202/pT205 is associated with fibrillar tau pathology	Kohler et al., 2013
	Cdk5	P25/Cdk5 transgenic mice	–	Increased pS202/pT205 is associated with aggregated tau filaments	Cruz et al., 2003
	–	TauE391 truncated transgenic mice	–	Truncation at E391 increases pS202/pT205; tau accumulation, mislocalization, tangle formation	McMillan et al., 2011
	Cdk5, GSK-3β	P25/P301L transgenic mice	–	Increased pS202 is associated with increased number of NFTs	Noble et al., 2003
	LRRK2	LRRK2/TauP301L transgenic mice	–	LRRK2 expression increases pS199/pS202/pT205 of insoluble tau	Bailey et al., 2013
	–	rTg4510 tau transgenic mice	–	pS202/pT205 in TBS-extractable tau which consists of granular aggregates and short filaments	Sahara et al., 2013
	–	IHC on paraffin-sections AD brain	–	Enhanced pS199/pS202/pT205 in mature NFTs	Mondragon-Rodriguez et al., 2014
	–	homogenates from AD brain tissue, AD synaptosomes	–	Oligomers positive for pS202/pT205 accumulate at synapses in AD	Henkins et al., 2012; Lasagna-Reeves et al., 2012; Tai et al., 2012
S262/S356 (12E8 epitope)	MARK2	*In vitro* biochemical assay	–	Acetylation on S262/S356 inhibits its phosphorylation and tau aggregation	Schwalbe et al., 2013; Cook et al., 2014
	MARK4	rat primary hippocampal neurons	Increased pS262/pS356 is associated with decrease in synaptic markers, loss of spines and synapses	–	Yu et al., 2012

(Continued)

Table 2 | Continued

Tau residue	Kinase	Model	Cytotoxicity	Aggregation	References
	–	rTg4510 tau transgenic mice	O-GlcNAcylation of tau lessens pS262/pS356, reduces the number of dystrophic neurons	O-GlcNAcylation of tau protects against tau aggregation	Graham et al., 2013
	–	rTg4510 tau transgenic mice	–	pS262/pS356 in TBS-extractable tau which consists of granular aggregates and short filaments	Sahara et al., 2013
S262	DAPK1	HEK293,N2a cells	Tau expression antagonizes DAPK1 induced apoptosis with simultaneous pS262, no up-regulation of kinases	–	Duan et al., 2013
	PKA	Rat hippocampal neurons	pS262 mediates toxicity via MT instability; accelerated degradation of synaptophysin	–	Qureshi et al., 2013
	GSK-3β	Cortical neurons, rat hippocampal slices	Stress-induced increase of pS262 reduces cell viability	–	Selvatici et al., 2013
	Par-1	*Drosophila*	pS262 contributes to tau-mediated neurodegeneration	–	Iijima-Ando et al., 2012
	MARK2 MARK4	Paraffin sections AD brain	–	MARK-tau interactions and pS262 correlate with Braak stages	Gu et al., 2013a
T231/S235 (AT180,PHF-6 epitopes)	–	*In vitro* NMR measurements	–	pT231/pS235 has a helix stabilizing role, potentially affecting tau function and aggregation	Sibille et al., 2011
	DAPK1	HEK293,N2a cells	tau expression antagonizes DAPK1 induced apoptosis with simultaneous pT231, no up-regulation of kinases	–	Duan et al., 2013
	GSK-3β	Rat hippocampal slices	NMDA receptor activation induces pT231/pS235 and facilitates LTD induction	–	Mondragon-Rodriguez et al., 2012
	–	TauE391 truncated transgenic mice	–	Truncation of tau at E391 increases pT231/pS235, tau accumulation, mislocalization, and tangle formation	McMillan et al., 2011
	–	rTg4510 tau transgenic mice	–	pT231/pS235 in TBS-extractable tau which consists of granular aggregates and short filaments	Sahara et al., 2013
	GSK-3β	SAMP8 mice	GSK-3β antisense treatment decreases pT231/pS235; reduced oxidative stress, improved learning and memory	–	Farr et al., 2013
	–	Homogenates from AD brain tissue	–	Identification of pT231-positive oligomers at early AD stages	Lasagna-Reeves et al., 2012
S396/S404 (PHF-1, AD2, PHF-13 epitopes)	MARK2	*In vitro* biochemical assay	–	Acetylation on S396/S404 inhibits its phosphorylation and tau aggregation	Cook et al., 2014
	–	*In vitro* biochemical assay	–	S396E/S404E affects MT binding, MT polymerization and aggregation of tau	Sun and Gamblin, 2009; Bibow et al., 2011
	GSK-3β	*In vitro* biochemical assay	–	pre-assembled pS396/pS404 tau filaments form large tangle-like structures	Rankin et al., 2008

(Continued)

Table 2 | Continued

Tau residue	Kinase	Model	Cytotoxicity	Aggregation	References
	LRRK2	*In vitro* biochemical assay, SH-SY5Y cells, LRRK2 tg mice	–	LRRK2 increases pS396, pT149, and pT153, and aggregation of tau	Bailey et al., 2013; Kawakami et al., 2014
	DAPK1	HEK293,N2a	Tau expression antagonizes DAPK1 induced apoptosis with simultaneous pS396, no up-regulation of kinases	–	Duan et al., 2013
	GSK-3β	Rat hippocampal slices	NMDA receptor activation induces pS396/pS404 and facilitates LTD induction	–	Mondragon-Rodriguez et al., 2012
	GSK-3β	Cortical neurons, rat hippocampal slices	Stress-induced increase of pS404 reduces cell viability	–	Selvatici et al., 2013
	–	rTg4510 tau transgenic mice	–	pS396/pS404 in TBS-extractable tau which consists of granular aggregates and short filaments	Sahara et al., 2013
	–	rTg4510 tau transgenic mice	O-GlcNAcylation of tau lessens pS396/pS404, reduces the number of dystrophic neurons	O-GlcNAcylation of tau protects against tau aggregation	Graham et al., 2013
	Cdk5, GSK-3β	p25/P301L transgenic mice	–	Increased pS396/pS404 is associated with increased number of NFTs	Noble et al., 2003
	Cdk5	p25/Cdk5 transgenic mice	–	Increased pS396/pS404 is associated with aggregated tau filaments	Cruz et al., 2003
	GSK-3β	P301L and GSK-3β/P301L transgenic mice	–	Increased pS396/pS404, increased tangle pathology but also longer survival than P301L mice.	Terwel et al., 2008
	–	AD material: homogenates, synaptosomes, paraffin sections	–	Oligomers positive for pS396 and/or pS404 accumulate at synapses in AD at different stages	Henkins et al., 2012; Lasagna-Reeves et al., 2012; Tai et al., 2012; Mondragon-Rodriguez et al., 2014
	–	IHC on paraffin sections AD brain	–	Content of tangles rather than phosphorylated tau lead to altered spine morphology and spine loss	Merino-Serrais et al., 2013
T212/S214/T217 (AT100 epitope)	–	*In vitro* biochemical assay	–	T212E/S214E/T217E affects MT binding, MT polymerization, and aggregation of tau	Bibow et al., 2011
	–	*C. elegans*	–	Inhibition of tau aggregation is paralleled by reduced pT212/pS214/pT217 and mitigates proteotoxicity	Fatouros et al., 2012
	GSK-3β	pR5 tau transgenic mice (P301L)	–	Increased pT212/pS214/pT217 is associated with fibrillar tau pathology	Kohler et al., 2013
	–	rTg4510 tau transgenic mice	–	pT212 in TBS-extractable tau which consists of granular aggregates and short filaments	Sahara et al., 2013
S422 residue	GSK-3β	pR5 tau transgenic mice	–	Increased pS422 is associated with fibrillar tau pathology	Kohler et al., 2013
	–	rTg4510 tau transgenic mice	–	pS422 in TBS-extractable tau which consists of granular aggregates and short filaments	Sahara et al., 2013
	–	AD synapses	–	Increased pS422 in AD synapses; SDS-stable tau oligomers and aggregates.	Henkins et al., 2012

phosphorylation within the repeat region, particularly at KXGS motifs, induced specific conformational changes that altered the MT binding properties of tau (Fischer et al., 2009). In other cases, structural changes were localized in the proximity of the phosphorylation sites without affecting the global conformation (Schwalbe et al., 2013).

Despite intensive research in the field, the contribution of phosphorylation to the formation of tau aggregates is still controversial (**Table 2**). Recent results from *in vitro* experiments, showing that recombinant unphosphorylated tau induced fibril formation similar to AD-derived PHFs, questioned the necessity of tau phosphorylation for the fibrillization process (Morozova et al., 2013). Furthermore, altered spine morphology and spine loss in tissue of AD cases were attributed to the content of tangles rather than to the amount of phosphorylated tau (Merino-Serrais et al., 2013). In a study using PS19 mice (tauP301S mutation), synthetic tau fibrils induced NFT pathology in the absence of tau hyperphosphorylation (Iba et al., 2013). The introduction of "pro-" and "anti-" aggregation mutations revealed that hexapeptide motifs of tau may function as a core to form local β-sheet structure and, subsequently, to induce PHF formation (Von Bergen et al., 2000; Eckermann et al., 2007). Enhanced tau levels, via stabilization of tau mRNA, may contribute to tau pathology independent of tau phosphorylation (Qian et al., 2013).

Other PTMs of tau might interfere with its phosphorylation, thereby influencing the structure, function and regulation of the protein, but the data are not consistent. KXGS motifs were found to be hypoacetylated and hyperphosphorylated in patients with AD, consistent with *in vitro* data showing that the acetylation of tau prevents its phosphorylation and inhibits tau aggregation (Irwin et al., 2013; Cook et al., 2014). In contrast, acetylation of tau at K280 was associated with phosphorylation at the AT8 epitope in tau aggregates of tau transgenic mice, and detected in post-mortem tissue of cases with AD or other tauopathies (Min et al., 2010; Cohen et al., 2011; Irwin et al., 2012). Recent evidence was provided that tau itself possesses acetyltransferase activity, and is capable of catalyzing self-acetylation (Cohen et al., 2013). *In vitro*, O-linked β-N-acetylglucosaminylation (O-GlcNAcylation) at S400 was inversely correlated with tau phosphorylation at S396 (Smet-Nocca et al., 2011). However, treatment of tau transgenic mice with an O-GlcNAcase inhibitor increased tau O-GlcNAcylation, hindered the formation of tau aggregates, and slowed neurodegeneration without affecting the phosphorylation of tau (Yuzwa et al., 2012; Graham et al., 2013).

Phosphorylation of tau at several residues mediates cellular toxicity (**Table 2**). Many data implicate that phosphorylation of tau needs to be well balanced. It was hypothesized that the detachment of tau from MTs results in impaired MT stability and excess amount of unbound hyperphosphorylated tau in the cytosol, thereby contributing to toxic insult. *In vitro* experiments provided evidence that phosphorylation of tau at S262, T231, and S214 is necessary for the full detachment of tau from MTs (Illenberger et al., 1998; Sengupta et al., 1998). Consistently, enhanced phosphorylation at S262 and T231 resulted in MT instability and cytotoxicity in cell and animal models (Steinhilb et al., 2007; Qureshi et al., 2013). Moreover, pathological processes were rescued by overexpression and activation of microtubule-affinity-regulating kinases (MARKs) that phosphorylate tau at KXGS motifs of the repeat domains (Mandelkow et al., 2004; Thies and Mandelkow, 2007). Aberrant phosphorylation of tau at pathological sites may result in altered tau-MT binding, thereby affecting the organization and dynamics of MT networks. This in turn may compromise axoplasmic flow and proper neuronal function, and ultimately cause cell death. The phosphorylation within KXGS motifs, especially at S262, and GSK-3β seem to take key roles among the phosphorylation sites and tau kinases, respectively.

Mitochondrial dysfunction and oxidative stress are both intimately associated with cell death in neurodegeneration. Mitochondrial oxidative stress in superoxide dismutase 2-deficient and APP expressing mice exacerbated amyloid burden and the hyperphosphorylation of tau at S396. Treatment with high doses of antioxidants prevented from tau hyperphosphorylation and neuropathology (Melov et al., 2007). Triple AD mice expressing mutant tau, APP and presenilin 1 developed tangles and Aβ plaques, and displayed deregulation of several mitochondrial proteins suggesting synergistic effects of Aβ and tau in perishing mitochondria (Rhein et al., 2009).

Undoubtedly, tau hyperphosphorylation is an important phenomenon in AD and other tauopathies and parallels the appearance of tau aggregates and NFTs, but despite great efforts, the underlying mechanisms that ultimately lead to toxicity and neurodegeneration remain elusive (Papanikolopoulou et al., 2010; Ambegaokar and Jackson, 2011). In recent years, it was hypothesized that the segregation of tau in intracellular aggregates is an escape route for the cell from excess amount of protein. Instead, tau oligomers were considered as toxic species that harm the cell and, ultimately, lead to cell death (Sahara and Avila, 2014).

Accumulation of phosphorylated (AT8, PHF-1, S422) tau oligomers was detected at human AD synapses concomitant with dysfunction of the UPS (Henkins et al., 2012; Tai et al., 2012). Use of a tau oligomer-specific antibody in human AD brain samples revealed that tau oligomers appear at early stages in AD, either before or after the manifestation of tau phosphorylation at specific epitopes (Lasagna-Reeves et al., 2012). Thus, aggregation of the hyperphosphorylated forms of tau into PHF structures could be neurotoxic by sequestering important cellular proteins, but it could also be neuroprotective by avoiding accumulation of toxic oligomeric tau.

PHYSIOLOGICAL AND PATHOLOGICAL IMPLICATIONS OF TAU PHOSPHORYLATION

At normal levels of phosphorylation, tau contains 2–3 moles phosphate/mole of protein and is a soluble cytosolic protein (Khatoon et al., 1992). From the overall 85 phosphorylatable residues, approximately 30 residues are phosphorylated in normal tau proteins (Morishima-Kawashima et al., 1995a; Hanger et al., 2009a). Most of the tau phosphorylation sites are clustered in the proline-rich region, the microtubule binding repeats (MTBR) or MTBR-flanking domains.

Tau expression and phosphorylation are developmentally regulated. A single tau isoform is expressed in fetal human brain whereas six isoforms are expressed in adult human brain, with fetal tau corresponding to the shortest adult tau isoform. The degree of tau phosphorylation decreases during embryogenesis

(Mawal-Dewan et al., 1994), which might be related to increasing neuronal plasticity in the early developmental process (Brion et al., 1993; Hanger et al., 2009a). PHF-tau contains 3–4 fold phosphates over the normal adult tau (Khatoon et al., 1992; Iqbal et al., 2013). In immature brain, as in PHFs, tau is phosphorylated at a large number of sites (Kenessey and Yen, 1993; Morishima-Kawashima et al., 1995b). However, as in adult brain, the phosphorylation in fetal tau is only partial. Phosphorylation of tau in PHFs is denominated as "hyperphosphorylation" which takes into account that other sites than the physiological ones are phosphorylated. This state is also referred to as "abnormal" or "pathological" phosphorylation.

MT dynamics are dependent on a balanced ratio between tau molecules and MT tracks. Either excess or poor binding of tau molecules, e.g., through dysregulation of the tau phosphorylation state, results in destabilization and breakdown of MT networks. This has a direct impact on MT function in the formation of the cytoskeletal architecture and as track for axonal and organelle transport, and is resumed in the "Tau-microtubule hypothesis" (Alonso et al., 1994).

Early studies clearly demonstrated that tau plays an important role in the establishment of neuronal polarity and axonal outgrowth (Caceres and Kosik, 1990). Neurite extension and retraction may be regulated by MARK and GSK-3β-mediated tau phosphorylation (Biernat et al., 2002; Sayas et al., 2002). It was speculated that phosphorylation of tau within the MTBR is necessary for appropriate neurite outgrowth whereas phosphorylation at SP and TP motifs within flanking domains retards neuronal differentiation (Biernat and Mandelkow, 1999). Tau, a cargo of kinesin, may displace other kinesin-based cargo indicating that the development and stabilization of axons are dependent on a balance of cytoskeletal elements (Dubey et al., 2008).

Overexpression of tau is known to compromise MT-dependent axonal transport in a phosphorylation-dependent manner (Sato-Harada et al., 1996). Co-expression of constitutively active GSK-3β exacerbated, whereas GSK-3β inhibition rescued vesicle aggregation and locomotor dysfunction in a *Drosophila* model (Mudher et al., 2004; Cowan et al., 2010b). Phosphorylation of tau at Y18 by the Fyn kinase was suggested to prevent the activation of the GSK-3β signaling cascade, thereby counteracting tau's inhibitory effect on anterograde fast axonal transport (Kanaan et al., 2012). These data suggest that the pathological over-activation of GSK-3β inhibits axonal transport through hyperphosphorylation of tau. In contrast, other studies showed that the inhibition of tau phosphorylation by GSK-3β inhibitors was associated with decreased mitochondrial transport and motility and increased mitochondrial clustering in cells (Tatebayashi et al., 2004; Llorens-Martin et al., 2011). Tau may control intracellular trafficking by affecting the frequencies of attachment and detachment of motors, in particular kinesin, to the MT tracks (Trinczek et al., 1999; Morfini et al., 2007). It was speculated that excess tau acts as transport block for vesicles and organelles which is reversed by removal of tau through MARK-mediated tau phosphorylation and subsequent detachment of tau from MTs (Thies and Mandelkow, 2007). However, detachment of tau from MT may also contribute to axonal transport blockage and neurodegeneration (Iijima-Ando et al., 2012). Dephosphorylation and phosphorylation cycles of tau, through the interplay of tau kinases and phosphatases, may serve as general mechanism to regulate tau's function to maintain a dynamic MT network for neurite outgrowth and axonal transport (Fuster-Matanzo et al., 2012; Mandelkow and Mandelkow, 2012). Interestingly, improper distribution of overexpressed tau in the somatodendritic compartment was shown to result in more numerous and densely packed MTs in axons and dendrites. Phosphomimic mutations of the AT8 epitope caused expansion of the space between MTs and may thereby contribute to axonal transport and mitochondrial movement defects (Thies and Mandelkow, 2007; Shahpasand et al., 2012). Furthermore, phosphorylated tau may sequester normal tau in neurites away from MTs leading to disruption of the microtubular cytoskeleton and demise of axonal transport (Niewiadomska et al., 2005; Cowan et al., 2010a; Iqbal et al., 2013).

Extracellular Aβ, shown to exacerbate the hyperphosphorylation of tau and NFT formation, was also suggested to modulate N-methyl-D-aspartate receptor (NMDAR) function and to induce excitotoxicity (Lauren et al., 2009). However, among the plethora of known Aβ-interacting molecules, the specific Aβ target and the intracellular propagation of the signal remain elusive. Prion protein was proposed as binding partner of Aβ but there is still controversy about the significance of this interaction (Balducci et al., 2010; Kessels et al., 2010; Chen et al., 2013a). Aβ induces the activation of Fyn which, in turn, increases the phosphorylation of a subunit of NMDARs dependent on the status of tau phosphorylation and tau localization at the post-synapse. After an initial increase, the number of surface NMDARs declined which resulted in dendritic spine loss and excitotoxicity (Um et al., 2012). The interaction of Fyn and tau, both forming a complex together with NMDAR, seems to modulate synaptic plasticity and to sensitize synapses to glutamate excitoxicity in AD (Ittner et al., 2010).

Phosphorylation of tau was also linked to altered turnover and proteolysis. The detection of ubiquitin immunoreactivity in tau inclusions was interpreted as failure of the ubiquitin proteasome system (UPS) to proteolytically degrade excess tau (Bancher et al., 1989). Proteasomal inhibition resulted in the accumulation of particularly hyperphosphorylated tau species (Shimura et al., 2004) and disruption of neuritic transport (Agholme et al., 2013). Inhibition of autophagy in neurons resulted in 3-fold accumulation of phosphomimic tau over wild type tau indicating that both, autophagic and proteasomal pathways, are responsible for the clearance of phosphorylated tau species (Rodriguez-Martin et al., 2013). Biochemical and morphological analysis of AD cortices revealed that tau becomes hyperphosphorylated and misfolded at presynaptic and postsynaptic terminals, in association with an increase in ubiquitinated substrates and proteasome components (Tai et al., 2012).

Many other mechanisms were suggested for the implication of tau hyperphosphorylation in tauopathies. Cell death was accompanied by expression of cell-cycle regulatory proteins in aged mice expressing human tau isoforms on a knockout background (Andorfer et al., 2003). Inappropriate re-entry to the cell cycle plays a role in AD and might be linked to hyperphosphorylation of tau via activation of cell-cycle relevant kinases (Delobel et al., 2002; Absalon et al., 2013). Abnormal interaction

with the mitochondrial fission protein Drp1 might be causative for mitochondrial dysfunction and neuronal damage (Manczak and Reddy, 2012). DNA damage resulted in the activation of the checkpoint kinases Chk1 and 2, subsequent tau phosphorylation at AD-related sites, and enhancement of tau-induced neurodegeneration in human tau expressing *Drosophila* (Iijima-Ando et al., 2010). Immunohistochemical analysis of AD brains revealed that tau is truncated at D421, and that this cleavage occurs after conformational changes detected by the Alz-50 antibody but precedes cleavage at E391 (Guillozet-Bongaarts et al., 2005). Accumulation of D421 and E391-truncated species occurs early in the disease and correlates with the progression in AD (Basurto-Islas et al., 2008). In transgenic mice, truncation of tau was shown to drive pre-tangle pathology (McMillan et al., 2011). In cells, hyperphosphorylation of tau at several residues and cleavage of tau at D421, the preferential cleavage site of caspase-3, enhanced the secretion of tau. This was suggested as potential mechanisms for the propagation of tau pathology in the brain and tau accumulation in the CSF (Plouffe et al., 2012).

KINASES INVOLVED IN TAU PHOSPHORYLATION

Similar to the pattern of tau hyperphosphorylation, the idea of a distinct signature-specific pattern of tau kinase activation emerged (Duka et al., 2013). Several attempts were done to identify the responsible kinases and the corresponding phosphorylation sites of tau (**Table 2**). However, most of the kinases phosphorylate several residues of tau, and most tau phosphorylation sites are targets of more than one kinase (**Figure 3**). In addition, the existence of priming, meaning that the phosphorylation at one site facilitates phosphorylation at another site, and feedback events to regulate the overall level of tau phosphorylation, hamper the assignment of a specific phosphorylation site to a particular (dys-)function of tau (Bertrand et al., 2010; Kiris et al., 2011).

Numerous kinases, including more than 20 serine/threonine kinases, were shown to phosphorylate tau *in vitro* but their relevance in AD is still under investigation (Hanger et al., 2009b; Cavallini et al., 2013).

The proline-directed kinase GSK-3β was particularly associated with the formation of PHFs and NFTs and proposed as key mediator in the pathogenesis of AD (Hooper et al., 2008; Terwel et al., 2008; Ma, 2014; Medina and Avila, 2014). GSK-3β targets tau at SP/TP sites, including the epitopes PHF-1, AT8, AT180, AT100, S404 and S413 (Pei et al., 1999; Medina and Avila, 2014). Alterations in GSK-3β levels were associated with changes in tau phosphorylation in several cell and animal models (Hernandez et al., 2013). Stress stimuli such as mitochondrial toxins or oxidative stress to mimic conditions in neurodegenerative disorders resulted in increased GSK-3β-mediated phosphorylation of tau, reduced cell metabolic activity and MT destabilization (Hongo et al., 2012; Selvatici et al., 2013). Other studies position GSK-3β as prominent player in the pathogenesis of AD beyond its role as tau phosphorylating kinase. Tau-P301Lx GSK-3β mice developed severe forebrain tauopathy with tangles in the majority of neurons but in the absence of tau hyperphosphorylation (Muyllaert et al., 2006). In a *Drosophila* model, co-expression of a GSK-3β homolog and human tau led to increased toxicity more likely due to the fact that GSK-3β is a pro-apoptotic protein than due to increased tau phosphorylation (Jackson et al., 2002).

The serine/threonine kinase Cdk5 plays important roles in neuronal development and migration, neurite outgrowth, and synaptic transmission, and is implicated in the pathogenesis of AD (Cheung and Ip, 2012; Shukla et al., 2012). Immunoreactivity of Cdk5 in several brain regions in AD was associated with pre-tangle and early NFT stages, and colocalized with AT8-positive tau in a subset of neurons (Pei et al., 1998; Augustinack et al., 2002). Cdk5 activity was found to be higher in AD than control cases probably due to the conversion of the Cdk5 activator p35 into the constitutive active form p25 (Lee et al., 1999; Patrick et al., 1999; Shukla et al., 2012).

Mice overexpressing human p25/Cdk5 displayed enhanced Cdk5 activity, hyperphosphorylation of tau, and cytoskeletal disorganization (Ahlijanian et al., 2000). The activation of Cdk5 along with overexpression of mutant tau was associated with tau hyperphosphorylation and tangle formation (Noble et al., 2003). APPswe mice showed increased Cdk5 activity due to increases in p25 levels, and substantial phosphorylation of tau at AT8 and PHF-1 epitopes linking Aβ pathology to tau hyperphosphorylation via increased Cdk5 activity (Otth et al., 2002). Furthermore, Cdk5 was suggested to be linked to GSK-3β. Mice expressing human p25 showed elevated Aβ levels but decreased phospho-tau levels and reduced GSK-3β activity. Administration of Cdk5 inhibitors reduced Aβ production but did not alter the phosphorylation of tau suggesting that Cdk5 predominantly regulates APP processing, whereas GSK-3β plays a dominant role in tau phosphorylation (Wen et al., 2008; Engmann and Giese, 2009). The crosstalk between Cdk5 and mitogen-activated protein kinase (MAPK) pathways suggests a connection with neuronal apoptosis and survival signaling (Sharma et al., 2002; Zheng et al., 2007). Dysregulation of the MAPK signaling pathways, comprising the three signaling cascades extracellular signal regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), was suggested to be implicated in AD and other neurodegenerative disorders (Kim and Choi, 2010). In the course of AD, ERK and JNK are activated throughout all stages, and p38 in mild to severe cases (Braak stages III to VI) (Pei et al., 2001; Zhu et al., 2001). p38 and JNK immunoreactivity were associated with neurons containing neuritic plaques, neuropil threads, and NFTs, structures that were also recognized by antibodies raised against phosphorylated PHF-tau (Hensley et al., 1999; Atzori et al., 2001).

The kinases MARK1-4 are non-proline directed kinases that are involved in the establishment of neuronal polarity and the regulation of neurite outgrowth (Biernat et al., 2002; Matenia and Mandelkow, 2009; Reiner and Sapir, 2014). MARKs are named after their ability to regulate the affinity of tau to MTs through phosphorylation (Drewes et al., 1997). Importantly, MARKs phosphorylate tau within the KXGS motifs, particularly at S262, which phosphorylation is detected early in the course of AD. Expression of MARK2 and MARK4, as well as the interactions of these kinases with tau, were significantly enhanced in AD brains, correlated with the Braak stages of the disease, and were associated with NFTs (Chin et al., 2000; Gu et al., 2013a).

In transgenic *Drosophila*, overexpression of the *Drosophila* homolog Par-1 was associated with increased phosphorylation

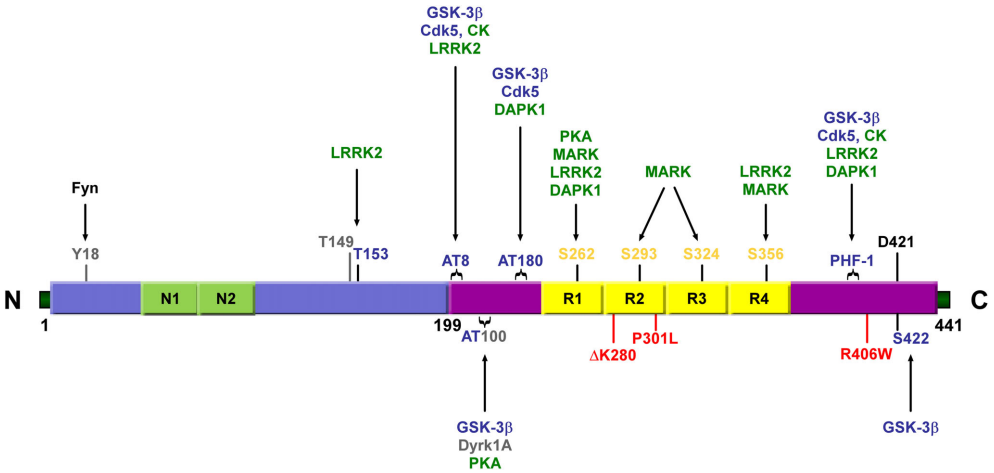

FIGURE 3 | Schematic representation illustrating the various residues in the longest isoform of tau that can be phosphorylated. SP/TP motifs (represented in blue), KXGS motifs (represented in yellow), and other sites (represented in gray) can be phosphorylated by proline-directed kinases (represented in blue) and non-proline directed Ser/Thr kinases (represented in green). Antibody epitopes AT8, AT100, AT180, and PHF-1 comprise dual and triple serine/threonine residues (indicated by brackets). Some mutations associated with FTDP-17 are shown in red. Alternative splicing of N1, N2, and R2 generates the six different isoforms of tau. N1, N2, N-terminal inserts 1 and 2; R1-R4,MT binding repeats 1–4; GSK-3β, Glycogen synthase kinase 3β; Cdk5, Cyclin-dependent kinase 5; CK, casein kinase; MARK, microtubule affinity-regulating kinase; LRRK2, leucine-rich repeat kinase 2; DAPK, Death-associated protein kinase; Dyrk1A, dual-specificity protein kinase.

and enhanced toxicity of human tau. Loss of Par-1 function and mutation of tau at the Par-1 directed phosphorylation sites (S262, S356) rescued from tau-induced toxicity. Interestingly, Par-1 phosphorylation of tau was a prerequisite for downstream phosphorylation through GSK-3β and Cdk5, and the generation of disease-associated phosphorylation epitopes (Nishimura et al., 2004). Activation of MARK2 rescued from synaptic decay caused by overexpression and improper distribution of tau in the somatodendritic compartment (Mandelkow et al., 2004; Thies and Mandelkow, 2007). However, overexpression of MARK4 resulted in tau hyperphosphorylation and loss of spines, which also manifested after Aβ treatment. Therefore, MARKs may have regulatory functions in spine morphology and synaptic transmission, but may also act as critical mediators in Aβ-induced toxicity on synapses and dendritic spines (Zempel et al., 2010; Hayashi et al., 2011; Yu et al., 2012). Furthermore, the phosphorylation of tau by MARK was suggested to inhibit tau's assembly into PHFs (Schneider et al., 1999), contradictory to the hypothesis that the pool of hyperphosphorylated, MT-unbound tau assembles into PHFs. Phosphorylation of tau at SP/TP sites has low impact on the tau-MT binding and is observed in AD, dissociating the detachment of tau from MTs from the likability to assemble into PHFs. GSK-3β was shown to phosphorylate MARK2 at two different sites, the activatory T208 and the inhibitory S212, thereby modulating the phosphorylation of tau, particularly at S262 (Kosuga et al., 2005; Timm et al., 2008). MARK1/2 activity was also regulated by the death domain of DAPK. DAPK activated MARK and promoted the phosphorylation of tau but also seems to act via MARK-independent pathways on T231, S262, and S396 of tau (Wang et al., 2010; Duan et al., 2013). Moreover, DAPK induced rough eye and loss of photoreceptor neurons in a *Drosophila* model, in part through the activation of the *Drosophila* ortholog Par-1 (Wu et al., 2011b). PKC was described as negative regulator of MARK2, playing an important role in neuronal polarity (Chen et al., 2006).

CK1 and CK2 are serine/threonine-selective protein kinases. Overall CK2 immunoreactivity is reduced in the brain of AD cases although NFTs stain very strong with anti-CK2 antibodies (Iimoto et al., 1990). CK-1δ is upregulated in AD brain, correlating with the degree of regional pathology. CK-1δ colocalizes with NFTs, neuropil threads and dystrophic neurites (Yasojima et al., 2000). In cells, CK1δ inhibition reduced the phosphorylation of tau at S396/S404 by more than 70%. Exogenous expression of CK1δ increased tau phosphorylation at S202/T205 and S396/S404 and reduced tau-MT binding (Lee and Leugers, 2012).

Several sites in PHF-tau are targeted by CK1 in concert with other kinases such as GSK-3β and protein kinase A (PKA). Moreover, three sites, S113, S238, and S433, were phosphorylated only by the action of CK1δ suggesting a relevant role of this kinase in tau pathology (Hanger et al., 2007). Synthetic Aβ was reported to stimulate the activities of CK1 and CK2 and to mediate phosphorylation of the substrate casein *in vitro* (Chauhan et al., 1993). Aβ production was increased in cells with exogenous expression of constitutively active CK1 and reduced by CK1 specific inhibition (Flajolet et al., 2007).

Dyrk1A is upregulated in AD, Down's syndrome, and Pick's disease. Dyrk1A immunoreactivity was observed in the cytoplasm and nucleus of scattered neurons, and detected in sarkosyl-insoluble PHF fractions. Overexpression of Dyrk1A in transgenic mice led to increased tau levels in the brain and accumulation in NFTs (Wegiel et al., 2008). Direct phosphorylation of tau at T212 by Dyrk1A still lacks evidence *in vivo*.

LRRK2, a putative kinase, gained interest in recent years due to its genetic association with both, inherited and sporadic PD,

and a possible overlap to AD (Zhao et al., 2011; Ujiie et al., 2012). The first hints for an involvement of LRRK2 in tau pathology were given in transgenic mice expressing mutant LRRK2. These animals showed increased tau phosphorylation at T149, T153, and the AT8, CP13, 12E8 and PHF-1 epitopes, tau mislocalization in cell bodies and the neuropil, and tau aggregation (Li et al., 2009, 2010; Melrose et al., 2010; Bailey et al., 2013). Correspondingly, phosphorylation of tau at the AT8 epitope was decreased in LRRK2 knock-out mice (Gillardon, 2009). Several studies imply that LRRK2 phosphorylates and activates other kinases and signal transduction pathways, thereby contributing to enhanced tau phosphorylation, mislocalization, and dendritic degeneration (Gloeckner et al., 2009; Lin et al., 2010; Chen et al., 2012).

Several other kinases may be implicated in the pathology of AD, anticipated from their aberrant activity in human brain (Martin et al., 2013). In general, dysregulation of kinases is likely to be responsible for the hyperphosphorylation of tau, abnormal tau-MT binding, tau mislocalization, and tau assembly into PHFs. However, it is still not known, which phosphorylation sites of tau are the most critical ones, which kinases are the main players, and how these processes are mechanistically linked to toxicity in AD and other neurodegenerative disorders.

PHOSPHORYLATION AS A TARGET FOR THERAPEUTIC INTERVENTION

Our current lack of understanding of the precise molecular mechanisms underlying neurodegenerative disorders has limited our ability to develop effective therapeutic strategies. Targeting phosphorylation of aSyn and tau can impact on several mechanisms associated with the pathogenesis of AD, PD and other neurodegenerative disorders. Thus, reducing aberrant protein phosphorylation and protein levels, preventing protein aggregation, eliminating amyloidogenic species and preventing spreading of pathology are all potentially beneficial and will be discussed below.

KINASE INHIBITION

Although the precise consequences of aSyn phosphorylation remain to be fully understood, it is evident that pS129 correlates with disease progression, is present in the pathological hallmark lesions of synucleinopathies and can have detrimental functional consequences. Thus, inhibition of the relevant kinases might constitute a possible therapeutic strategy. Inhibitors of PLKs have been developed for oncology indications and tested *in vivo*. A PD mouse model of subject to a treatment with the specific PLK inhibitor BI2356 presented reduced pS129 aSyn (Inglis et al., 2009). However, the long-term safety of such strategy is currently unclear, as the kinases that phosphorylate aSyn have ubiquitous distribution and because there is evident redundancy in the types of kinases phosphorylating same residues in aSyn. In the particular case of PLK2, recent data suggest that enhancing the kinase activity, instead of inhibiting, might also prove worth investigating further, as PLK2 suppresses aSyn toxicity *in vivo* by promoting autophagy-mediated degradation of pS129 aSyn (Oueslati et al., 2013).

The pharmacological inhibition of c-Abl is also emerging as an attractive therapeutic strategy, as it was found to be neuroprotective in animal models of PD (Ko et al., 2010; Imam et al., 2011, 2013; Hebron et al., 2013a; Mahul-Mellier et al., 2014), by promoting aSyn degradation (Hebron et al., 2013a,b; Mahul-Mellier et al., 2014). Interestingly, c-Abl inhibition also targets hyperphosphorylated tau for degradation (Hebron et al., 2013a) and inhibits β-amyloid production in rat neuronal primary cultures and in guinea pig brains (Netzer et al., 2003). Therefore, this kinase is a promising target for the treatment of both, PD and AD.

Inhibition of GSK-3β activity by chemical compounds, antisense RNAs and kinase-dead mutations or reduction of GSK-3β levels were the most promising attempts to decrease the phosphorylation of tau at critical residues. This was shown to counteract neuronal death, reduce oxidative stress, and improve learning and memory (Hernandez et al., 2013; Medina and Avila, 2014). GSK-3β was suggested as missing link between Aβ and tau pathology. Small molecule inhibitors of GSK-3β might be potent to reduce Aβ-induced tau hyperphosphorylation (Noh et al., 2013; Ye et al., 2013). Given the contribution of other kinases to tau hyperphosphorylation, effective treatment may require multiple kinase targeting (Mazanetz and Fischer, 2007; Tell and Hilgeroth, 2013; Pinsetta et al., 2014).

IMMUNOLOGICAL TARGETING OF PHOSPHORYLATED PROTEINS

The consequences of loss of aSyn function in PD are not completely clarified. Some studies suggest that a substantial reduction in the levels of aSyn could have potential harmful effects in the CNS. In fact, aSyn knockout mice show some loss of dopaminergic nigrostriatal terminals with aging (Al-Wandi et al., 2010). Moreover, aSyn seems to play an important role holding together the SNARE complex, suggesting that excessive reduction of this abundant protein in the nervous system could lead to deleterious effects. Nevertheless, therapeutic strategies involving immunization or promoting clearance of aSyn excess are attractive and have been recently considered. Studies exploring immunization as a potential therapeutic were performed in mice models of PD and achieved promising results (Masliah et al., 2005, 2011). However, to our knowledge, the use of phospho-specific antibodies against aSyn was not explored so far.

Targeting specific phospho-tau sites through passive immunization may be useful to slow, or even reverse, the progression of a disease. Antibody uptake resulted in reduced tau phosphorylation, and clearance of pathological tau protein in brain slices up to significantly improved cognitive performance in Thy-Tau22 transgenic mice (Troquier et al., 2012; Gu et al., 2013b). However, repeated immunization of mice with phospho-tau peptides may cause neuroinflammation (Rozenstein-Tsalkovich et al., 2013).

ACTIVATION OF PHOSPHATASES

Another possible therapeutic strategy could involve restoring or increasing the activity of specific phosphatases. Although phosphatases are thought to be less appealing drug targets, since they are considered less specific than kinases, increasing evidence suggests that they might be "druggable" proteins. On the other hand, the lower level of redundancy may be seen as an advantage.

In fact, pharmacological induction of the phosphoprotein phosphatase 2A (PP2A) by eicosanoyl-5-hydroxytryptamide resulted in dephosphorylation of aSyn at S129, inhibition of aSyn aggregation with concomitant improved neuronal integrity, reduction in inflammation and amelioration of behavioral deficits in an aSyn transgenic mouse model, (Lee et al., 2011).

Dysregulated phosphatase activity also seems to be partially responsible for tau pathology. A number of pharmacological agents, such as the FDA-approved drug memantine, prevented okadaic acid or calyculin-induced PP2A inhibition and tau phosphorylation (Li et al., 2004; De Los Rios et al., 2010; Kickstein et al., 2010; Yang et al., 2011).

MODULATION OF PROTEIN CLEARANCE VIA PHOSPHORYLATION

The induction of aSyn degradation through clearance pathways is also seen as an attractive therapeutic strategy. aSyn can be degraded by direct proteolysis, by the UPS, by chaperone-mediated autophagy, or by general autophagy (Lashuel et al., 2013). As discussed earlier, phosphorylation of Y39 by c-Abl impairs aSyn degradation by autophagy and proteasome (Mahul-Mellier et al., 2014), while PLK2 phosphorylation at S129 promotes selective autophagic aSyn clearance (Oueslati et al., 2013). Likewise, inhibiting c-Abl and enhancing PLK2 activity are two promising therapeutic approaches.

Several studies indicate that the reduction of tau phosphorylation was paralleled by an overall decrease in protein levels. Moreover, targeting unphosphorylated tau protein had deleterious effects since tau is a normal component of the cytoskeleton (Rosenmann et al., 2006). Additionally, reduced protein levels were achieved through interference with the UPS and the lysosomal / autophagic pathways. Immunization using phospho-specific antibodies against tau epitopes resulted in reduced levels of tau protein and clearance of tau aggregates (Asuni et al., 2007; Boimel et al., 2010; Boutajangout et al., 2010, 2011). Interestingly, memantine that inhibited the phosphorylation of tau at some epitopes in hippocampal slices also reduced tau aggregation (Li et al., 2004). The small molecule IU1, a potent and selective inhibitor of the deubiquitinating enzyme ubiquitin specific peptidase 14 (USP14), enhanced the degradation of tau (Lee et al., 2010). The underlying mechanisms are not clear because USP14-deficient mice showed no alterations in tau degradation and actually increased amounts of phosphorylated tau (Jin et al., 2012). Chronic treatment with lithium chloride, a direct inhibitor of GSK-3β, reduced tau pathology by promoting ubiquitination (Nakashima et al., 2005).

Positive lysosomal modulation was described for several factors and may be an attempt of cells to clear amyloidogenic species such as tau and aSyn oligomers (Lee et al., 2004b; Butler et al., 2006; Bahr et al., 2012). Methylene blue (MB) was shown to induce autophagy and to reduce total and phosphorylated levels of tau. Although MB administration improved cognitive performance in tau transgenic mice, a reversal of already existing NFTs was disputed (Congdon et al., 2012; Spires-Jones et al., 2014). Interestingly, MB was the first identified direct tau aggregation inhibitor (Duff et al., 2010). Modified versions of this substance with greater tolerability and better absorption are underway in clinical trials (Wischik et al., 2013). Lithium chloride prevented tau aggregation in cultured neurons probably through decreased tau protein levels as outlined above (Rametti et al., 2008). In recent studies, pre-filamentous aSyn and tau oligomers rather than aSyn fibrils and NFTs were considered as toxic species questioning the usefulness of aggregation inhibitors (Castillo-Carranza et al., 2013; Crowe et al., 2013; Lesne, 2013). Furthermore, at higher doses, some of the *in vitro* tested substances showed severe side effects on the normal biology of tau and its MT stabilization in cell culture and brain slices (Duff et al., 2010).

MODULATION OF PATHOLOGY SPREADING VIA PHOSPHORYLATION

Recent studies strongly suggest that disease progression in AD, PD, and other neurodegenerative disorders may, at least in part, be due to cell-to-cell transmission of amyloidogenic species. Thus, the elimination of these toxic species may help to slow or contain neurodegeneration.

The mechanisms behind the spreading of aSyn are not fully understood. However, aSyn secreted by neurons could strongly contribute to cell-to-cell propagation (Marques and Outeiro, 2012; Eisbach and Outeiro, 2013). The relationship between the phosphorylated status of aSyn and its secretion are currently unknown and require investigation, but if correlation exists then phosphorylation might be targeted to prevent the spreading of aSyn pathology.

Immunization with cell-penetrating phospho-tau antibodies was already discussed as a therapeutic approach to clear from toxic tau species. Similarly, antibodies that remain in the extracellular space may inhibit spreading and this includes both, anti-tau and anti-Aβ antibodies (Giacobini and Gold, 2013; Liu et al., 2014). Lowering the intracellular amyloid burden through RNA interference or by drugs may entail reduced secretion of Aβ and thereby lessen tau pathology (Chen et al., 2013b; Spilman et al., 2013). However, some of these treatments failed in clinical trials (Doody et al., 2014). Importantly, extracellular soluble tau was also shown to initiate spread of tau pathology and may be a plausible target for treatment (Michel et al., 2014).

CONCLUSIONS AND FUTURE PERSPECTIVES

While it is clear that phosphorylation of aSyn and tau is relevant in the context of their aggregation and toxicity, there is still no final consensus on the precise contribution this type of PTM has toward the disease process. For example, there is still no consensus on whether aSyn phosphorylation is a cause or a consequence of aggregation, or whether phosphorylation is neurotoxic or neuroprotective. Furthermore, only a few phosphorylation sites have been confirmed in human tissue so far, so there may be other sites relevant to human pathology that remain to be identified. Tau phosphorylation regulates tau's function in many ways while abnormal phosphorylation of tau is neurotoxic. The relationship between tau phosphorylation and aggregation is clearly complex with evidence that tau phosphorylation precedes, prevents or is irrelevant to its aggregation. It will also be important to explore the cross-talk between different PTMs, as this will likely have a strong impact on our understanding of the biology/pathobiology of different proteins associated with neurodegeneration. In addition, the identification of the kinases and phosphatases involved

in the phosphorylation/dephosphorylation of aSyn and tau will certainly open novel possibilities for pharmacological intervention. Ultimately, solving the problems and inconsistencies surrounding the phosphorylation of these important players in AD and PD will be essential for advancing the development of novel therapeutic strategies.

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Emerging modes of PINK1 signaling: another task for MARK2

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PTEN-induced kinase 1 (PINK1) acts at multiple levels to promote mitochondrial health, including regulatory influence on ATP-synthesis, protein quality control, apoptosis, mitochondrial transport, and destiny. PINK1 mutations are linked to Parkinson disease (PD) and mostly result in loss of kinase activity. But the molecular events responsible for neuronal death as well as the physiological targets and regulators of PINK1 are still a matter of debate. This review highlights the recent progress evolving the cellular functions of the cytosolic pool of PINK1 in mitochondrial trafficking and neuronal differentiation. Regulation of PINK1 signaling occurs by mitochondrial processing to truncated forms of PINK1, differentially targeted to several subcellular compartments. The first identified activating kinase of PINK1 is MAP/microtubule affinity regulating kinase 2 (MARK2), which phosphorylates T313, a frequent mutation site linked to PD. Kinases of the MARK2 family perform diverse functions in neuronal polarity, transport, migration, and neurodegeneration such as Alzheimer disease (AD). This new protein kinase signaling axis might provide a link between neurodegenerative processes in AD and PD diseases and opens novel possibilities in targeting pathological signaling processes.

Keywords: PINK1, MARK2, mitochondria, transport, differentiation, neurodegeneration, Alzheimer disease, Parkinson disease

INTRODUCTION

Many neurodegenerative disorders, such as Alzheimer (AD) and Parkinson disease (PD) show mitochondrial abnormalities during their pathogenesis. Neurons, due to their size and complex geometry, are particularly dependent on the proper functioning and distribution of mitochondria, which are the powerhouses of the cells. Beside ATP production, they perform a variety of functions that are important for cell life and death, including reactive oxygen species (ROS) generation, intracellular calcium homeostasis, and apoptosis. In the nervous system, mitochondrial dynamics are crucial to guarantee long distance delivery and balanced distribution of energy to axons, dendrites and synapses (Jacobson and Duchen, 2004; DiMauro and Schon, 2008). Tau and other microtubule associated proteins promote the assembly and stabilization of neuronal microtubule tracks and ensure microtubule dependent transport. Pathological changes of tau may lead to the breakdown of microtubules observed in AD while elevated tau on microtubules can compete with motor proteins, resulting in inhibition of traffic (Mandelkow et al., 2004; Dixit et al., 2008). This suggests that a strict regulation is needed to maintain the flow of material. Phosphorylation of tau, especially at the KXGS motifs of the repeat domain, decreases its affinity to microtubules and provides a mechanism for regulating microtubule stability as well as axonal transport (Matenia and Mandelkow, 2009). Enhanced phosphorylation of tau at multiple sites is an early hallmark of AD, followed by abnormal aggregation of tau protein into paired helical filaments (PHFs) and neurofibrillary tangles (NFTs). The microtubule-affinity regulating kinase 2 (MARK2) was originally discovered by its ability to phosphorylate tau protein and related microtubule-associated proteins (MAPs; Drewes et al., 1997; Schwalbe et al., 2013). Furthermore, active MARK2 co-localizes with NFTs in AD brain, and MARK2 target sites on tau are elevated in transgenic mouse models of tauopathy, emphasizing the importance of MARK2 in this disease (Matenia and Mandelkow, 2009). Recently, MARK2 was identified as an upstream regulator of PTEN-induced kinase 1 (PINK1; Matenia et al., 2012). This provides insights into the regulation of mitochondrial trafficking in neurons and a potential link between neurodegenerative processes in AD and PD.

PTEN-INDUCED KINASE 1

Familial cases of PD can be caused by mutations in different genes, such as PINK1 or Parkin. PINK1 is a mitochondria-targeted serine/threonine kinase promoting cell survival, particularly under conditions of oxidative/metabolic stress (Valente et al., 2004; Deng et al., 2005; Wood-Kaczmar et al., 2008). In particular, PINK1 regulates mitochondrial transport, morphology, biogenesis, function, calcium buffering capacity, and mitochondrial clearance (Petit et al., 2005; Wood-Kaczmar et al., 2008; Dagda and Chu, 2009; Gandhi et al., 2009; Gegg et al., 2009; Van Laar and Berman, 2009; Matsuda et al., 2010; Narendra et al., 2010; Sun et al., 2012). Most of the reported PD-linked PINK1 mutations result in a loss of kinase activity (Cookson and Bandmann, 2010).

The molecular events responsible for PINK1-induced neuronal death as well as its physiological substrates or regulators are still a matter of debate (Deas et al., 2009; Pogson et al.,

2011). Upon entry to the mitochondria the PINK1 protein is proteolytically cleaved by mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL) to produce two N-terminally truncated protein fragments of 54 and 45 kDa without mitochondrial localization sequence (Deas et al., 2009; Narendra et al., 2010; Greene et al., 2012). The cleaved ΔN-PINK1 forms localize preferentially in the cytosolic instead of the mitochondrial fraction (Lin and Kang, 2008). ΔN-PINK1 is constitutively degraded in the cytosol by the proteasomal pathway (Yamano and Youle, 2013), indicating that only the mitochondrially targeted PINK1^{FL} has a cellular function. But expression of ΔN-PINK1 protects neurons against the neurotoxin 1-methyl-4-pheny-1,2,3,6-tertahydropyridin (MPTP). This suggests that the mitochondrial import sequence of PINK1 is not strictly necessary for neuroprotection and that cytosolic targets and signal transduction pathways may be modified by cleaved PINK1 (ΔN-PINK1) to affect neuronal survival (Haque et al., 2008). Recent studies validate this hypothesis. PINK1 cleavage-products localized in the cytosol are degraded by proteasomes but also bind Parkin, repress Parkin translocation to mitochondria and prevent mitophagy (Fedorowicz et al., 2014; **Figure 1A**). Furthermore, cytosolic ΔN-PINK1 influences mitochondrial mobility. The kinase enhances anterograde movements of mitochondria, both in dendrites and axons (Matenia et al., 2012; Dagda et al., 2013). However, the mechanisms of these ΔN-PINK1 functions are mostly unknown. So far only one upstream regulating kinase was identified: MARK2 phosphorylates PINK1 and thereby regulates mitochondrial transport parameters (Matenia et al., 2012). This new signaling axis might help to clarify common mechanisms in neurodegenerative diseases, although future studies are required to understand the exact functional relationship of these kinases.

REGULATION OF PINK1 AND MITOCHONDRIAL MOTILITY IN NEURONS

Recent studies have investigated the PINK1/Parkin pathway for sensing and selectively eliminating damaged mitochondria from the mitochondrial network. Parkin is a cytoplasmic E3 ubiquitin ligase and can be phosphorylated by PINK1 (Kim et al., 2008). Both proteins cooperate to control mitochondrial clearance, known as mitophagy. Full length PINK1 (PINK1^{FL}) is stabilized on mitochondria with low membrane potential and recruits cytosolic Parkin, which becomes enzymatically active and initiates the lysosomal degeneration of defective mitochondria via ubiquitination of mitochondrial target proteins (Youle and Narendra, 2011; Grenier et al., 2013; **Figure 1B**).

Another aspect of PINK1 concerns its role in the regulation of mitochondrial transport in neurons (Wang et al., 2011; Liu et al., 2012; Matenia et al., 2012; Dagda et al., 2013). Mitochondria are transported along microtubules by the motor proteins kinesin (anterograde, toward the microtubule plus ends) and dynein (retrograde). The kinesin-adaptor complex attached to the outer mitochondrial membrane comprises the GTPase Miro, kinesin heavy chain, and the adaptor protein Milton (Goldstein et al., 2008). PINK1^{FL} is also attached to this complex, and even ΔN-PINK1 can be targeted to it despite the lack of the mitochondrial targeting sequence (Weihofen et al., 2009). PINK1^{FL} phosphorylates the GTPase Miro, and thus induces Parkin-dependent degeneration of Miro. The resulting decrease in mitochondrial movement may represent a quality control mechanism of defective

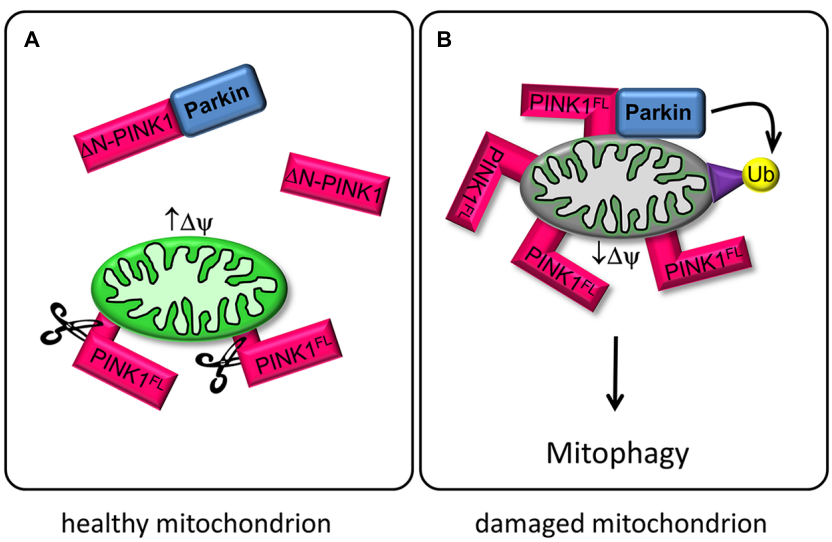

FIGURE 1 | PINK1 and Parkin regulate mitophagy. (A) In healthy mitochondria with high mitochondrial membrane potential (↑ΔΨ), PINK1^{FL} is maintained at low levels by the sequential proteolytic actions of mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL; Greene et al., 2012). The resulting ΔN-PINK1 is partially located in the cytosol and interacts directly with Parkin, thereby preventing Parkin-mediated mitophagy (Fedorowicz et al., 2014). **(B)** Upon mitochondrial depolarization (↓ΔΨ) PINK1^{FL} is stabilized and selectively accumulates in the outer membrane of defective mitochondria with its kinase domain facing the cytoplasm. This accumulation is a crucial signal for Parkin recruitment to impaired mitochondria, promoting ubiquitination of mitochondrial outer membrane proteins and subsequent disposal of the damaged organelle (Narendra et al., 2010).

mitochondria (Wang et al., 2011). Active mitochondria with a high membrane potential tend to cause cleavage of $PINK1^{FL}$ to ΔN-PINK1, which is released to the cytoplasm, where it is not only destined for degradation by the proteasome but also binds to Parkin (Miller and Sheetz, 2004; **Figure 1A**). The interaction of cytosolic ΔN-PINK1 with Parkin represses Parkin translocation to the mitochondria and subsequent mitophagy (Fedorowicz et al., 2014). The question is therefore how the different isoforms of PINK1 become active.

Only few studies have examined the regulation of PINK1 and its consequences for mitochondria. The serine/threonine kinase MARK2 phosphorylates PINK1, activates the kinase activity of ΔN-PINK1 (with regard to the artificial substrate histone H4) and enhances protein stability of both, $PINK1^{FL}$ and ΔN-PINK1, arguing for a physiological relevance of this kinase-substrate interaction. The primary phosphorylation site is threonine 313 (T313; Matenia et al., 2012). This residue is mutated to a non-phosphorylatable form (T313M) in a frequent variant of PD (Mills et al., 2008). Residue T313 is located in β-strand 5 of PINK1 (based on a structural model of PINK1 by Swiss-Model using CaMK1 as a structure template; Matenia et al., 2012). Phosphorylation of this residue could therefore result in the interaction of this strand with helix C which is part of the scaffold that fixes the Mg-ATP underneath the P-loop. Stabilization of this part of the catalytic domain is a requirement for the activity of the kinase. In fact, mutation of T313 to glutamate further enhances the phosphorylation and activation of ΔN-PINK1 by MARK2, suggesting that this residue could be a priming phosphorylation site, changing the conformation of the kinase and preparing it for further modifications.

The importance of the PINK1 phosphorylation site T313 is further emphasized by the fact that expression of $PINK1^{T313M}$ causes severe toxicity for cells. $\Delta N\text{-}PINK1^{T313M}$ leads to abnormal mitochondrial accumulation in the cell soma, whereas $PINK1^{FL/T313M}$ causes degradation of mitochondria. Within neurons endogenous PINK1 and MARK2 colocalize partly on mitochondria, especially in axons and dendrites, changing mitochondrial transport parameters (mitochondrial density and movement direction in axons). MARK2 interacts with and preferentially phosphorylates the cytosolic ΔN-PINK1, thereby increasing its kinase activity and promoting anterograde mitochondrial motility (Matenia et al., 2012). Consistent with this, a high membrane potential enhances the anterograde transport of mitochondria (Miller and Sheetz, 2004) and also promotes the proteolysis of $PINK1^{FL}$ into ΔN-PINK1 (Narendra et al., 2010), thereby inhibiting Parkin-dependent mitophagy of active mitochondria (**Figure 2**). Phosphorylation and activation of ΔN-PINK1 by MARK2 possibly enhances the stability of the mitochondrial transport-complex and ensures the supply of energy at the growth cone. Conversely, retrograde transport is favored for mitochondria with low membrane potential destined for mitophagy (Jin et al., 2010). In this case the cleavage of $PINK1^{FL}$ is inhibited. Thus, Parkin decorated mitochondria assemble as large clusters primarily in the lysosome-rich perinuclear area (Narendra et al., 2010). This effect is influenced by MARK2 (Matenia et al., 2012). MARK2 could phosphorylate $PINK1^{FL}$, consequently enhance the binding and possibly the phosphorylation of Parkin and Miro by $PINK1^{FL}$. This results in accumulation of mitochondria around the perinuclear region and suggests that failure of the MARK2-PINK1 signaling cascade could contribute to PD. Thus, our study revealed the existence of two cellular pools of PINK1 that differently modify and regulate mitochondrial movement direction.

PINK1, MARK2, AND DIFFERENTIATION

MAP/microtubule affinity regulating kinase 2 is involved in several regulatory processes of the cell such as determination of polarity, cell cycle control, intracellular signal transduction, transport, and cytoskeletal stability (Matenia and Mandelkow, 2009). MARK2 and its homolog par-1 (for "partition defective") belongs to a set of conserved proteins in *Drosophila* and *Caenorhabditis elegans*, which are essential for cellular polarity, with roles in establishing the embryonic body axis and in maintaining cell differentiation (Kemphues et al., 1988; Tomancak et al., 2000). The par-1-dependent cell polarization is based on a tight network of cross-reactive and feedback interactions of the par proteins, other regulators of polarity and the cytoskeleton (Munro, 2006). MARK/par-1 is a central player in localization of the cell polarity proteins. In mammalian epithelial cells the overexpression of inactive MARK2 disturbs the polarity, suggesting a similar mechanism of governing polarization (Böhm et al., 1997).

Microtubules are important determinants of cell polarity. MARK2 plays a significant role in axon formation, which requires dynamic instability of microtubules (Biernat et al., 2002). This is in part related to the phosphorylation of axonal Tau protein in its "repeat domain" which decreases its affinity for microtubules. The reduction of MARK2 via RNA interference (RNAi) induces multiple axons in hippocampal neurons, whereas enhanced MARK2 expression inhibits axon formation altogether (Chen et al., 2006). Following the establishment of an axon MARK2 promotes its elongation (Uboha et al., 2007). In dendrites, the predominant MAP is MAP2 which has a similar repeat domain as Tau and can also be phosphorylated by MARK2 (Illenberger et al., 1996). In this case MARK2 inhibits the development of dendrites in hippocampal neurons through phosphorylation of MAP2. In particular, MARK2 shortens the length and decreases branching of dendrites (Terabayashi et al., 2007).

Interestingly, transient expression of ΔN-PINK1 promotes dendritic outgrowth and neurite length in dopaminergic midbrain neurons. This effect seems to be kinase dependent, since a kinase deficient mutant of PINK1 fails to influence neurite length. The action of ΔN-PINK1 on neurite length was not related to its activity at mitochondria, since an outer mitochondria membrane (OMM)-targeted ΔN-PINK1 construct, which exhibits cytosolic localization, failed to enhance neuronal differentiation. These data indicate divergent roles for cytosolic and mitochondrial targeted forms of PINK1. Furthermore, PINK1 deficiency reduces dendritic length of primary neurons isolated from PINK1 knockout mice. To clarify the mechanism underlying the regulation of neurite outgrowth induced by cytosolic ΔN-PINK1, Dagda et al. (2013) examined the expression of various neuronal differentiation proteins as a function of PINK1. PINK1 increases levels of MAP2 and activates protein kinase A (PKA)-regulated signaling

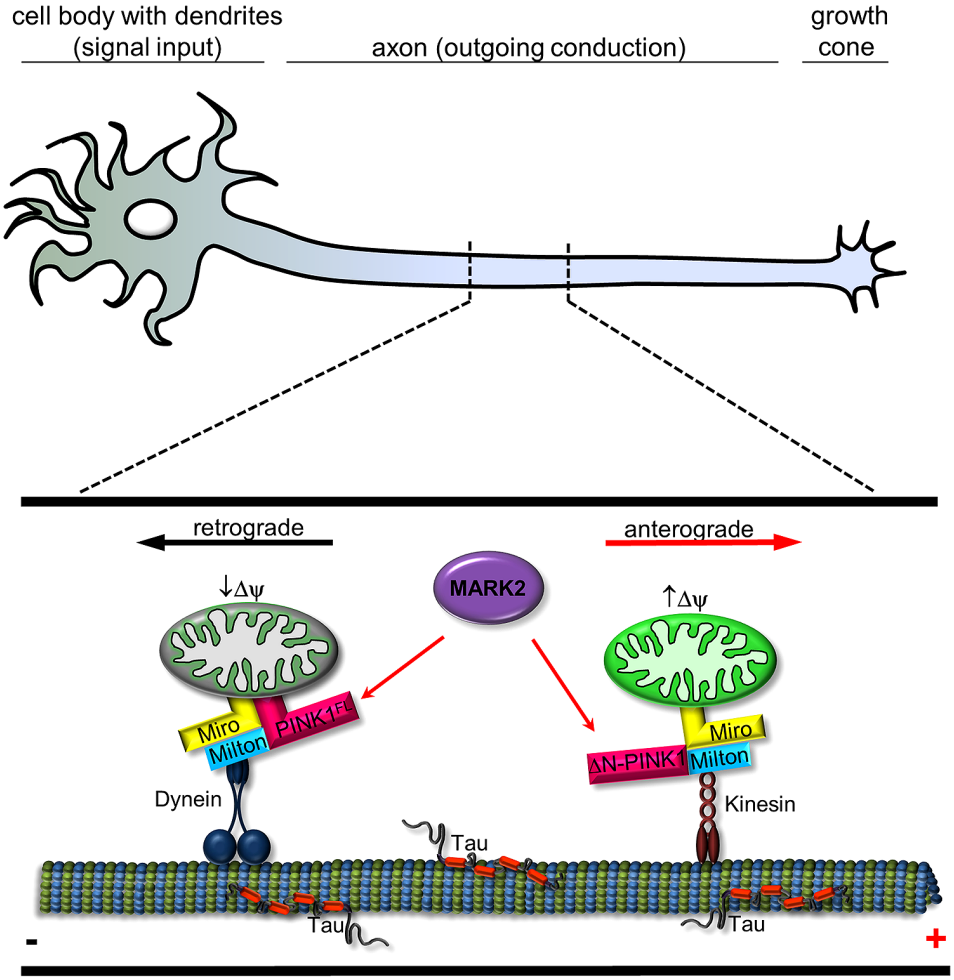

FIGURE 2 | Schematic representation of interplay between MARK2 and PINK1^{FL}/ΔN-PINK1 to regulate mitochondrial transport. In a healthy neuron, mitochondria are carried along by motor proteins dynein (retrograde) and kinesin (anterograde). PINK1 is a molecular switch that changes the probability between anterograde and retrograde mitochondrial transport. Transport direction of neuronal mitochondria is regulated by PINK1 cleavage and binding/phosphorylation by MARK2. Kinesin motors are linked to mitochondria by adaptor proteins like Miro and Milton (Weihofen et al., 2009) and regulate in association with ΔN-PINK1 the anterograde movement (*red arrow*; Matenia et al., 2012; Dagda et al., 2013), whereas Miro also has an effect on dynein-mediated retrograde movement (Russo et al., 2009; *black arrow*). Active mitochondria tend to cause cleavage of PINK1^{FL} (Narendra et al., 2010), which is phosphorylated at Thr-313 by MARK2 (Matenia et al., 2012); both events promote anterograde movement by kinesin. Retrograde movement by dynein is promoted by PINK1^{FL} and further increased by MARK2 (Matenia et al., 2012).

pathways. Since MAP2 is an anchoring protein of PKA in dendrites (Obar et al., 1989; Harada et al., 2002), this data suggests that PINK1 is an upstream regulatory kinase of this pathway to influence dendritic morphology. On the other hand, the ability of microtubule-associated PKA to promote elongation of dendrites is independent of MAP2 phosphorylation. This suggests other proteins in close proximity to the microtubule cytoskeleton are involved in this process (Huang et al., 2013). Since KXGS is not only a kinase consensus motif for targets of MARK2 but also of PKA, both kinases share some substrate preferences (Drewes et al., 1997). The microtubule binding affinity of tau as well as that of doublecortin (Dcx) is regulated via phosphorylation by MARK2 and PKA (Drewes et al., 1997; Schaar et al., 2004). This provides the clue, that MARK2 signaling pathways could be involved in ΔN-PINK1 mediated neurite outgrowth regulation. Due to substrate competition ΔN-PINK1 could inhibit MARK2 by binding, thereby enhancing dendritic length.

CONCLUSION AND OUTLOOK

This review summarizes and evaluates recent findings in PINK1 biology and focuses on emerging aspects concerning the novel role of cytosolic ΔN-PINK1 that has not yet received adequate attention as compared to mitochondrial PINK1^{FL}. In the case of mitochondria the full-length PINK1 regulates the transport and clearance of defective mitochondria through phosphorylation of Miro and recruitment of Parkin, respectively (**Figure 1**). These protective activities of PINK1^{FL} are dependent on its localization at the mitochondrial surface and have been studied extensively. But even the N-terminally truncated enzyme ΔN-PINK1 lacking the mitochondrial localization signal can be found in close proximity

to mitochondria, probably via binding to mitochondrial membrane localized protein adaptor complexes (**Figure 2**), controlling their health status and distribution. Beside this task, ΔN-PINK1 released from mitochondria via proteolytic cleavage by mitochondrial enzymes shows neurite promoting activity. This outgrowth effect was specific to dendrites as axonal length did not change significantly (Tieu and Xia, 2013).

Microtubule-affinity regulating kinase 2, the upstream regulator of ΔN-PINK1 and PINK-1^{FL}, activates and regulates a diverse range of cellular activities and participates in several signaling cascades. Since the discovery of MARK2 as a kinase of Tau and MAP2 (Drewes et al., 1997), several other substrates have been identified. Some of these affect mitochondrial transport, presumably by regulating the affinity to microtubules (Thies and Mandelkow, 2007; Pluciñska et al., 2012). Neurons are particularly dependent on mitochondrial function, so disrupting the transport of these organelles can cause neurological disease (Schon and Przedborski, 2011). The vulnerability results from the high metabolic demands of neurons, their dependence on proper calcium handling and their susceptibility to local ROS signaling, processes in which mitochondria are critically involved. In the context of transport PINK1 acts as a molecular switch between anterograde and retrograde mitochondrial transport. As mentioned above, transport direction is regulated by PINK1 cleavage depending on the mitochondrial membrane potential (**Figure 1**) and PINK1 binding/phosphorylation by MARK2 (**Figure 2;** Matenia et al., 2012). Since MARK2 is an upstream regulatory component in PINK1 signaling, this extends the complexity of its biological function. Mitochondria are enriched at synapses and play a critical role in both pre- and post-synaptic functions (Hollenbeck, 2005). Accurate regulation of mitochondrial motility and maintenance of neuronal plasticity are closely related.

Increasing evidence implicates that dysfunction of kinase activities and phosphorylation pathways are involved in the pathogenesis of neurodegenerative diseases. PINK1 mutations linked to PD are mostly accompanied by loss of kinase activity; therefore an effective therapy would have to replace functional PINK1-signaling. The limiting factor is that the details of the PINK1 signaling network are not yet fully elucidated. An initial step in the right direction is the identification and characterization of a PINK1/Parkin independent mitophagy pathway (Allen et al., 2013). Selective induction of mitophagy could prove beneficial as a potential therapy for several neurodegenerative diseases in which mitochondrial clearance is advantageous.

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Structural biology of the LRRK2 GTPase and kinase domains: implications for regulation

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Human leucine rich repeat kinase 2 (LRRK2) belongs to the Roco family of proteins, which are characterized by the presence of a Ras-like G-domain (Roc), a C-terminal of Roc domain (COR), and a kinase domain. Mutations in LRRK2 have been found to be thus far the most frequent cause of late-onset Parkinson's disease (PD). Several of the pathogenic mutations in LRRK2 result in decreased GTPase activity and enhanced kinase activity, suggesting a possible PD-related gain of abnormal function. Important progress in the structural understanding of LRRK2 has come from our work with related Roco proteins from lower organisms. Atomic structures of Roco proteins from prokaryotes revealed that Roco proteins belong to the GAD class of molecular switches (G proteins activated by nucleotide dependent dimerization). As in LRRK2, PD-analogous mutations in Roco proteins from bacteria decrease the GTPase reaction. Studies with Roco proteins from the model organism *Dictyostelium discoideum* revealed that PD mutants have different effects and most importantly they explained the G2019S-related increased LRRK2 kinase activity. Furthermore, the structure of *Dictyostelium* Roco4 kinase in complex with the LRRK2 inhibitor H1152 showed that Roco4 and other Roco family proteins can be important for the optimization of the current, and identification of new, LRRK2 kinase inhibitors. In this review we highlight the recent progress in structural and biochemical characterization of Roco proteins and discuss its implication for the understanding of the complex regulatory mechanism of LRRK2.

Keywords: LRRK2, Roco, structure, kinase, G-protein, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) affects 1–2% of the population above the age of 65 and is the second most common neurodegenerative disease (Lees et al., 2009). PD causes the loss of dopaminergic neurons in the substantia nigra and is associated with the formation of fibrillar aggregates that are composed of α-synunclein and other proteins. The loss of those neurons leads to the major hallmarks of PD: tremor, bradykinesia, rigidity, and postural instability. Today several genes have been found to be involved in PD, among them the PARK8 locus that encodes for Leucine rich repeat kinase 2 (LRRK2). Mutations in LRRK2 have been found to be the most frequent cause of late onsets PD and are found in both hereditary and sporadic forms of PD (Paisán-Ruíz et al., 2004; Zimprich et al., 2004; Bekris et al., 2010). LRRK2 has been linked to a multitude of cellular functions and pathways, including regulation of neurite outgrowth, Wnt signaling, mitochondrial disease, and autophagy (Dächsel et al., 2010; Winner et al., 2011; Berwick and Harvey, 2012; Papkovskaia et al., 2012). Several studies have identified interaction partners of LRRK2, including 14-3-3, Tubulin, ArfGAP1, Rac1, and DVL (Sancho et al., 2009; Chan et al., 2011; Kawakami et al., 2012; Xiong et al., 2012; Dzamko et al., 2013; Fraser et al., 2013). Despite all this accumulating data, substantial gaps remain in the knowledge about the underlying pathways of LRRK2 mediated PD.

LRRK2 belongs to the Roco superfamily of proteins, which constitutes a novel multi-domain family of Ras-like G-proteins (Bosgraaf and Van Haastert, 2003; Marín et al., 2008). LRRK2 consists of armadillo repeats (ARM), ankyrin repeats (ANK), leucine-rich repeats (LRR), Ras of complex (Roc), C-terminal of Roc (COR), kinase and a WD40 domains (Mills et al., 2014). PD mutations are accumulated around the central core of the protein, two mutations are found in the Roc domain, one in the COR domain, and two in the kinase domain. In addition, two variants have been identified that act as risk factors for sporadic PD, one in the COR domain and one in the WD40 repeats (Cookson, 2010; Cookson and Bandmann, 2010). The multiple disease-linked mutations in LRRK2 represent a unique opportunity to explore the activation mechanism of the protein and its mis-regulation in PD. In this review we will focus on the recent progress in the structural and biochemical characterization of LRRK2 and discuss this in context of the LRRK2 activation mechanism.

HOMOLOGOUS ROCO PROTEINS AS STRUCTURAL MODEL FOR LRRK2

So far it has been a major challenge to isolate sufficient high-quality recombinant protein of full-length LRRK2 and/or domains thereof. Therefore, important structural understanding has come from work with related Roco proteins from bacteria and *Dictyostelium discoideum* (Gotthardt et al., 2008; Gilsbach et al., 2012). Roco proteins are characterized by the occurrence of a Roc domain, which has high homology to proteins of the Ras superfamily

and possesses all five G motifs that are required for guanine nucleotide binding. Roc always forms an inseparable tandem with the COR domain, a 300–400 long stretch of amino acids with no significant homology to other described domains. Roco proteins were first described in *D. discoideum* and have since been identified in prokaryotes, plants and metazoans (Bosgraaf and Van Haastert, 2003; Marín et al., 2008). However, they seem to be absent in yeast and Plasmodium. Four Roco proteins are identified in vertebrates, called LRRK1, LRRK2, death-associated protein kinases-1 (DAPK1), and malignant fibrous histiocytoma amplified sequences with leucine-rich tandem repeats 1 (MASL) (**Figure 1**). Remarkably, the slime mold *D. discoideum* contains 11 Roco family members. Based on domain topology, Roco proteins can be divided into three separate groups (Bosgraaf and Van Haastert, 2003). MASL belongs to the first group of Roco proteins, which is also found in other metazoan, plants and prokaryotes. In these proteins the RocCOR tandem is always preceded by an LRR domain. The human proteins LRRK2 and LRRK1 belong to the second group of Roco proteins. These proteins, which are also present in *D. discoideum* and metazoans, always have in addition to the RocCOR tandem an N-terminal LRR and C-terminal kinase domain. The third group of Roco proteins, which is only found in metazoans, is characterized by the presence of a tumor-suppressor DAPK domain. Besides this general domain composition, individual Roco proteins are found to be combined with a diversity of additional domains such as protein–protein interaction domains, Guanine nucleotide exchange factor (GEF), and Regulator of G-protein Signalling (RGS) domains. Although there is a high variation in these additional regulatory domains among the Roco proteins, as described below, previous studies have shown that the structure, function and regulation of the catalytic core is conserved.

THE LRRK2 KINASE DOMAIN

The kinase domain of LRRK2 has been extensively studied after its discovery. Kinases transfer the γ-phosphate of ATP to a target protein. Phosphorylation of proteins as a regulatory mechanism was discovered by Krebs and Fisher (1956), in their studies of glycogen phosphorylase. Nowadays, it is known that kinases are essential regulators of almost every signal transduction cascade. Kinases can be classified into three groups, the majority belongs to the group of serine/threonine kinases, a much smaller amount to the class of tyrosine kinases and only a few are classified as atypical kinases (Manning et al., 2002; Taylor and Kornev, 2011; Endicott et al., 2012).

LRRK2 and Roco proteins are serine/threonine specific kinases. Our previous solved structure of the kinase domain of *Dictyostelium* Roco4 in its active and inactive state, gave insight into the regulation mechanism of this group of kinases (Gilsbach et al., 2012). *Dictyostelium* Roco4 has the same domain architecture as LRRK2, but is biochemically and structurally more tractable than LRRK2. Like almost all kinases, the Roco4 kinase structure consists of a canonical, two-lobed kinase structure, with an adenine nucleotide bound in the conventional nucleotide-binding pocket

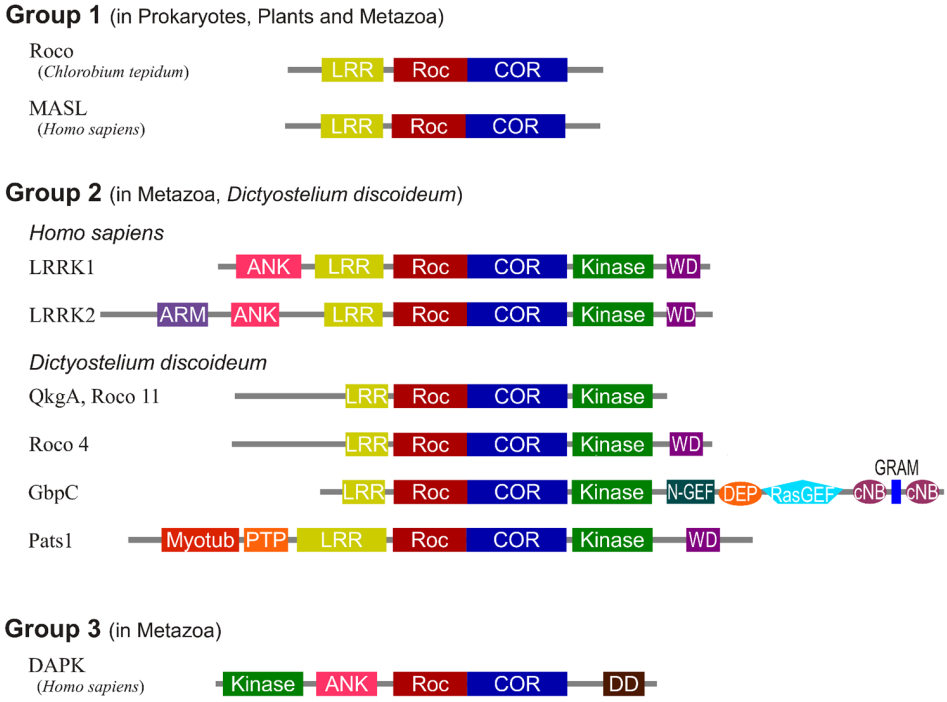

FIGURE 1 | Domain topology of the Roco family proteins. The domains are Leucine rich repeats (LRR), Ras of complex proteins (Roc), C-terminal of Roc (COR), Ankyrin repeats (ANK), WD40 repeats (WD), Armadillo repeats (ARM), N-terminal motif of RasGEF (N-GEF), Ras guanine nucleotide exchange factor domain (RasGEF), cyclic nucleotide binding domain (cNB), glucosyltransferases, Rab-like GTPase activators and myotubularins domain (GRAM), N-terminal myotubularin-related domain (myotub), protein tyrosine phosphatase domain (PTP), and death domain (DD).

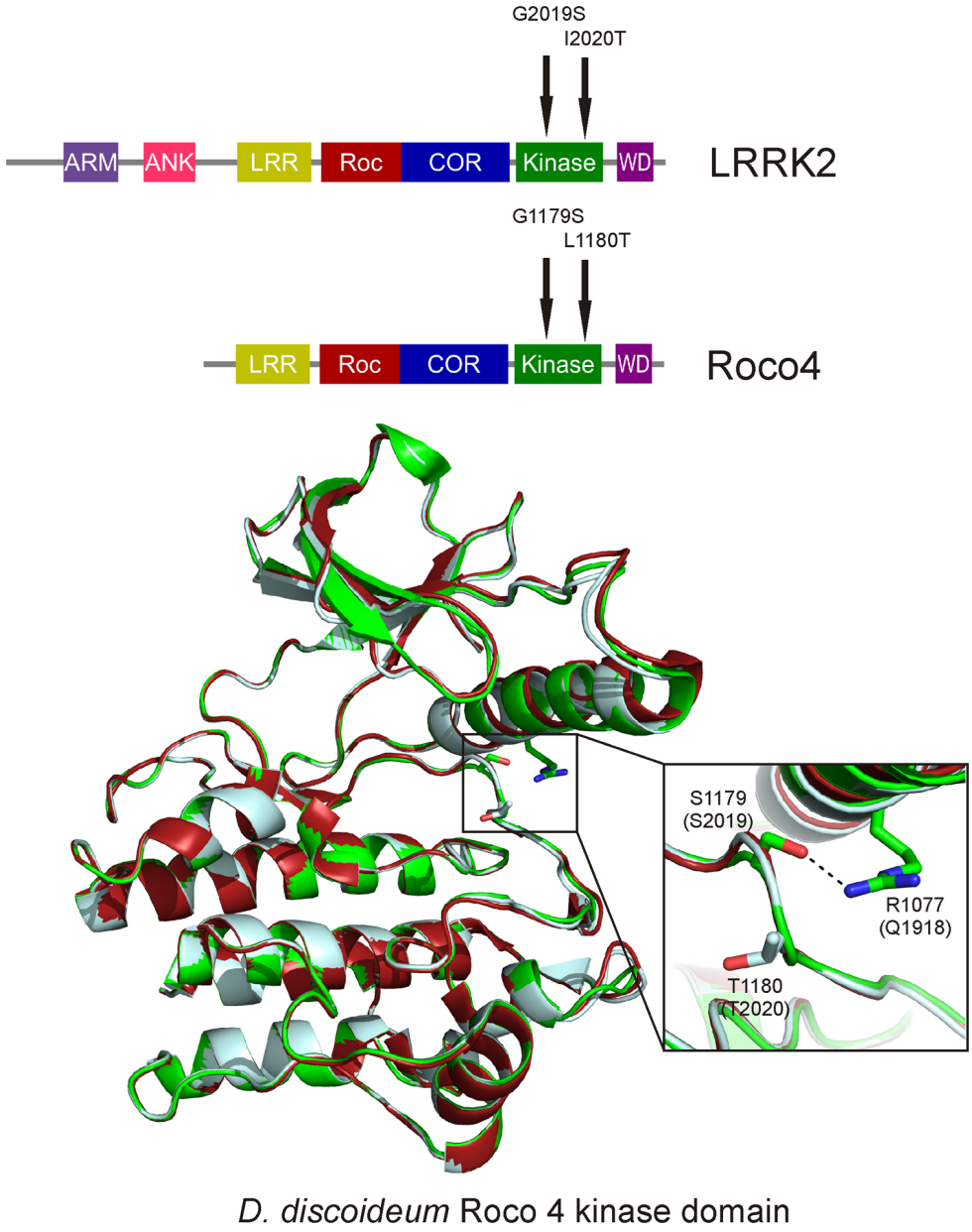

FIGURE 2 | Overlay of the *Dictyostelium* Roco4 kinase wt, the G1179S (G2019S) and the L1180T (I2020T) structures. The wt structure is shown in red, the G1179S (G2019S) in green and the L1180T (I2020T) in light blue. Enlarged; PD mutation shown as sticks. The dashed line indicates the stabilizing hydrogen-bond.

(**Figure 2**). The smaller N-terminal lobe is mostly composed of anti-parallel β sheets and contains the conserved αC-helix. The bigger C-terminal lobe mostly consists of α-helices and contains the activation loop with the conserved N-terminal DFG motif. The ATP binding site is formed by a cleft between those lobes and forms the catalytic site of the kinase together with the activation loop and αC-helix. The formation of a polar contact between Roco4 Lys 1055 from the β3-strand and Glu1078 from the αC-helix is essential for correct positioning of the αC-helix. The DFG motif is essential for catalysis: the Asp makes contact with all three ATP phosphates either directly or via coordination of a magnesium ion; the Phe makes hydrophobic contacts to the αC-helix and the HxD motif and is responsible for the correct positioning of the DFG motif. One can distinguish two conformations: a DFG-in (active) and a DFG-out (inactive) conformation. In the structure of active (phosphorylated) Roco4 kinase, the activation loop is visible and ordered. In contrast, the activation loop is not visible and is flexible in the structure of inactive (dephosphorylated) Roco4 kinase (Huse and Kuriyan, 2002; Taylor and Kornev, 2011).

This mechanism to switch from an inactive to an active state is conserved in most kinases and often involves autophosphorylation

of one or more residues in the activation loop. Autophosphorylation not only results in the reorientation of the activation loop, but often also alters ATP binding and/or interaction with substrates (Huse and Kuriyan, 2002; Kornev et al., 2006). Autophosphorylation of LRRK2 and related Roco proteins was shown by various studies (Lobbestael et al., 2012; Zhao et al., 2012). LRRK2 possesses three potential phosphorylation sites in the activation loop (Thr2031, Ser2032, and Thr2035), while four putative phosphorylation sites (Ser1181, Ser1184, Ser1187, and Ser1189) are present in the same region of Roco4. *In vitro* and *in vivo* analysis revealed that in both LRRK2 and Roco4, only the two latter phosphorylation sites are important for function *in vivo* (Li et al., 2010; Gilsbach et al., 2012). In addition, several LRRK2 autophosphorylation sites outside the activation loop have been identified, most of which are located in the Roc domain. Importantly, mutation of several of these residues, or inhibition of kinase activity with inhibitors, completely rescues neurite outgrowth in LRRK2 PD mutant strains (MacLeod et al., 2006; Herzig et al., 2011; Yao et al., 2013). This suggests that LRRK2 kinase-mediated phosphorylation events are important for both the intramolecular activation mechanism as well for downstream signaling.

MECHANISM OF PD-MUTATION IN THE LRRK2 KINASE DOMAIN

It has been shown that kinase activity is essential for LRRK2-induced neuronal toxicity (Greggio et al., 2006; Smith et al., 2006). However, conflicting data regarding kinase activity of PD-related mutants have been published. Increased kinase activity only has been consistently shown for the most prevalent LRRK2 Gly2019Ser mutation, whereas for the other mutations, either no effect or even a decreased kinase activity has been reported (Reviewed by Greggio and Cookson, 2009). LRRK2 Gly2019 is located within the conserved DFG motif and critically linked to a 2- to 4-fold increase in kinase activity (West et al., 2005; Greggio et al., 2006; Jaleel et al., 2007; Anand et al., 2009). The molecular mechanism by which this mutation enhances the catalytic activity of LRRK2 was resolved by our study with *Dictyostelium* Roco4 as a model (Gilsbach et al., 2012). LRRK2 Gly2019 corresponds to Roco4 Gly1179. As expected, introducing the PD mutation at this position leads to increased kinase activity. The Roco4 Gly1179Ser mutation does not result in large changes in the overall structure, however, closer observation reveals that Ser1179 makes a new hydrogen bond with Arg1077, thereby presumably stabilizing the activation loop and the αC-helix in their active conformation (**Figure 2**). Roco4 Arg1077 is conserved in almost all Roco proteins and corresponds to LRRK2 Gln1918. Consistent with the proposed mechanism: the Roco4 double mutant Gly1179Ser/Arg1077Ala and the homologous LRRK2 double mutant Gly2019Ser/Gln1918Ala, in which the new hydrogen bond is no longer possible, have again normal wild-type kinase activity (Gilsbach et al., 2012).

Both the LRRK2 Ile2020Thr PD mutant and the corresponding Roco4 Leu1180Thr mutant have a slightly decreased kinase activity (Jaleel et al., 2007; Gilsbach et al., 2012). The structure of Roco4 Leu1180Thr does not directly explain this decreased kinase activity: the Thr1180 side-chain points into the solvent and most likely does not directly interfere with active site configuration. It has been speculated that the higher neurotoxicity of this mutant might be due to a higher susceptibility of the mutant to intracellular degradation (Smith et al., 2006; Ohta et al., 2010). Others postulated that in analogy to what has been shown for B-RAF mutations, LRRK2 works in tandem such that the interaction between wild-type and LRRK2-Thr2020 might increase kinase activity (Wan et al., 2004). Alternatively, the Ile2020Thr could affect intramolecular interactions with other domains and thereby indirectly influence kinase activity. Importantly, the Roco4 structures show that the PD-related effect of LRRK2 mutations result from different defects in the LRRK2 activation mechanism.

STRUCTURAL-BASED OPTIMIZATION OF KINASE INHIBITORS

Kinases are one of the most potent classes of drug targets and have been effectively used in the treatment of cancer, and for immunological, neurological and infectious diseases (Cohen, 2002). Several kinase inhibitors have been identified that are selective for LRRK2 and brain penetrant (Deng et al., 2011; Ramsden et al., 2011; Choi et al., 2012; Reith et al., 2012; Zhang et al., 2012). However, long-term inhibition of LRRK2 by many of these inhibitors leads to kidney abnormality, similar to what has been observed in LRRK2 knock-out mice (Herzig et al., 2011; Tong et al., 2012; Ness et al., 2013). Most likely, the ATP binding pocket is the direct target of many of these inhibitors, but the exact binding mechanism is unknown. Previously, the structure of the *Dictyostelium* Roco4 kinase in complex with the LRRK2 inhibitor H1152 was solved (Gilsbach et al., 2012). This shows that Roco4 can be used as an important tool to biochemically and structurally characterize LRRK2 inhibitor binding in more detail (Gilsbach et al., 2012). Furthermore, Roco4 structures will allow the building of a reliable model of LRRK2 for computer-aided drug development, while the biochemical tractability of Roco4 allows the *in vitro* screening of inhibitor libraries.

STRUCTURAL CHARACTERIZATION OF THE Roc-COR TANDEM

So far, the function of the Roc domain of LRRK2 is not completely understood. However, it has been shown that the G-domain of LRRK2 functions as a bona fide GTP-binding protein and that GTP binding is essential for the regulation of kinase activity (Taymans, 2012; Biosa et al., 2013). Harvey and Kirsten already showed the oncogenic effect of mutated Ras in the 1960s, and since the function of small G-proteins has been extensively studied (Vetter and Wittinghofer, 2001; Bos et al., 2007; Csépányi-Kömi et al., 2012). G-proteins switch between an active GTP- and inactive GDP-bound state. G domains, including Roco proteins, contain the five highly conserved motifs, G1–G5, which are responsible for nucleotide binding. The G1 motif, also called p-loop, is essential for the binding of the α- and β-phosphate of the nucleotide, as well as for the interaction with a magnesium-ion in the nucleotide binding pocket. G-domains have a universal switch mechanism that carries out the basic function of nucleotide binding and hydrolysis (Vetter and Wittinghofer, 2001). The structures of the GDP- and GTP- bound state of Ras only differ in the switch regions, which are in an active and inactive conformation, respectively

(Vetter and Wittinghofer, 2001). Despite this small conformational change, only GTP-bound G-protein has a high affinity for effector proteins.

In Roco family members, the G-domain always occurs in tandem with the COR domain. There are two crystal structures comprising the Roc G-domain available: one structure of the LRRK2 Roc domain and one of the Roc-COR tandem of the Roco protein from *Chlorobium tepidum* (Deng et al., 2008; Gotthardt et al., 2008). Surprisingly, the structure of the LRRK2 Roc domain revealed a swapped dimer: in which the N-terminal part of one G-domain interacts with the C-terminal of the other, thereby forming a constitutive dimer (Deng et al., 2008). In contrast, the Roc domain in the *C. tepidum* RocCOR dimer structure shows the typical small G protein fold with six β-strands and helices on both sides and an additional N-terminal helix, termed α_0-helix (**Figure 3**). The COR domain consists of two parts: the highly conserved N-terminal part interacts with the Roc domain and the less conserved C-terminal part functions as dimerization device. It seems rather unlikely that the human RocCOR tandem has a different folding than that of the bacterial Roco protein. Importantly, an overlay of the human Roc and the bacterial RocCOR structure revealed major clashes of the highly conserved N-terminal part of the COR domain with the swapped Roc dimer [**Figure 3**, (Gotthardt et al., 2008)]. Furthermore, Deng et al. (2008) could not convincingly show dimer formation of the Roc domain in solution, while Liao et al. (2014) showed that human Roc forms primarily a monomer in solution with low GTPase activity. (Deng et al., 2008; Liao et al., 2014) Together, this strongly suggest that, like all previously observed swapped G-protein structures (Chavas et al., 2007), the LRRK2 Roc structure is a crystallographic artifact.

REGULATION OF THE G-DOMAIN AND EFFECT OF PD-MUTATIONS

The switch between the active and inactive state of small G proteins is dependent on regulatory proteins. Small G-proteins have a very high nucleotide affinity (nM–pM range), GEFs reduce this affinity by many orders and thereby promote nucleotide release. This subsequently facilitates binding of GTP, which is present in about 30-fold excess over GDP in the cytosol of the cell (Bernards and Settleman, 2004). The intrinsic GTPase activity of small G-proteins is extremely low; therefore GTPase activating proteins (GAPs), which increase the intrinsic GTPase activity by a thousand fold or more, are necessary to switch the protein off (Scheffzek, 1997; Bernards and Settleman, 2004).

There are a few reports describing GAPs and GEFs for LRRK2. Surprisingly none of these putative regulators directly bind to the Roc domain (Stafa et al., 2012; Xiong et al., 2012; Häbig et al., 2013). LRRK2 and all Roco proteins studied so far have a much

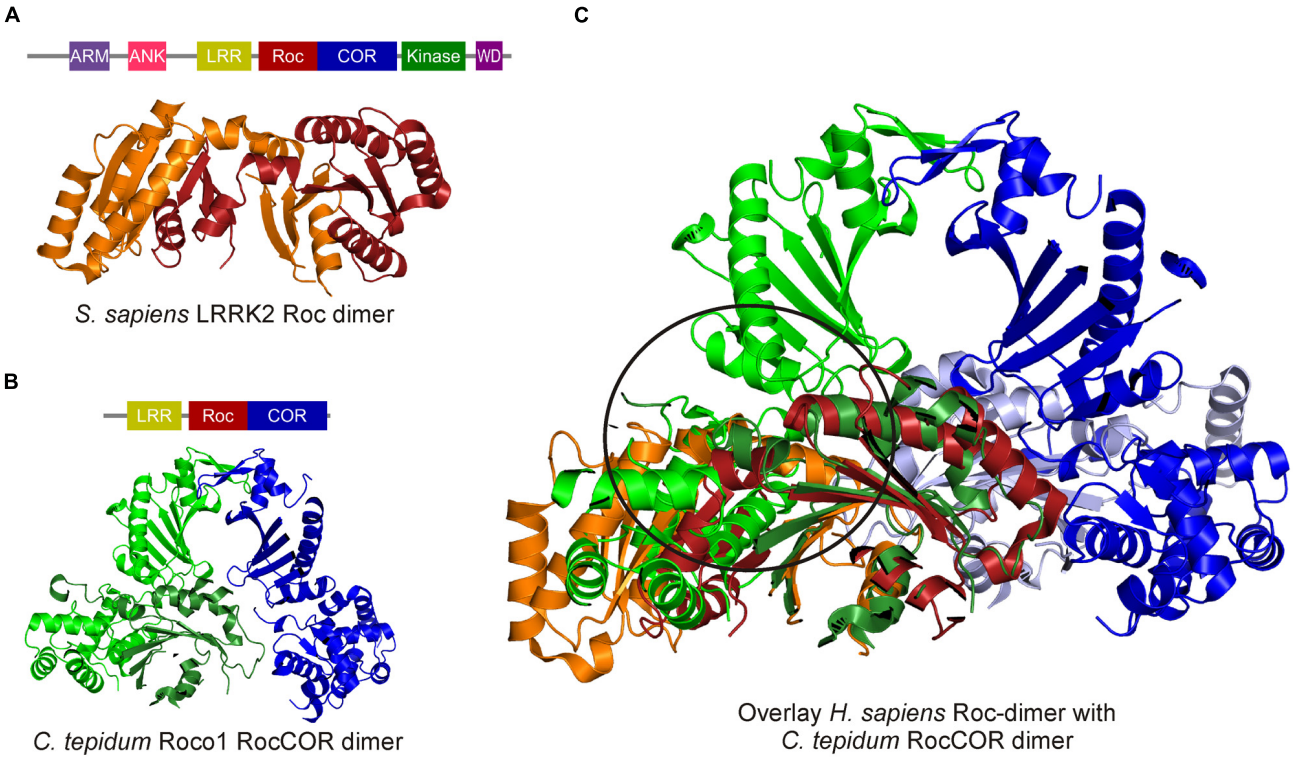

FIGURE 3 | Crystal structures of the human swapped Roc dimer and the *C. tepidum* RocCOR dimer. (A) Human Roc Dimer depicted as a cartoon with Roc-A in orange and Roc-B in red. Above the domain representation of LRRK2 is shown. **(B)** Domain representation of *C. tepidum* and below a cartoon representation of the *C. tepidum* RocCOR structure with RocCOR-A in green and COR-B in blue. **(C)** Overlay of the two structures Roc-A (orange) of the human protein clashes with the N-terminal part of the *C. tepidum* COR-A (green). [PDB: 2ZEJ (human Roc), 3DPU (C. tepidum RocCOR)].

lower nucleotide affinity (μM range) compared to other small G-proteins and are therefore not strictly dependent on GEFs for activation (Ito et al., 2007; Gotthardt et al., 2008; Liao et al., 2014). However, in some transient responses, as previously shown for *Dicyostelium* Roco1, additional stimulation of the already high intrinsic nucleotide exchange rate by GEFs might be required. It is well known that LRRK2 and other Roco proteins are active as a dimer (Greggio et al., 2008; Berger et al., 2010).

The *C. tepidum* RocCOR structure showed that COR is the dimerization device and that Roco proteins that are not able to dimerize, are subsequently also not able to hydrolyze GTP (Gotthardt et al., 2008). This suggest that Roco proteins belong to the GAD class of molecular switches (G proteins activated by nucleotide dependent dimerization; Gotthardt et al., 2008). Important proteins such as signal recognition particle, dynamin and septins also belong to this class of G-proteins (Gasper et al., 2009). GADs possess usually low nucleotide affinity and the stimulation of the low GTPase activity is completely dependent on dimerization. Consistently, the hydrolysis rate of the monomeric LRRK2 Roc domain is more than 700-fold slower than that of dimeric full-length LRRK2 (Liu et al., 2010; Liao et al., 2014). In GADs, stimulation of GTPase activity is accomplished by nucleotide dependent dimerization; within the complex, one monomer completes the catalytic machinery of the other monomer (Gasper et al., 2009). *C. tepidum* Roco uses, like classical Ras-GAPs, an Arginine finger that is essential for stimulating GTP hydrolysis in the neighboring Roc domain (Gotthardt et al., 2008).

Two common PD-related mutations have been found in the RocCOR domain: Arg1441 with multiple substitutions (Cys/Gly/His) in the Roc domain and Tyr1699Cys in the COR domain (Zimprich et al., 2004). Due to the lack of stable purified recombinant protein it has been so far a challenge to study if these mutations affect the GTPase activity of LRRK2. However, recent data strongly suggest that both the PD mutations in the Roc and the COR result in decreased GTPase activity (Lewis et al., 2007; Li et al., 2007). Importantly, the structure of the *C. tepidum* Roco protein showed that the PD-analogous mutations of the Roc and COR domains are in close proximity to each other at the dimer interface and most likely alter the interaction in the dimer between the Roc and COR domains (Gotthardt et al., 2008). Furthermore, these mutations are present in a region of the protein that is strongly conserved between bacteria and man. Subsequently, the Arg1441Cys and Tyr1699Cys PD mutations, as well as the PD-analogous mutations in the *C. tepidum* protein, do not affect nucleotide binding, but significantly decrease GTPase activity (Guo et al., 2007).

STRUCTURE OF THE N- AND C-TERMINUS OF LRRK2

In addition to the central core of the protein, Roco proteins contain a large variety of additional C- and N-terminal domains. The N- terminal part of LRRK2 consists of ARM, ANK, and LRR, while a WD40 domain is present at the C-terminus of LRRK2 (Cardona et al., 2014). All these domains are commonly found in signaling proteins, in which they have often a role in protein–protein interaction. Although there are no structures of the N- or C-terminal LRRK2 domains available, these protein–protein domains have a highly conserved fold (Cardona et al., 2014). ARM repeats are about a 42 amino acid long tandem repeat that form a super-helical bundle (Tewari et al., 2010). One mutation within the ARM domain (Glu334Lys) is associated with PD. *In silico* modeling predicts that this mutation changes the electrostatic surface of the domain (Cardona et al., 2014). ANK contains seven repetitive motifs which form helix-loop-helix structures that end in a loop or hairpin (Mosavi and Cammett, 2004). Modeling of the PD-related Pro755Leu and Arg793Met mutants predict that these mutations would affect protein stability or the electrostatic surface, respectively (Cardona et al., 2014). LRRs are made of an 11 amino acid long conserved motif LxxLxLxxNxL (Leucines can be replaced by isoleucine, valine or phenylalanine). These repeats form a parallel β-sheet and end with a α-helix. Multiple LRR repeats together form a characteristic horseshoe like structure (Bella et al., 2008). The recombinant purified LRR domain of LRRK2 is monomeric in solution and PD variants in the LRRs do not alter the overall folding of the protein, suggesting that most likely these mutation affect inter- and intramolecular interactions (Vancraenenbroeck et al., 2012). The LRR domains of LRRK2 and *Dictyostelium* Roco4 are essential for function *in vivo*, but are not required for kinase activity *in vitro* (Iaccarino et al., 2007; van Egmond and van Haastert, 2010). This suggests that the LRRs most likely determine the specificity of the protein by interacting with an upstream activator or downstream target of the protein. Previously, it was shown that the N-terminus of LRRK2 binds in a phosphorylation-dependent manner to 14-3-3 proteins (Nichols et al., 2010). Inhibiting phosphorylation of two LRRK2 residues, Ser910 and Ser935, disrupts binding to 14-3-3 and subsequently leads to strong defects in LRRK2 signaling; the protein is delocalized and accumulates in inclusion-like bodies instead of being transported to the cell membrane (Dzamko et al., 2010).

WD40 domains form a seven-blade propeller-like structure. Each propeller blade is made of four antiparallel β-strands (Xu and Min, 2011). WD40 domains usually have a highly hydrophilic surface and are often involved in membrane binding. Disruption of the LRRK2 WD40 domain results in abolished dimer formation, impaired kinase activity and aberrant protein localization (Jorgensen et al., 2009). Furthermore, the Gly2385Arg PD risk factor mutation causes a decrease in kinase activity and loss of 14-3-3 binding to the N-terminus (Rudenko et al., 2012). Together these results suggest an important role for the WD40 domain in the intramolecular regulation of LRRK2 activity.

LRRK2 ACTIVATION MODEL

Altogether, the structural and biochemical data suggest that LRRK2 activity is regulated by at least two different mechanisms: intramolecular activation and binding of input/substrate to the N- and C- terminal domains (**Figure 4**). The COR domain functions as a dimerization device. Within the dimer, the Roco G-domains are flexible in the GDP-bound inactive state, but in the active form the G-domains come in a more fixed state in close proximity to each other. This conformational change is transmitted to other parts of the protein, which subsequently allows the activation loops of the two kinase protomers to be autophosphorylated and activated. The GTPase reaction is also critically dependent on

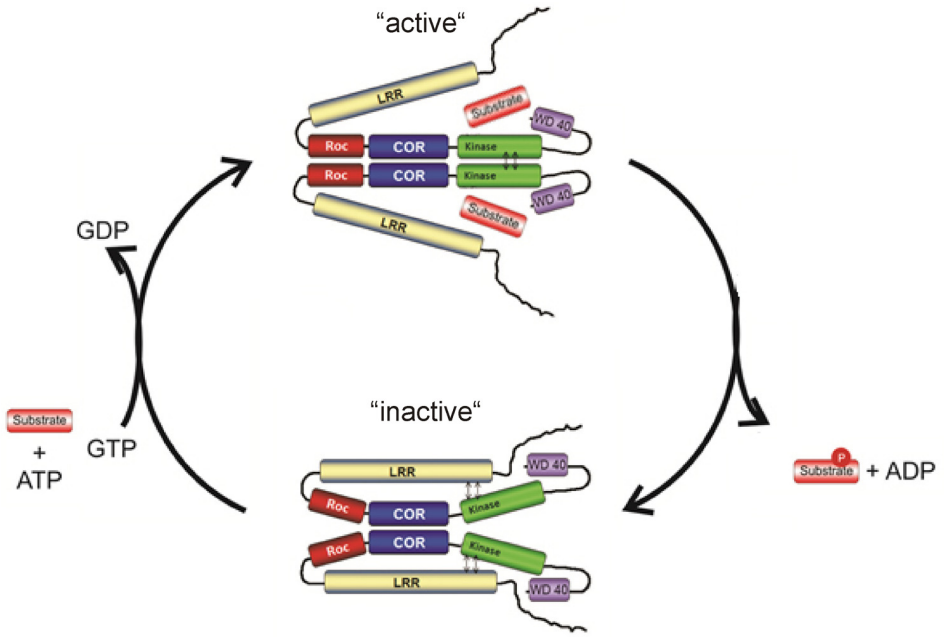

FIGURE 4 | Working model for the complex LRRK2 activation mechanism.

dimerization, because efficient catalytic machinery is formed by complementation of the active site of one protomer with that of the other protomer. In this way the intramolecular GTPase reaction functions as a timing device for the activation and biological function of Roco proteins. The N- and C-terminal segments of LRRK2 regulate this intramolecular signaling cascade and are important for kinase activity, oligomerization, and/or localization. Most likely the N- and C-terminal protein-protein interaction domains are directly binding upstream proteins and/or downstream effector proteins and thereby determine the specificity of the Roco proteins.

CONCLUSION

The multiple allosteric and enzymatic functions within one protein make LRRK2 an excellent therapeutic target. So far the major focus has been to develop kinase domain inhibitors as potential PD therapeutics. However, most of the specific LRRK2 inhibitors lead to kidney and lung abnormality. Furthermore, an increased kinase activity has only been thus far reported for G2019S. All other pathogenic mutations show inconsistent, modest or no effect on kinase activity. Importantly, the Roco structures show that PD-mutations have different defects in the LRRK2 activation mechanism. Therefore, alternative approaches that target other domains of LRRK2, including LRRK2, localization, dimerization, or allosteric modulation of the kinase domain may have significantly improved therapeutic benefits. To fully explore these potential targets more knowledge about the complex intramolecular activation mechanism of LRRK2, upstream and downstream regulators, and the cellular function of LRRK2 is needed. A high-resolution structural map of LRRK2 and related Roco proteins is essential in this enterprise.

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In silico, *in vitro* and cellular analysis with a kinome-wide inhibitor panel correlates cellular LRRK2 dephosphorylation to inhibitor activity on LRRK2

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Leucine-rich repeat kinase 2 (LRRK2) is a complex, multidomain protein which is considered a valuable target for potential disease-modifying therapeutic strategies for Parkinson's disease (PD). In mammalian cells and brain, LRRK2 is phosphorylated and treatment of cells with inhibitors of LRRK2 kinase activity can induce LRRK2 dephosphorylation at a cluster of serines including Ser910/935/955/973. It has been suggested that phosphorylation levels at these sites reflect LRRK2 kinase activity, however kinase-dead variants of LRRK2 or kinase activating variants do not display altered Ser935 phosphorylation levels compared to wild type. Furthermore, Ser910/935/955/973 are not autophosphorylation sites, therefore, it is unclear if inhibitor induced dephosphorylation depends on the activity of compounds on LRRK2 or on yet to be identified upstream kinases. Here we used a panel of 160 ATP competitive and cell permeable kinase inhibitors directed against all branches of the kinome and tested their activity on LRRK2 *in vitro* using a peptide-substrate-based kinase assay. In neuronal SH-SY5Y cells overexpressing LRRK2 we used compound-induced dephosphorylation of Ser935 as readout. *In silico* docking of selected compounds was performed using a modeled LRRK2 kinase structure. Receiver operating characteristic plots demonstrated that the obtained docking scores to the LRRK2 ATP binding site correlated with *in vitro* and cellular compound activity. We also found that *in vitro* potency showed a high degree of correlation to cellular compound induced LRRK2 dephosphorylation activity across multiple compound classes. Therefore, acute LRRK2 dephosphorylation at Ser935 in inhibitor treated cells involves a strong component of inhibitor activity on LRRK2 itself, without excluding a role for upstream kinases. Understanding the regulation of LRRK2 phosphorylation by kinase inhibitors aids our understanding of LRRK2 signaling and may lead to development of new classes of LRRK2 kinase inhibitors.

Keywords: docking, MOE, LRRKtide, Parkinson's disease, kinase, phosphorylation, inhibitor, receiver operator characteristic

INTRODUCTION

Leucine-rich repeat kinase 2 (LRRK2) is a 2527 amino-acid long complex multidomain protein which is a member of the ROCO protein family. This family of proteins is derived from a signature homologous region including a domain encoding for a GTPase of the Ras family, termed ROC (for Ras of complex proteins) (Taymans, 2012), followed by a characteristic COR (C-terminal of ROC) domain. The ROC-COR bidomain is flanked C-terminally by a kinase domain and a WD40 domain and N-terminally by an armadillo repeat domain (ARM), ankyrin repeat domain (ANK), and the namesake leucine-rich repeat (LRR) domain. LRRK2 has primarily been studied for its role in Parkinson's disease (PD) (Cookson, 2010), but is also reported to play a role in cancer, Crohn's disease, and leprosy (Lewis and Manzoni, 2012). LRRK2 is the single most prevalent genetic cause of PD known to date (Paisan-Ruiz et al., 2008). Together with alpha-synuclein, LRRK2 has been both linked to familial PD and associated to sporadic PD (Singleton et al., 2013). Also, PD patients carrying the LRRK2 mutations show a clinical and neuropathological profile which is indistinguishable from sporadic PD, indicating that LRRK2 may contribute to a PD pathway common to both familial and sporadic PD (Healy et al., 2008).

The kinase activity of LRRK2 has been proposed as a promising target for developing disease modifying therapy for PD (Greggio and Singleton, 2007; Vancraenenbroeck et al., 2011; Lee et al., 2012) and deletion of LRRK2 kinase activity has been shown to be protective in cellular (Greggio et al., 2006; Smith et al., 2006) or *in vivo* models (Lee et al., 2010; Yao et al., 2013) of LRRK2 mediated toxicity. Currently, several compounds have been reported that are capable of inhibiting LRRK2 kinase activity (reviewed previously; Vancraenenbroeck et al., 2011; Deng et al., 2012; Kramer et al., 2012). Of these examples, staurosporine,

K252A, and sunitinib are promiscuous kinase inhibitors, known to bind several classes of kinases. Other described compounds are active on specific classes of kinases such as Ro-31-8220, H1152, and Y-27632 (Davies et al., 2000; Bain et al., 2007). Recently, several inhibitors for LRRK2 with an *in vitro* potency in the low nanomolar range have been described including LRRK2-IN1 (Deng et al., 2011), CZC-25146 (Ramsden et al., 2011), TAE684 (Zhang et al., 2012), GSK2578215A (Reith et al., 2012), or HG-10-102-01 (Choi et al., 2012). These compounds are currently being implemented as tool compounds in basic research studies on LRRK2 and indicate the feasibility of developing LRRK2 inhibitors for other applications such as implementation as an imaging tracer or clinical testing.

One key question in assessing LRRK2 kinase inhibitors for these various applications involves understanding the molecular consequences of kinase inhibition in cells. Some clues are given recently from the effects of various inhibitors on the phosphorylation state of LRRK2 in cells. LRRK2 is a highly phosphorylated protein in cells with a notable cluster of phosphorylation sites in the interdomain region between the ANK and LRR domains, including sites Ser910/S935/S955/S973 (West et al., 2007; Gloeckner et al., 2010; Nichols et al., 2010; Lobbestael et al., 2012). Interestingly, these sites are dephosphorylated in cells or tissues following treatment by inhibitors of LRRK2 kinase activity (Dzamko et al., 2010; Choi et al., 2012; Doggett et al., 2012). It is tempting to conclude from these studies that phosphorylation levels at these sites reflects LRRK2 kinase activity, however kinase-dead variants of LRRK2 (K1906M or D2017A) or kinase activating variants (G2019S, T2031S) do not display altered Ser935 phosphorylation levels compared to wild type (Nichols et al., 2010; Lobbestael et al., 2013). Furthermore, Ser910/935/955/973 are not autophosphorylation sites but are rather sites phosphorylated by other kinases (West et al., 2007; Dzamko et al., 2010; Gloeckner et al., 2010; Nichols et al., 2010; Doggett et al., 2012), therefore, it is unclear if inhibitor induced dephosphorylation of LRRK2 wildtype depends on the activity of compounds on LRRK2 or on yet to be identified upstream kinases. We have recently shown that LRRK2 regulates its own dephosphorylation through protein phosphatase 1, including dephosphorylation induced by the LRRK2 kinase inhibitor LRRK2-IN1 (Lobbestael et al., 2013). It remains to be verified that LRRK2 inhibitor-induced dephosphorylation is generalized across multiple chemical classes and whether dephosphorylation is correlated to inhibitor binding to LRRK2 kinase.

In the present study, we addressed these issues using a chemical biology approach. A panel of cell permeable kinase inhibitors targeting all branches of the kinome was tested for its activity on LRRK2 *in vitro* as well as in cells. Using an optimized LRRK2 kinase homology model, selected compounds were docked *in silico* to assess binding at the ATP-binding site.

RESULTS

TESTING OF A KINASE INHIBITOR PANEL ON LRRK2 *IN VITRO* KINASE ACTIVITY

The assay employed here is based on phosphorylation of a peptide termed LRRKtide derived from the cytoskeleton-associated moesin protein (Jaleel et al., 2007) and is adapted to a phosphor imaging readout (Asensio and Garcia, 2003; Taymans et al., 2011), as described in Materials and Methods and shown in **Figure 1**. The quality of the chosen assay is given by the average Z′ factor for this assay which we determined to be 0.82 (**Figure 2**), well within the range of 0.5–1 which is considered an excellent value for screening assays (Zhang et al., 1999). A panel of 160 kinase inhibitors was tested in the LRRK2 *in vitro* kinase activity assay using GST-LRRK2$_{970-2527}$ at one concentration (10 μM) (**Figure 1**, quantifications given in **Table 1**). Of these 160 compounds, 35 compounds from three compound classes (A) 9-methyl-N-phenylpurine-2,8-diamine, (B) N-phenylquinazolin-4-amine, and (C) 1,3-dihydroindol-2-one analogs were selected for further testing in the *in vitro* assay as well as for *in silico* analysis (see further). These three compound classes were chosen because they contain compounds with a common core or scaffold possessing a wide variety of activities. Additionally, known LRRK2 reference compounds were tested including LRRK2-IN1, CZC-25146 and the pan-kinase inhibitor staurosporine as well as compound CDK1/2 inhibitor III which displayed highest potency in inhibiting LRRK2. The selected compounds were retested with full length recombinant enzyme at 100 μM, 10 μM, 1 μM and 100 nM. For those compounds that inhibited LRRK2 kinase activity more than 50% at 1 μM, a pIC$_{50}$ [$= -\log(\text{IC50})$] value was determined as described in Materials and Methods (**Figure 2** and **Table 2**). It can be noted that for known LRRK2 inhibitory compounds that we included in our testing, our adapted LRRKtide phosphorylation assay yielded similar potencies to those previously published (comparative examples of obtained IC50 values are: LRRK2-IN1 3.54 nM vs. 13 nM (Deng et al., 2011), CZC-25146 1.78 nM vs. 4.76 nM (Ramsden et al., 2011), with values given from this study and from published studies, respectively; for the LRRK2-IN1 comparison, the slightly lower value obtained here may be due to the ATP concentrations used which are 10 μM in the present study and 100 μM in the original study).

TESTING OF KINASE INHIBITOR PANEL IN LRRK2 CELLULAR PHOSPHORYLATION ASSAY

Using the same panel of 160 kinase inhibitors, cellular activity was assayed by monitoring dephosphorylation of LRRK2 at Ser935 induced by kinase inhibitor treatment (5 μM for 2 h) of the SH-SY5Y cell line with stable expression of LRRK2 as described in Materials and Methods and shown in **Figure 3**. The mean Z′ factor for the dual detection immune-dotblot assay used here is 0.65 (**Figure 4**). A total of 20 compounds were found to reduce Ser935 phosphorylation levels to less than 50% of control levels (**Tables 1, 2**), all are ATP-binding site competitive compounds. None of the 20 non ATP-competitive compounds of the panel (see Materials and Methods) induce more than 50% dephosphorylation of LRRK2 at 5 μM although AG490 shows 49.32% dephosphorylation of LRRK2 at 5 μM. Representative dot blot images and bar diagrams are depicted in **Figure 3**, exact quantifications are given in **Table 1**. For the selected compounds, the IC50 was determined (**Figure 4** and **Table 2**).

CORRELATION BETWEEN *IN VITRO* AND CELLULAR ACTIVITY OF COMPOUNDS

Correlations between the *in vitro* and cellular activities for each compound were investigated by drawing up correlation plots for

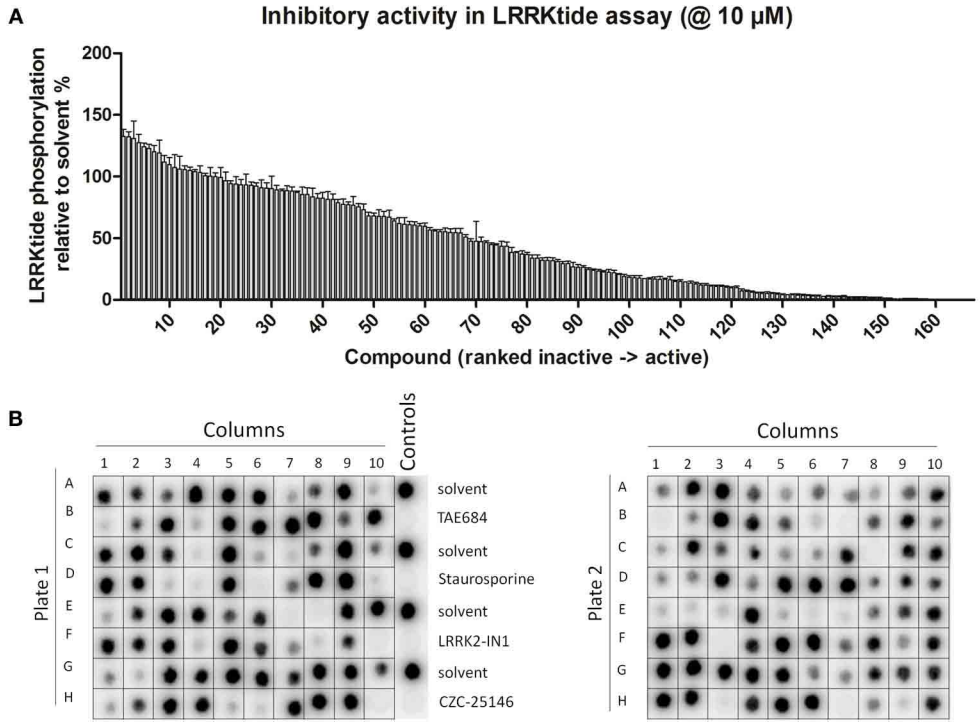
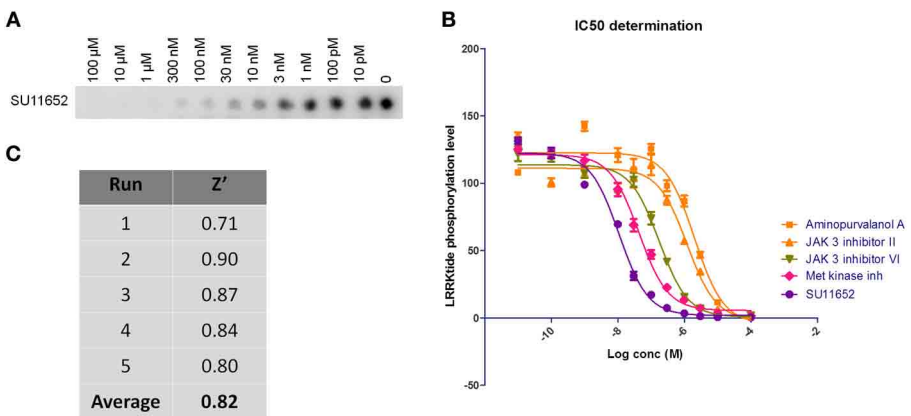


FIGURE 1 | Testing of effect of reference kinase inhibitors on *in vitro* LRRK2 kinase activity. (A,B) 160 kinase inhibitors from a panel of inhibitors known to target kinases in all branches of the kinome were tested for their ability to inhibit LRRK2 at 10 µM in an *in vitro* kinase assay using the LRRKtide model peptide substrate, as described in Materials and Methods. **(A)** Quantification of the LRRKtide phosphorylation level for each kinase reaction. Signal intensity per reaction was quantified via densitometry as described in Materials and Methods and values are normalized to phosphorylation levels measured in solvent controls (control values are set at 100%). Values obtained (mean ± s.e.m., $N = 3$) are depicted as histogram bars ordered from least active to most active compound, showing that the panel comprises a broad range of activity on LRRK2 kinase function. Exact values are given in **Table 1**. **(B)** Representative autoradiograms of P81 paper spotted with kinase reactions from testing of the 160 compounds which were used for densitometric quantification given in A. Also shown at the right of the first panel are the solvent controls as well as positive controls using potent inhibitors of the LRRK2 kinase, TAE684, staurosporine, LRRK2-IN1, and CZC-25146. Illustration of detailed IC_{50} determination for active compounds is given in **Figure 2**.



Run	Z′
1	0.71
2	0.90
3	0.87
4	0.84
5	0.80
Average	**0.82**

FIGURE 2 | Dose-response curves and Z′ factor determination for *in vitro* LRRKtide assay. (A,B) Detailed IC_{50} determinations were performed for selected active compounds by testing LRRKtide phosphorylation in the presence of a range of inhibitor doses, as described in Materials and Methods. A representative autoradiogram for compound SU11652 is given **(A)** as well as the fitted inhibition curves obtained for five compounds **(B)** *In vitro* IC50 values for each tested compound are given in **Table 2**. **(C)** LRRKtide phosphorylation values were used to calculate the Z′ of the *in vitro* LRRKtide phosphorylation assay using the formula given in Materials and Methods.

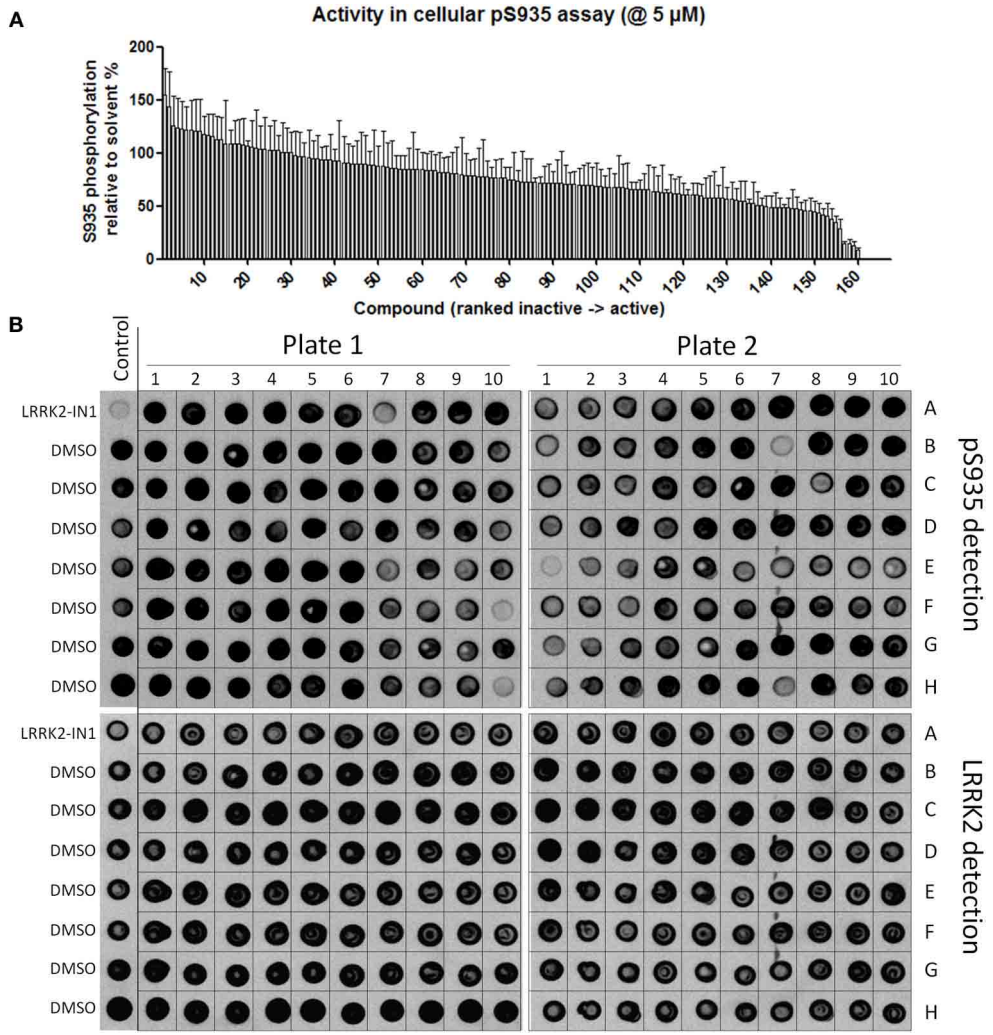


FIGURE 3 | Testing of effect of reference kinase inhibitors on cellular LRRK2 pS935 levels. 160 kinase inhibitors from a panel of inhibitors known to target kinases in all branches of the kinome were tested for their ability to dephosphorylate LRRK2 at phosphoserine 935 in a spotblot assay, as described in Materials and Methods. **(A)** Quantification of the cellular phosphorylation level at Ser935 for each compound and ranked from least to most active (exact values per compound can be found in **Table 1**). **(B)** Representative spot blots detecting phospho-LRRK2 (pS935 detection in upper panels) and total-LRRK2 (LRRK2 detection in lower panels) for 160 kinase inhibitors tested at 5 µM. Illustration of detailed IC_{50} determination for active compounds is given in **Figure 4**.

these two parameters and performing linear regression analysis as described in Materials and Methods (**Figure 5**). This analysis showed a significant correlation between *in vitro* and cellular activity (with Pearson's r coefficient of −0.7953).

LRRK2 KINASE STRUCTURAL MODEL

We constructed, optimized and quality improved a 3D homology model of the LRRK2 kinase domain as described in detail in Materials and Methods. Based on their sequence identity with the LRKK2 kinase domain, the tyrosine-kinase like (TKL) kinases B-Raf (PDB 3OG7; Bollag et al., 2010), MLK1 (PDB 3DTC; Hudkins et al., 2008), and IRAK-4 (PDB 2NRU; Wang et al., 2006) were selected as templates to model LRRK2 kinase (see **Table 3** for an overview of TKL kinases with available 3D structures and their sequence identity with the LRRK2 kinase domain). The alignment between the LRRK2 kinase domain and these three kinases is given in **Figure 6A**. The final homology model colored by conserved kinase motifs is shown in **Figure 6B**; the final model colored by quality of each predicted amino-acid position is given in **Figure 6C** and was determined as described in the Materials and Methods section. The ATP-binding groove lies at the interface of the N-and C-terminal lobes (Huse and Kuriyan, 2002; Nolen et al., 2004).

IN SILICO ANALYSIS OF LRRK2 KINASE—LIGAND INTERACTIONS

A preliminary docking step, where staurosporine was docked in the LRRK2 ATP-binding site, was applied to optimize the local environment to get the most optimal binding pose during the subsequent docking step. 35 compounds from three compound classes (A) 9-methyl-N-phenylpurine-2,8-diamine, (B)

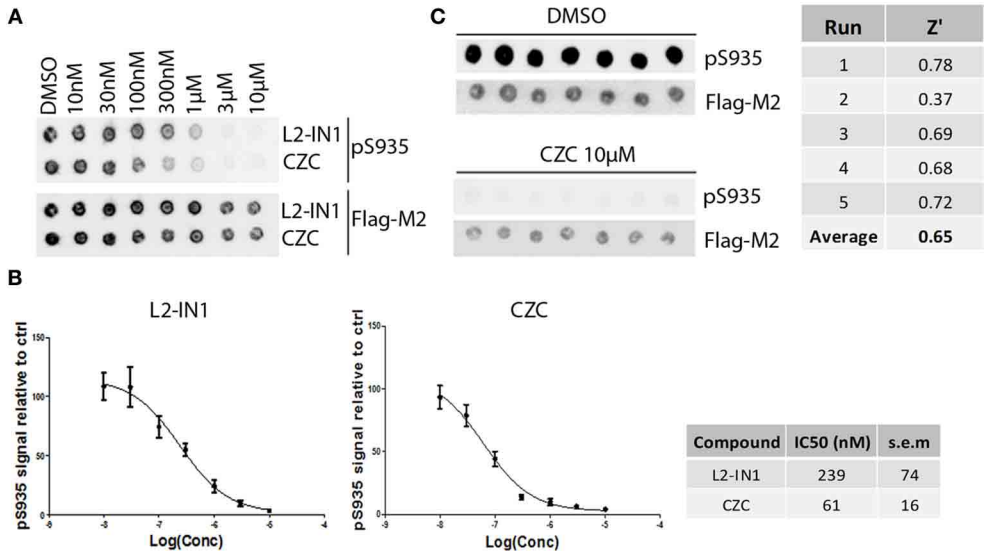


FIGURE 4 | Dose-response curves and Z′ factor determination for Ser935 cellular dephosphorylation assay. (A) Representative spotblot of cell lysates of SH-SY5Y stably overexpressing 3flag-LRRK2 WT and treated with a dose range of reference compounds LRRK2-IN1 or CZC using concentrations as indicated. DMSO treatment was included as a control. Total LRRK2 protein was detected with Flag-M2 antibody, phosphorylation at Ser935 with a monoclonal LRRK2 PS935 antibody. **(B)** Fitting of inhibition curves based on spot intensity in **(A)** (determined by densitometric analysis and used to quantify phosphorylation levels relative to total LRRK2 levels) and IC50 determination of phosphorylation at LRRK2 Ser935 for reference compounds LRRK2-IN1 and CZC. $N \geq 5$. Cellular pS935 dephosphorylation IC50 values for each tested compound are given in **Table 2**. **(C)** Representative spotblot of cell lysates of SH-SY5Y stably overexpressing 3flag-LRRK2 WT and treated with DMSO or 10 μM CZC. Phosphorylation level values derived from total LRRK2 and phospho-LRRK2 levels quantified via spotblot were used to calculate the Z′ of the pS935 dephosphorylation assay using the formula given in Materials and Methods.

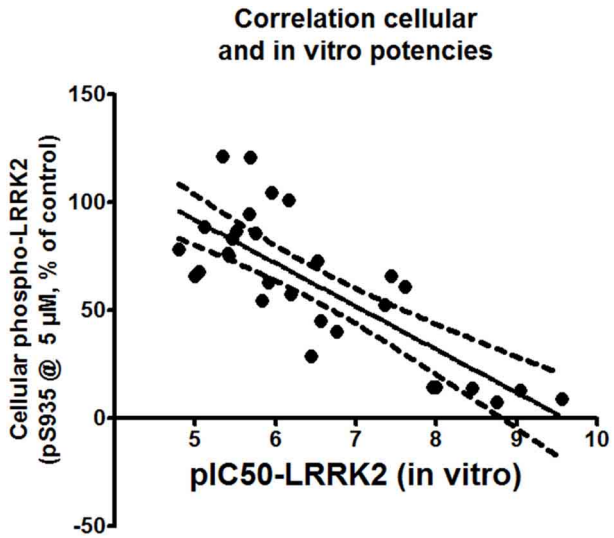


FIGURE 5 | Correlation plot of cellular activity (pS935 spotblot assay) and *in vitro* potency (pIC50 of LRRKtide assay). The assessment is performed for compounds with *in vitro* pIC50 of 5 or better. Exact values of the plotted data are given in **Table 2**.

N-phenylquinazolin-4-amine, and (C) 1,3-dihydroindol-2-one analogs were docked into this active site using three class-specific pharmacophore models (**Figures 7A–C**) as described in detail in Materials and Methods. A summary of the *in silico* docking scores, given as the Generalized-Born Volume Integral/Weighted Surface area dG (GBVI/WSA dG) is given in **Table 2**. To evaluate the correlation between docking and *in vitro* and cellular compound activities, receiver operating characteristic plots describing the trade-off between sensitivity and specificity were constructed using the GVBI/WSA dG computed values and the measured *in vitro* activities in the LRRKtide assay (IC50 of 1 μM or better is scored active) or the measured pS935 cellular dephosphorylation (a measure of greater than 50% dephosphorylation at 5 μM is scored as active). Receiver operating characteristic plots which trace above the diagonal signify docking enrichment, with best docking for those receiver operating characteristic plots furthest above the diagonal. An indicative parameter of the receiver operating characteristic plot is the area under the curve (AUC) with values above 0.5 indicating a valid correlation between *in silico* and measured activity values. The receiver operating characteristic plots were determined for the LRRK2 kinase model as well as the three separate kinase structures which were used as templates for constructing the LRRK2 kinase model. These receiver operating characteristic plots, displayed in **Figures 7D–K** clearly show that of these four models, only docking results obtained with the LRRK2 kinase domain model itself have predictive value for *in vitro* kinase activity and cellular pS935 dephosphorylation activity for at least three different structural classes of LRRK2-active compounds. Illustrative LRRK2 kinase—ligand binding poses for representative active compounds representing the three different classes are given in **Figure 8**.

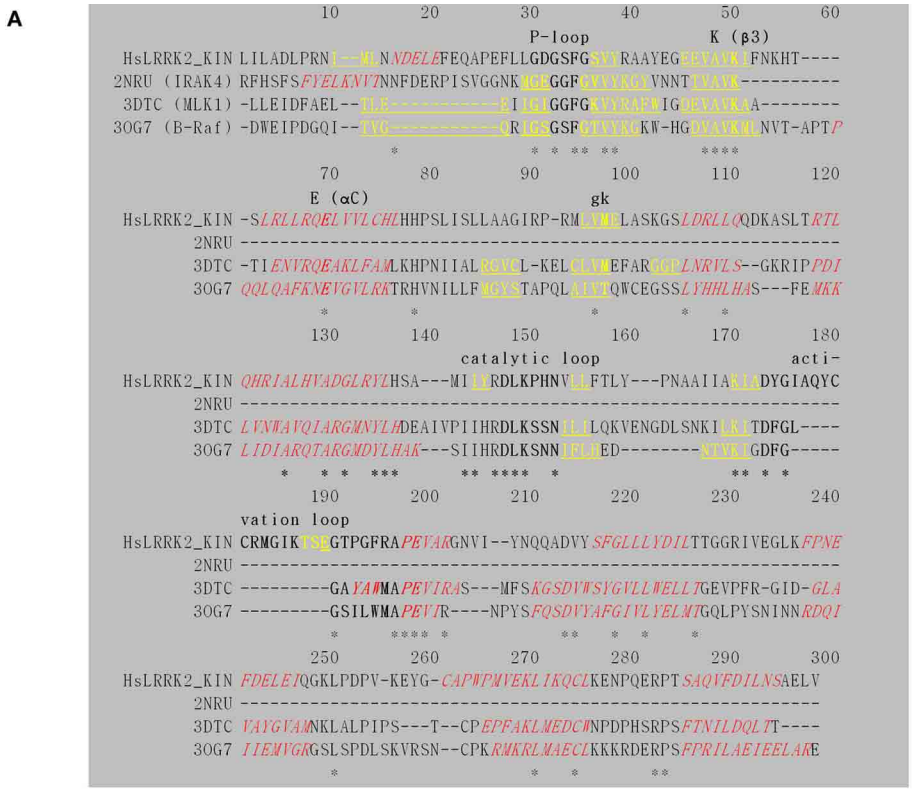


FIGURE 6 | Structural modeling of the LRRK2 kinase domain (residues 1859–2138). (A) Target (the LRRK2 kinase domain)—template (2NRU, 3DTC, and 3OG7) alignment (see **Table 3** for an overview of available structural templates of TKL kinases). Secondary structure elements (predicted for LRRK2 via NetSurfP or obtained from the 3D PDB structure) are indicated: α-helices are red *italic*, and β-strands yellow underlined. The conserved motifs are highlighted in **bold** and labeled. Identical residues are marked with an asterisk (*). **(B)** Illustration of the LRRK2 kinase homology model depicting key residues and functional features. The P-loop is shown in red, the conserved K in β3 and E in αC in green, the catalytic loop in purple, the activation loop in yellow, the gatekeeper (gk), gk + 1, and gk +3 residues from the hinge region in blue. Sticks colored by CPK convention correspond to the residues that are affected by the G2019S and I2020T mutations segregating with PD. G2019S is part of the beginning of the activation loop. **(C)** Graphical representation of potential errors in the homology model as predicted by Meta-MQAPII. The color spectrum from blue to red reflects the accuracy of the 3D residue prediction from correct to incorrect respectively. The spheres indicate the position of the residues in the disallowed and generously allowed regions of the Ramachandran Plot and residues with unfavorable bond angles and dihedrals. Figures generated with PyMol.

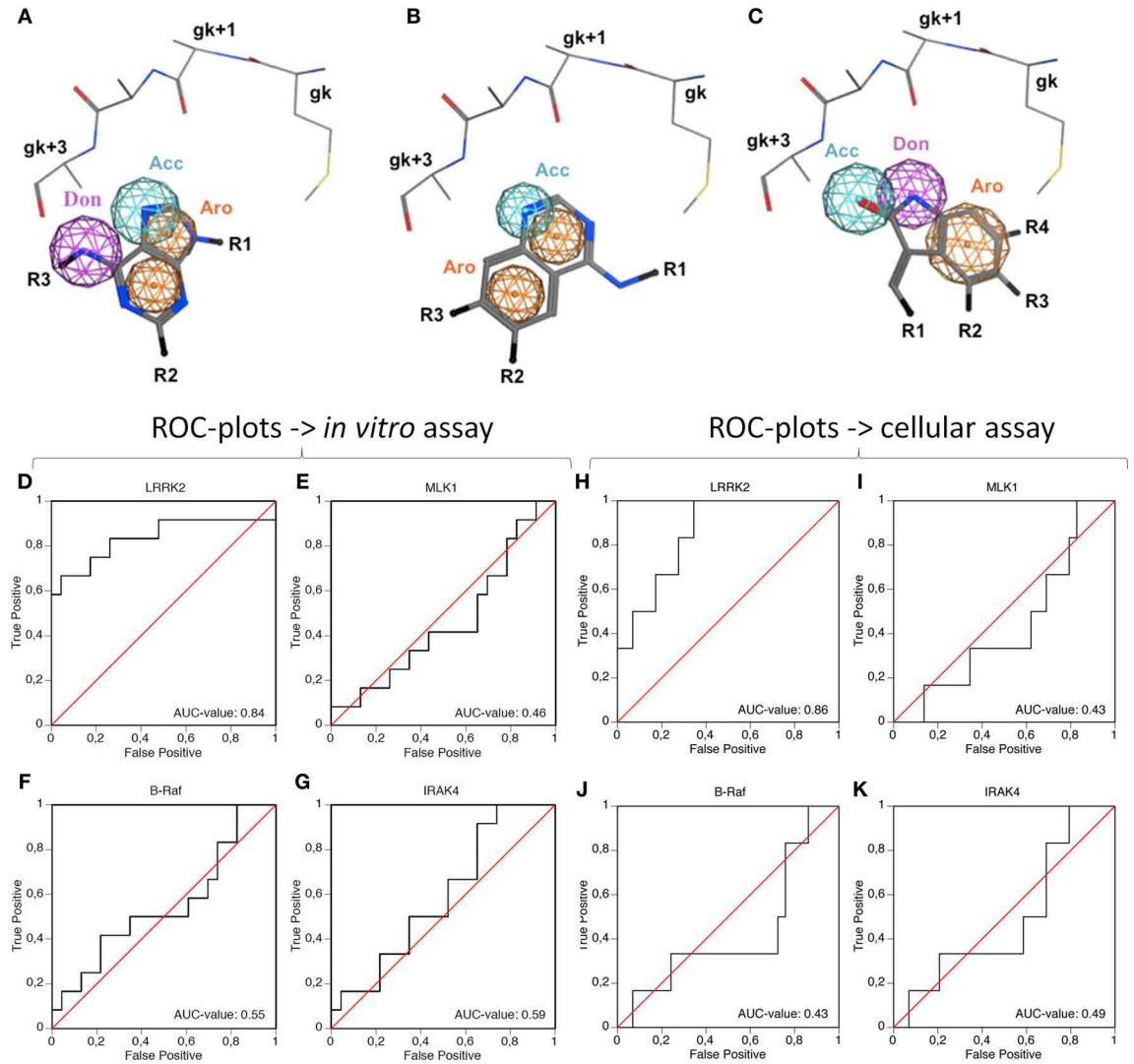


FIGURE 7 | Illustration of the pharmacophore queries used during "pharmacophore filtering" and receiver operating characteristic plots assessing the correlation between inhibitor docking and *in vitro* or cellular activity. (A–C) Pharmacophore queries are illustrated for purine, quinazoline and oxindol derivatives [termed compound classes **(A–C)**, respectively, panels **(A–C)**, respectively]. Shown are the hinge region residues, pharmacophore features and the substructure of the respective class. Only the main chain and Cβ side chain atoms are shown for the gk-1, gk+1, gk+2, gk+3, and gk+4 residues. Depicted pharmacophore features include aromatic rings (orange spheres), hydrogen bond acceptors (cyan spheres), or donors (purple spheres). The substructures of the respective compound classes are shown in stick representation. All molecules are colored by CPK convention. Abbreviations: Acc, acceptor; Aro, aromatic; Don, donor; gk, gatekeeper. Figures generated with MOE. **(D–K)** The receiver operating characteristic plots were determined to evaluate the predictive power of the docking method in the LRRK2 model compared to the separate templates used to generate the LRRK2 model, i.e., B-Raf (3OG7), MLK1 (3DTC), and IRAK-4 (2NRU). Using the activity measures for *in vitro* activity **(D–G)** or cellular activity **(H–K)**, true positive rate (measure for sensitivity) as function of false positive rate (indication of specificity) is plotted. The Area Under Curve (AUC)-value of each receiver operating characteristic plot is indicated on the plot, with an AUC > 0.5 indicating predictive value of the docking. **(D,H)** receiver operating characteristic plots of LRRK2 docking, **(E,I)** receiver operating characteristic plots of MLK1 docking, **(F,J)** receiver operating characteristic plots of B-Raf docking, **(G,K)** receiver operating characteristic plot of IRAK4 docking. Docking scores are given in **Table 2**.

DISCUSSION

The relationships between binding of kinase inhibitors to the LRRK2 kinase pocket, *in vitro* inhibition of LRRK2 kinase activity and inhibitor-induced cellular dephosphorylation of LRRK2 hold clues for understanding LRRK2 signaling and interpreting LRRK2 cellular activity assays. Here, we assessed the interrelationship between these parameters, using *in silico*, *in vitro* and cellular activity assays. Using a panel of 160 reference kinase inhibitors targeting all branches of the kinome, we found a broad range of potencies to inhibit LRRK2 *in vitro* kinase activity, ranging from inactive compounds to compounds inhibiting LRRK2 in the subnanomolar range. Similarly, the panel of kinase inhibitors displayed a broad range of cellular potencies with strongest compounds active in the low nanomolar range. Finally, the picture of activities was completed by determining *in silico* docking of compounds selected from three different structural classes which

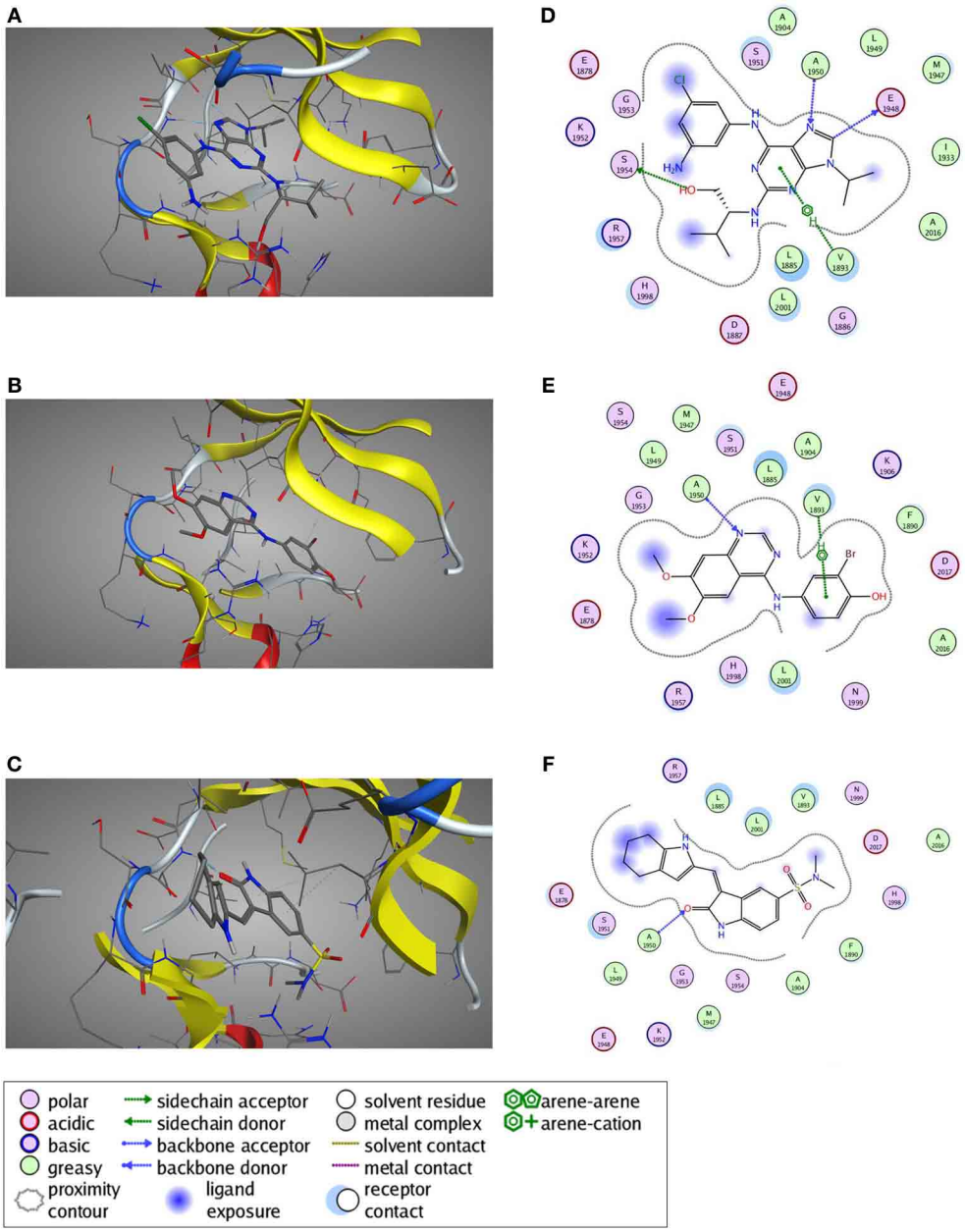


FIGURE 8 | Predicted ligand binding poses. (A–C) Selected active compounds of the three different compound classes are depicted in the LRRK2 kinase ATP-binding pocket. Compounds shown are **(A)** Aminopurvalanol A (purine derivative), **(B)** JAK3 Inhibitor II (quinazoline derivative), and **(C)** SU6656 (oxindole derivative). LRRK2 kinase is given in cartoon and line representation, the ligands in stick representation. The blue dotted lines denote hydrogen bond interactions. The green dotted lines denote π—cation interactions. **(D–F)** Schematic representation of the interactions between the LRRK2 kinase active site and the compound from panels **(A–C)**: **(D)** Aminopurvalanol A, **(E)** JAK3 Inhibitor II, and **(F)** SU6656. See box for the explanation of colors and arrows. Figures generated with MOE.

included compounds with identical scaffolds but varying activities ranging from potent to inactive in the *in vitro* kinase assay and cellular dephosphorylation assay.

The activity assays selected and optimized here are suitable to predict activity of compounds on LRRK2. First, for the *in vitro* activity and cellular activity assays, we used established assays with slight modifications, and we could demonstrate excellent Z'-factors for these assays in our hands (Z' of 0.82 for the *in vitro* assay and of 0.65 for the cellular assay). The results obtained with LRRKtide *in vitro* phosphorylation assay as performed here yielded potencies similar to those reported for compounds tested in previously published studies. Using LRRK2-IN1 as a benchmark, the IC50 value obtained here of 3.54 nM is comparable to the published value (13 nM) (Deng et al., 2011). The same

holds true for the second assay testing cellular activity which is based on kinase inhibitor induced dephosphorylation of LRRK2 at Ser935 and adapted here using a spotblot readout. For instance, for LRRK2-IN1 the IC50 value obtained here (279.9 nM) is in the same range as IC50 values obtained with other readouts of pS935 levels such as western blot (about 100 nM; Dzamko et al., 2010; Deng et al., 2011), time-resolved FRET (90–200 nM; Hermanson et al., 2012), or ELISA (50–100 nM; Delbroek et al., 2013). Thirdly, in the absence of a physical 3D atomic structure of the LRRK2 kinase domain, an optimized 3D homology model was generated for *in silico* docking. Because the available templates with closest homology showed only 30% sequence identity, we took great care in optimizing the model. Homology models of the LRRK2 kinase domain have been constructed before (see for example references; Marin, 2006; Mata et al., 2006; Nichols et al., 2009; Deng et al., 2011; Yun et al., 2011), however here we refined the model extensively for use in molecular docking studies. In particular, alignments between LRRK2 kinase and potential templates were performed using alternative sequence-to-structure alignments guided by conserved residues Also, multiple templates were used to employ the most appropriate template for each structural segment. Finally, the best model was chosen according to the model evaluation rather than the alignment score.

Our results show a strong correlation between the potency of compounds to inhibit LRRK2 *in vitro* kinase activity and potency to dephosphorylate LRRK2 in cells (Pearson's $r = -0.7953$, **Figure 5**). Kinase inhibitor induced dephosphorylation of LRRK2 in cells could be seen for compounds with an *in vitro* IC50 of 10 μM or better. Conversely, the majority of the compounds which were inactive *in vitro* (no activity at 10 μM) were also inactive in cells. This is consistent with the notion that kinase inhibitors induce dephosphorylation through inhibition of LRRK2 kinase activity and/or through binding to the LRRK2 kinase ATP binding pocket. This conclusion is supported by receiver operating characteristic plots generated with the compound docking scores and the *in vitro* potency of each tested compound. It is often observed that in many cases docking based virtual screening performs no better than random selection. Inclusion of pharmacophore methods however has shown to significantly improve the virtual screening performance (Voet et al., 2014). As such we also combine pharmacophore models in our virtual screening setup. Via this approach, docking into our modeled LRRK2 kinase structure is clearly better in discriminating actives from decoys than docking into the three other kinases which were used to generate the LRRK2 model. Therefore, we can conclude that the *in vitro* and cellular activities of the compounds of the three structural classes tested are likely dependent on their binding to the LRRK2 ATP-binding site.

While it is not surprising that LRRK2 kinase activity is inhibited more potently by compounds that bind more tightly to the kinase, this is less evident for the correlation between binding and cellular dephosphorylation of LRRK2. Indeed, kinase inhibition of LRRK2 would be expected to reduce the phosphorylation rate of LRRK2 autophosphorylation sites (Greggio et al., 2009; Gloeckner et al., 2010), however the Ser935 used in the assay is not an autophosphorylation site (Nichols et al., 2010; Doggett et al., 2012; Lobbestael et al., 2012), rather it is a site phosphorylated by other kinases. This has implications for the signaling properties of LRRK2. Indeed, the direct regulation of the phosphorylation/dephosphorylation equilibrium in cells involves two partners, a phosphatase and a kinase. Using the kinase inhibitor signaling panel with broad coverage of the kinome, we expected to identify at least a few compounds which would be inactive on the LRRK2 kinase activity *in vitro*, but active in dephosphorylating LRRK2 as these active compounds would point to the upstream kinases of LRRK2. Looking to **Table 1**, only a handful of *in vitro* inactive compounds (such as LY303511, DNA-PK inhibitor V, Aurora kinase inhibitor 3, BAY11-7082) could affect moderate dephosphorylation of LRRK2 (∼50%). These four compounds are directed against kinases of different branches of the kinome: LY303511 (lipid kinases branch), DNA-PK inhibitor V (atypical kinases branch), Aurora kinase inhibitor 3 (other kinase branch), BAY11-7082 (other kinases branch). Although these compounds were not among the top hits of the study, further characterization of these compounds on their effects in regulating LRRK2 phosphorylation may provide more information on putative upstream kinases of LRRK2. For example, BAY11-7082 is reported to be a specific inhibitor of inducible IκB-alpha phosphorylation (Pierce et al., 1997) which is in line with the finding that IκB-alpha shown to phosphorylate LRRK2 in immune cells (Dzamko et al., 2012). However, none of the other *in vitro* inactive compounds displayed significant dephosphorylation of LRRK2 in cells. Also, it should be noted that full dephosporylation of LRRK2 (>70–80%) was not displayed by any of the *in vitro* inactive compounds and was only observed for the most potent *in vitro* inhibitors of LRRK2.

Taking the overall results into account, we conclude that LRRK2 dephosphorylation involves an important contribution of the activity of compounds on LRRK2 itself. Therefore, the regulation of LRRK2 phosphorylation at Ser935, as well at other sites of this phosphorylation cluster such as Ser910/955/973, involves at least three partners, i.e., a phosphatase, a phosphorylating kinase as well as LRRK2 itself. We recently showed that treatment of cells with the LRRK2 kinase inhibitor LRRK2-IN1 induced LRRK2 dephosphorylation by recruitment of protein phosphatase 1 (PP1) (Lobbestael et al., 2013). Our findings presented here suggest that this is a more generalized phenomenon for LRRK2 kinase inhibitors from multiple kinase classes. The activity of these compounds may possibly require conformational changes of the LRRK2 kinase domain to allow proper binding of the inhibitor. Experimental evidence for this has recently been reported, whereby detection of LRRK2 with a monoclonal antibody targeting the activation loop of the LRRK2 kinase domain was altered upon binding of kinase inhibitors (Gillardon et al., 2013). A compound induced conformational change of LRRK2 is likely to regulate binding affinities between LRRK2 and its cellular interactors and is consistent with our previous observation that PP1 is recruited to LRRK2 under conditions of dephosphorylation (Lobbestael et al., 2013). It remains to be determined whether compounds can be developed which inhibit LRRK2 kinase activity but which do not induce major conformational changes in LRRK2 leading to its dephosphorylation in cells. This has important implications as the pS935 dephosphorylation is not only observed after LRRK2 kinase inhibition (Deng et al., 2011;

Choi et al., 2012; Reith et al., 2012; Zhang et al., 2012), but also in at least some LRRK2 disease mutants (Nichols et al., 2010; Li et al., 2011; Lobbestael et al., 2012; Rudenko et al., 2012), therefore it is not yet clear whether dephosphorylation is a desired effect of a potential PD therapeutic based on LRRK2 kinase inhibition.

In conclusion, we report here the correlations between the *in vitro*, cellular and *in silico* activities of a kinome-wide panel of kinase inhibitors on LRRK2. Our results indicate that cellular LRRK2 dephosphorylation induced by kinase inhibitors involves a strong component of inhibitor activity on LRRK2 itself, without excluding a role for upstream kinases. Understanding the regulation of LRRK2 phosphorylation by kinase inhibitors has implications for cellular activity assays of LRRK2 and may lead to development of new classes of LRRK2 kinase inhibitors.

MATERIALS AND METHODS

IN VITRO KINASE ASSAY

LRRK2 kinase activity was assessed using an isotopic peptide substrate assay essentially as described in reference (Taymans et al., 2011). In short, recombinant LRRK2 was incubated with 6 μCi γ-^{32}P-ATP [3000 Ci/mmol; Perkin Elmer (USA)], 200 μM LRRKtide (RLGRDKYKTLRQIRQ) (Jaleel et al., 2007) [Enzo Life Sciences (USA)], 10 μM ATP and kinase inhibitor (see below) or dimethylsulfoxide (DMSO) solvent per 40 μl reaction in 1× kinase buffer for 30 min at 30°C. The composition of 1x kinase buffer is: Tris 25 mM pH 7.5, $MgCl_2$ 10 mM, dithiothreitol (DTT) 2 mM, Triton 0.02%, beta-glycerophosphate 5 mM, Na_3VO_4 0.1 mM. DMSO content of each *in vitro* kinase reaction was 1 %. For the single dose testing (at 10 μM of kinase inhibitor), the LRRK2 enzyme used was GST-tagged truncated LRRK2 containing residues 970 to 2527 (Life Technologies). Reactions were stopped by the addition of 500 mM EDTA containing bromophenol blue. Reactions were spotted to P81 Whatman phosphocellulose paper (GE Healthcare) and washed four times 10 min in 75 mM phosphoric acid. LRRKtide phosphorylation levels were measured via autoradiography (Asensio and Garcia, 2003).

A commercially available panel of 160 kinase inhibitors (EMD4Biosciences, Inhibitor select panel) was initially screened at one concentration (10 μM). The inhibitor panel contains cell-permeable and previously characterized inhibitors which together target all branches of the kinome. All inhibitors are confirmed cell permeable, with the exception of PKCβ Inhibitor, PKR Inhibitor—Negative Control, Alsterpaullone—2-Cyanoethyl, Cdk1/5 Inhibitor and JNK Inhibitor IX which are of unknown permeability. Compounds in the inhibitor panel are mostly ATP-binding site competitive inhibitors, although 20 compounds are labeled as non ATP-competitive compounds (including 1 allosteric compound). These compounds are: Bcr-abl Inhibitor, AG 490, AG 112, Akt Inhibitor X, AG 1024, Akt Inhibitor V—Triciribine, Akt Inhibitor VIII, Isozyme-Selectiv—Akti-1/2, Chelerythrine Chloride, MEK1/2 Inhibitor, MNK1 Inhibitor, KN-62, Cdk4 Inhibitor II—NSC 625987, ERK Inhibitor III, MK2a Inhibitor, MEK Inhibitor I, Sphingosine Kinase Inhibitor, PD 98059, GSK-3b Inhibitor I, KN-93 and the allosteric inhibitor IGF-1R Inhibitor II. Information on the branch of the kinome targeted is provided together with experimental results in **Table 1**; further details on each inhibitor are available from the supplier. Then, selected molecules were further tested at lower doses. For those selected compounds that were found to inhibit >50% at 1 μM, an IC_{50} (inhibitor concentration yielding 50% inhibition) was determined. To this end, compounds were tested in triplicate with full length LRRK2 protein (Taymans et al., 2011; Civiero et al., 2012) at concentrations of 10 μM, 1 μM, 300 nM, 100 nM, 30 nM, 10 nM, 3 nM, 1 nM, 100 pM, 10 pM, and solvent. An inhibition curve was fitted and IC50s calculated using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). IC50s are expressed as pIC50 [= −log(IC50)].

LRRK2 CELLULAR ACTIVITY AND PHOSPHOSERINE 935 SPOTBLOT DETECTION ASSAY

To assess LRRK2 cellular activity, an SH-SY5Y stable cell line was first generated. SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and 1× non-essential amino acids (Gibco) at 37°C and 5% CO_2. Lentiviral vectors (LVs) encoding human 3xFlag-LRRK2 under control of the cytomegalovirus (CMV) promoter and co-expressing a hygromycin selection marker via an internal ribosomal entry site element were prepared and used for cellular transduction as previously described (Civiero et al., 2012). Following selection in medium containing 200 μg/ml hygromycin, cells were expanded for use in experiments.

Cells were plated out into 96-well plates. When wells were >80% confluent, cells were treated with kinase inhibitors by dilution of the compounds into the cell culture medium to the desired final concentration. Following a 2 h incubation of the cells with kinase inhibitors, cells were immediately rinsed in PBS and lysed in lysis buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Tween 20 or 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1 mM $NaVO_4$, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche)]. Lysates were centrifuged for 30 min at 14000 × g. Supernatant was spotted to hydrated pvdf membranes and LRRK2 phospho-Ser935 levels as well as total LRRK2 levels were sequentially determined by immunoblot detection using the rabbit monoclonal anti-phospho-S935-LRRK2 antibody [Epitomics, clone UDD2 10(12)] and the mouse monoclonal anti-LRRK2 antibody N138/6 (Neuromab) or flag-M2 antibody followed by incubation with appropriate secondary horseradish peroxidase coupled antibodies and chemiluminescent detection using ECL plus HRP substrate (Thermo Scientific, Rockford, IL, USA). Densitometric analysis of the immunoreactive spots was performed using Aida analyzer v1.0 (Raytest, Straubenhardt, Germany). Phosphorylation levels were determined by the ratio of phospho-LRRK2 to total LRRK2, normalized to solvent controls.

Z′ DETERMINATION

To determine Z′ of the *in vitro* LRRKtide assay or the cellular Ser935 dephosphorylation assay, the following formula was used:

$$Z' = 1 - \left(\frac{3\sigma_{+c} + 3\sigma_{-c}}{|\mu_{-c} - \mu_{+c}|} \right)$$

Table 1 | Overview of *in vitro* and cellular activities of 160 kinase inhibitors tested at 10 and 5 μM, respectively.

						LRRKtide assay (@ 10 μM)		Cellular pS935 assay (@ 5 μM)	
Plate	**Position**	**Class**	**No.**	**Kinome branch(es) targeted**	**Compound name**	**% LRRKtide phosphorylation relative to solvent**	***s.e.m.***	**Phosphorylation level relative to solvent (%)**	***s.e.m.***
1	A1		1	TK	AG 1024	51.38	1.48	94.37	22.32
1	A2		2	TK	AGL 2043	29.68	1.80	90.06	21.92
1	A3		3	AGC	Akt inhibitor IV	18.59	1.11	76.44	17.96
1	A4		4	AGC	Akt inhibitor V, Triciribine	94.50	5.48	84.75	13.28
1	A5		5	AGC	Akt inhibitor VIII, isozyme-selective, Akti-1/2	99.69	7.69	77.80	26.82
1	A6		6	AGC	Akt inhibitor X	61.91	4.89	69.13	21.18
1	A7		7	AGC, ATYPICAL, TK	PDK1/Akt/Flt dual pathway inhibitor	5.76	0.58	57.20	29.84
1	A8	A	8	Other	Aurora kinase inhibitor II	22.60	1.34	69.85	18.74
1	A9		9	TK	Bcr-abl inhibitor	82.04	5.71	78.83	20.72
1	A10		10	AGC	Bisindolylmaleimide I	4.46	0.37	73.14	28.84
1	B1		11	AGC	Bisindolylmaleimide IV	3.07	0.13	84.51	19.01
1	B2	B	12	TK	BPIQ-I	17.30	1.70	88.55	33.49
1	B3		13	AGC	Chelerythrine chloride	77.91	3.86	67.25	21.94
1	B4	B	14	TK	Compound 56	4.52	0.20	86.56	22.77
1	B5		15	Atypical	DNA-PK inhibitor II	86.06	4.78	81.93	18.54
1	B6		16	Atypical	DNA-PK inhibitor III	68.41	2.69	71.78	18.12
1	B7		17	LIPID	PI-103	88.58	2.94	71.34	30.03
1	B8		18	CAMK	Diacylglycerol kinase inhibitor II	119.82	9.67	84.54	35.55
1	B9	C	19	TK	DMBI	19.33	1.47	54.34	18.86
1	B10	B	20	TK	EGFR/ErbB-2 inhibitor	68.24	4.64	67.43	30.39
1	C1		21	TK	EGFR inhibitor	64.37	1.33	57.76	21.33
1	C2		22	TK	EGFR/ErbB-2/ErbB-4 inhibitor	81.86	4.11	69.18	17.60
1	C3		23	TK	Flt-3 inhibitor	38.91	0.21	63.54	15.05
1	C4		24	TK	Flt-3 inhibitor II	2.16	0.06	53.00	3.65
1	C5		25	TK	cFMS receptor tyrosine kinase inhibitor	110.22	5.16	60.62	10.60
1	C6		26	AGC	Gö 6976	5.79	0.50	56.41	16.56
1	C7		27	AGC	Gö 6983	3.04	0.27	70.11	15.77
1	C8		28	TK	GTP-14564	18.03	1.74	71.63	22.34
1	C9		29	TK	Herbimycin A, Streptomyces sp.	103.97	4.57	92.79	38.16
1	C10		30	TK	Flt-3 inhibitor III	12.56	0.64	54.21	19.01
1	D1		31	TK	IGF-1R inhibitor II	93.23	2.56	80.40	24.89
1	D2		32	TKL	IRAK-1/4 inhibitor	55.78	2.47	71.83	15.88
1	D3	*	33	TK	**JAK inhibitor I**	3.20	0.18	47.17	11.26
1	D4	B	34	TK	JAK3 inhibitor II	1.86	0.03	63.02	3.14
1	D5		35	TK	JAK3 inhibitor IV	90.96	4.12	63.71	22.55
1	D6	C,*	36	TK	**JAK3 inhibitor VI**	1.30	0.14	40.36	12.03
1	D7		37	TK	Lck inhibitor	15.36	1.11	61.54	14.04
1	D8		38	LIPID	LY 294002	90.73	9.66	74.17	26.07
1	D9	*	39	LIPID	**LY 303511**	107.82	9.97	47.60	18.49

(Continued)

Table 1 | Continued

Plate	Position	Class	No.	Kinome branch(es) targeted	Compound name	LRRKtide assay (@ 10 μM)		Cellular pS935 assay (@ 5 μM)	
						% LRRKtide phosphorylation relative to solvent	*s.e.m.*	Phosphorylation level relative to solvent (%)	*s.e.m.*
1	D10	C	40	TK	Met kinase inhibitor	2.05	0.17	52.43	20.73
1	E1		41	TK	PD 158780	6.06	0.14	80.85	19.34
1	E2	B	42	TK	PD 174265	32.67	1.40	67.74	12.45
1	E3		43	TK	PDGF receptor tyrosine kinase inhibitor II	93.77	3.92	65.41	14.85
1	E4	B	44	TK	PDGF receptor tyrosine kinase inhibitor III	55.86	1.01	72.83	21.48
1	E5		45	TK	PDGF receptor tyrosine kinase inhibitor IV	23.12	0.70	57.30	25.20
1	E6		46	TK	PDGF RTK inhibitor	34.30	2.18	77.68	34.73
1	E7	C,*	47	Other	**PKR inhibitor**	1.18	0.09	29.00	8.27
1	E8	C	48	Other	PKR inhibitor, negative control	1.07	0.12	72.69	22.04
1	E9		49	LIPID	PI 3-Kg inhibitor	62.51	4.12	79.50	35.45
1	E10		50	LIPID	PI 3-KbInhibitor II	93.55	8.61	66.60	23.57
1	F1		51	TK	PP3	68.38	1.84	84.67	19.69
1	F2		52	TK	PP1 analog II, 1NM-PP1	44.14	2.64	68.59	16.24
1	F3		53	TK,AGC	PKCbII/EGFR inhibitor	67.41	5.09	56.68	11.09
1	F4		54	AGC	PKCb inhibitor	3.56	0.26	86.12	25.21
1	F5		55	AGC	Rapamycin	101.20	1.44	65.24	23.11
1	F6		56	AGC	Rho kinase inhibitor III, rockout	32.15	1.88	63.04	25.77
1	F7		57	AGC	Rho kinase inhibitor IV	10.29	0.77	59.98	10.49
1	F8		58	AGC, CMGC, TK	Staurosporine, N-benzoyl-	2.31	0.25	67.82	18.22
1	F9	B	59	TK	Src kinase inhibitor I	27.12	3.23	61.51	16.41
1	F10	C,*	60	TK	**SU11652**	0.79	0.07	14.19	4.14
1	G1	C	61	TK	Syk inhibitor	11.29	0.58	57.29	12.10
1	G2		62	TK	Syk inhibitor II	3.50	0.26	76.71	17.08
1	G3		63	TK	Syk inhibitor III	82.95	4.12	68.01	10.39
1	G4		64	TKL	TGF-b RI kinase inhibitor	47.94	15.83	71.73	11.39
1	G5		65	TKL	TGF-b RI inhibitor III	104.37	1.21	57.95	19.17
1	G6		66	TK	AG 9	82.97	3.32	55.25	10.97
1	G7		67	TK	AG490	34.24	2.12	50.68	7.31
1	G8		68	TK	AG 112	77.45	6.23	60.39	11.24
1	G9		69	TK	AG 1295	89.08	0.86	49.08	10.23
1	G10		70	TK	AG 1296	22.76	1.77	50.93	13.13
1	H1	B	71	TK	AG 1478	5.72	0.15	75.11	11.11
1	H2	C	72	TK	VEGF receptor 2 kinase inhibitor I	26.67	1.15	60.83	10.52
1	H3		73	TK	VEGF receptor tyrosine kinase inhibitor II	91.34	6.14	65.97	6.96
1	H4	B	74	TK	VEGF receptor tyrosine kinase inhibitor III, KRN63	68.07	3.47	62.48	16.81
1	H5	C,*	75	TK	**VEGF receptor 2 kinase inhibitor II**	4.13	0.33	37.26	10.69
1	H6	C,*	76	TK	**VEGF receptor 2 kinase inhibitor III**	2.19	0.31	45.08	9.20
1	H7	*	77	TK	**VEGF receptor 2 kinase inhibitor IV**	77.83	1.68	48.48	3.32
1	H8	*	78	Atypical	**DNA-PK inhibitor V**	89.01	3.54	48.36	9.41

(Continued)

Table 1 | Continued

Plate	Position	Class	No.	Kinome branch(es) targeted	Compound name	LRRKtide assay (@ 10 μM)		Cellular pS935 assay (@ 5 μM)	
						% LRRKtide phosphorylation relative to solvent	*s.e.m.*	Phosphorylation level relative to solvent (%)	*s.e.m.*
1	H9	*	79	Other	**Aurora kinase inhibitor III**	83.96	6.39	44.09	8.25
1	H10	*	80	AGC, CAMK, TK	**Staurosporine, Streptomyces sp.**	0.67	0.02	8.89	2.14
2	A1		81	CAMKII	KN-62	17.20	0.61	84.73	12.45
2	A2		82	Atypical	ATM kinase inhibitor	86.09	5.28	103.28	30.67
2	A3		83	Atypical	ATM/ATR kinase inhibitor	131.38	13.68	88.64	12.78
2	A4		84	CMGC	Alsterpaullone	31.34	1.30	108.54	22.06
2	A5		85	CMGC	Alsterpaullone, 2-Cyanoethyl	10.82	0.24	96.84	12.86
2	A6		86	CMGC	Aloisine A, RP107	23.85	0.78	102.87	22.84
2	A7		87	CMGC	Aloisine, RP106	17.45	1.08	109.11	40.35
2	A8	A	88	CMGC	Aminopurvalanol A	7.36	0.33	121.05	29.89
2	A9		89	CAMK	AMPK inhibitor, compound C	24.40	0.45	112.70	21.77
2	A10		90	Other	Aurora kinase inhibitor III	44.16	3.24	125.60	28.32
2	B1	*	91	Other, CMGC	**Aurora kinase/Cdk inhibitor**	1.21	0.04	45.67	9.90
2	B2	C	92	CMGC	Indirubin-3′-monoxime	11.18	0.60	85.53	12.46
2	B3	*	93	Other	**BAY 11-7082**	132.94	5.43	47.14	6.06
2	B4	A	94	CMGC	Bohemine	56.61	0.28	83.36	17.83
2	B5	C	95	CMGC	Cdk1 inhibitor	29.84	1.18	78.35	16.32
2	B6	A	96	CMGC	Cdk1 inhibitor, CGP74514A	3.91	0.21	83.35	15.80
2	B7	*	97	CMGC	**Cdk1/2 inhibitor III**	0.73	0.04	13.15	4.00
2	B8		98	CMGC	Cdk1/5 inhibitor	25.04	0.95	123.99	27.63
2	B9		99	CK1, CMGC, TKL	Casein kinase I inhibitor, D4476	60.00	2.43	97.88	23.12
2	B10		100	CK1	Casein kinase II inhibitor III, TBCA	16.78	2.18	116.47	20.08
2	C1		101	CMGC	Cdk4 inhibitor	6.82	0.21	49.88	9.67
2	C2		102	CMGC	Cdk4 inhibitor II, 625987	76.06	2.17	70.36	11.01
2	C3	*	103	CMGC	**Cdk4 inhibitor III**	26.80	1.93	48.63	8.94
2	C4		104	CMGC	Cdc2-like kinase inhibitor, TG003	37.41	1.21	81.30	15.73
2	C5		105	CAMK	Chk2 inhibitor II	11.58	0.20	93.25	24.28
2	C6	A	106	CMGC	Compound 52	7.76	1.06	76.44	10.34
2	C7	A	107	CMGC	Cdk2 inhibitor III	60.51	1.54	100.49	19.65
2	C8	*	108	CMGC	**Cdk2 inhibitor IV, NU6140**	0.98	0.10	34.63	5.97
2	C9		109	CMGC	Cdk/Crk inhibitor	54.91	3.05	97.11	22.67
2	C10		110	CMGC	ERK inhibitor III	55.32	2.27	90.93	24.24
2	D1		111	AGC	ROCK inhibitor, Y-27632	10.36	0.18	69.28	21.03
2	D2		112	CMGC	ERK inhibitor II, FR180204	14.06	1.25	65.88	6.29
2	D3		113	CMGC	ERK inhibitor II, negative control	97.07	6.45	77.12	9.52
2	D4	*	114	CMGC	**Fascaplysin, synthetic**	17.51	0.65	45.44	12.66
2	D5		115	CMGC, AGC, TK	GSK-3b inhibitor I	106.35	10.27	95.90	26.02
2	D6		116	CMGC	GSK-3b inhibitor II	100.90	6.56	84.51	16.29

(Continued)

Table 1 | Continued

Plate	Position	Class	No.	Kinome branch(es) targeted	Compound name	LRRKtide assay (@ 10 μM)		Cellular pS935 assay (@ 5 μM)	
						% LRRKtide phosphorylation relative to solvent	*s.e.m.*	Phosphorylation level relative to solvent (%)	*s.e.m.*
2	D7		117	CMGC	GSK-3b inhibitor VIII	128.15	6.14	115.21	21.46
2	D8	C	118	CMGC	GSK-3 inhibitor IX	18.72	0.92	104.53	35.69
2	D9	C	119	CMGC	GSK-3 inhibitor X	38.94	3.57	106.06	24.67
2	D10		120	CMGC	GSK-3b inhibitor XI	32.34	2.16	87.77	32.91
2	E1	C,*	121	TK	**SU6656**	4.57	0.08	14.34	2.80
2	E2		122	CMGC	GSK-3 inhibitor XIII	3.34	0.09	73.51	10.09
2	E3		123	CAMK, CMGC, ATYPICAL	Isogranulatimide	4.64	0.26	77.38	14.89
2	E4	C	124	CK1	IC261	89.62	3.16	78.81	15.27
2	E5		125	Other	IKK-2 inhibitor IV	5.13	0.41	108.38	23.53
2	E6	C	126	CMGC, TK	Indirubin derivative E804	2.23	0.13	101.13	27.96
2	E7		127	CMGC	JNK inhibitor II	2.32	0.07	81.52	14.12
2	E8		128	CMGC	JNK inhibitor, negative control	17.88	0.08	107.46	25.08
2	E9		129	CMGC	JNK inhibitor V	37.61	2.10	71.85	6.04
2	E10	*	130	CMGC	**JNK inhibitor IX**	61.45	2.35	42.05	5.61
2	F1		131	CAMK	MK2a inhibitor	123.21	2.99	60.73	4.94
2	F2		132	CMGC	JNK inhibitor VIII	105.45	2.05	93.08	10.18
2	F3		133	AGC	K-252a, Nocardiopsis sp.	1.13	0.07	72.18	4.83
2	F4		134	CMGC, CK1, TK	Kenpaullone	47.16	0.79	94.10	11.30
2	F5		135	CAMK	KN-93	120.59	4.60	102.70	28.01
2	F6		136	STE	MEK inhibitor I	124.71	2.56	100.55	23.22
2	F7		137	STE	MEK inhibitor II	21.13	0.29	108.79	13.13
2	F8		138	STE	MEK 1/2 inhibitor	61.26	1.91	117.92	16.91
2	F9		139	CAMK	MNK1 inhibitor	12.59	0.83	70.32	11.88
2	F10	B	140	Other	NF-KB activation inhibitor	57.28	1.39	65.67	8.71
2	G1		141	CMGC	p38 MAP kinase inhibitor III	112.29	4.78	83.74	15.83
2	G2		142	CMGC	p38 MAP kinase inhibitor	132.68	3.68	93.68	13.31
2	G3		143	STE	PD98059	92.53	2.37	106.55	5.54
2	G4		144	CMGC	PD 169316	87.56	0.73	90.07	17.02
2	G5		145	CMGC	SB 220025	73.68	4.02	70.97	17.27
2	G6	A	146	CMGC	Purvalanol A	15.64	0.37	121.26	28.75
2	G7		147	AGC,CK1,CAMK	GSK-3b inhibitor XII, TWS119	13.54	0.68	122.47	26.62
2	G8		148	AGC, CK1, CAMK	H-89, Dihydrochloride	45.29	0.88	155.11	24.98
2	G9		149	INACTIVE	SB 202474, Neg Con for p38 MAPK inhibition studies	47.64	3.34	112.24	20.96
2	G10		150	CMGC	SB 202190	45.07	0.27	88.12	18.23
2	H1		151	CMGC	SB 203580	100.48	2.60	90.32	17.97
2	H2		152	AGC	HA 1077, Dihydrochloride Fasudil	79.40	2.08	89.37	27.47
2	H3		153	CAMK	SB 218078	1.94	0.17	89.51	29.97
2	H4		154	CMGC	SC-68376	48.07	1.45	104.03	21.61

(Continued)

Table 1 | Continued

Plate	Position	Class	No.	Kinome branch(es) targeted	Compound name	LRRKtide assay (@ 10 μM)		Cellular pS935 assay (@ 5 μM)	
						% LRRKtide phosphorylation relative to solvent	*s.e.m.*	Phosphorylation level relative to solvent (%)	*s.e.m.*
2	H5		155	CMGC	SKF-86002	94.81	1.98	121.09	29.45
2	H6		156	LIPID	Sphingosine kinase inhibitor	106.21	2.40	144.00	32.87
2	H7	*	157	AGC, CAMK, TK	**Staurosporine, Streptomyces sp.**	0.79	0.05	48.71	13.60
2	H8		158	CAMK	STO-609	15.01	0.13	121.51	22.26
2	H9	C	159	CMGC	SU9516	2.99	0.40	94.62	17.52
2	H10		160	CMGC	Tpl2 kinase inhibitor	55.04	2.87	102.54	14.22

160 kinase inhibitors from a panel of inhibitors known to target kinases in all branches of the kinome were tested for their ability to (i) inhibit LRRK2 at 10 μM in an in vitro kinase assay using the LRRKtide model peptide substrate and (ii) dephosphorylate LRRK2 at phosphoserine 935 in a spotblot assay. Listed are the exact values (expressed as a % relative to the control) obtained in these tests for each compound. A general overview of these tests are given in ***Figures 1***, ***3*** *and the plate and position references given in the first 2 columns correspond to those given in* ***Figures 1B***, ***3B****. Color codes to facilitate identification of compounds from the three selected chemical classes are: (A) 9-methyl-N-phenylpurine-2,8-diamine compounds highlighted in green, (B) N-phenylquinazolin-4-amine analogs given in orange, and (C) 1,3-dihydroindol-2-one analogs in blue. The 20 most potent compounds in the cellular assay are indicated in bold. The 3rd column of the table (labeled "class") indicates the compound classes (A–C) or the 20 most active compounds in the cellular assay (asterisk), as applicable.*

where σ_{+c}, σ_{-c}, μ_{+c}, and μ_{-c} are the standard deviation (σ) and mean (μ) of the positive control samples (+c, LRRK2-IN1 10 μM for the LRRKtide assay, CZC 10 μM treated samples in the pS935 dephosphorylation assay) or negative control samples (−c, DMSO treated samples). Results are based on values from 3–10 replicates from the same assay run.

MODELLING THE LRRK2 KINASE DOMAIN BASED ON MULTIPLE TEMPLATES

All the following steps were conducted using MODELLER 9v9 (Sali and Blundell, 1993), unless otherwise stated. First, human TKL kinases with a DFG-in activation loop conformation (i.e., active conformation) available at that time in the "Kinase Database" implemented in Molecular Operating Environment (MOE, Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2013) were structurally aligned using the alignment.salign command. Afterwards, this structure domain was superimposed on the LRRK2 kinase domain. The TKL kinases B-Raf (Rapidly accelerated fibrosarcoma) (PDB 3OG7; Bollag et al., 2010) and MLK1 (Mixed Lineage Kinase 1) (PDB 3DTC; Hudkins et al., 2008) and—for one short stretch of 11 amino-acids in the N-terminal region of LRRK2 kinase (corresponding to LRRK2 amino acids 1872–1882 found just N-terminal of the P-loop, see **Figure 6A**)—IRAK-4 (Interleukin-1 Receptor–Associated Kinase 4) (PDB 2NRU; Wang et al., 2006) displayed the highest identity with the LRRK2 kinase domain and were chosen as templates. The final alignment between B-Raf, MLK1, IRAK-4, and the LRRK2 kinase domain is shown in **Figure 6A**. A 3D model of LRRK2 kinase domain was calculated by satisfaction of spatial restraints and screened for unfavorable regions by computing the Discrete Optimized Protein Energy (DOPE) score per residue. The alignment was modified using iterative alignment-modeling-evaluation steps until no improvement could be found. For the top-scoring alignment, multiple models were computed and subjected to a rough refining procedure: each model is optimized with the variable target function method with conjugate gradients and further refined using molecular dynamics with simulated annealing. The LRRK2 kinase domain model with the best DOPE score, GA341 score and Modeler objective function was selected.

Explicit hydrogen atoms were added and the model was subjected to a more thorough refining procedure with MOE using the AMBER99 force field with Born solvation model. First, all inconsistencies and outliers were selected (as observed from values in the Ramachandran plot, backbone bond angles and lengths, the rotamer strain energy, and atom clashes), the other residues were potentially fixed and an energy minimization (EM) was performed with backbone atoms restrained to 100. The EM was terminated when the RMS gradient fell below 0.1. A final EM was performed on all atoms with all backbone atoms restrained to 10. This minimization was terminated when the RMS gradient fell below 0.1.

QUALITY

Model quality has been checked by computational methods, giving us a good validation of the reliability of the model. The Ramachandran Plot assured very good confidence: only 0.7%

Table 2 | Overview of *in silico, in vitro* and cellular activities of selected kinase inhibitors.

Compound	Compound class	GBVI/WSA dG Score	pIC50 *in vitro* (lrrktide)	IC50 *in vitro* (lrrktide, nM)	Cellular pS935 (% @ 5 μM)	pIC50 cellular (pS935)	IC50 cellular (pS935, nM)
AG 1478	B	0.6074	5.43	3732.50	75.11	NA	NA
Aminopurvalanol A	A	−5.7089	5.69	2027.68	121.05	NA	NA
Aurora kinase inhibitor II	A	0.0905	<5	>1E4	69.85	NA	NA
Bohemine	A	−4.9590	<5	>1E4	83.36	NA	NA
BPIQ-I	B	−3.5711	5.12	7649.22	88.55	NA	NA
Cdk1 inhibitor	C	−5.6508	<5	>1E4	78.35	NA	NA
Cdk1 inhibitor. CGP74514A	A	−5.4352	5.46	3467.37	83.35	NA	NA
CDK2 inhibitor III	A	−5.9534	<5	>1E4	100.49	NA	NA
Compound 52	A	−4.9190	5.41	3926.45	76.44	NA	NA
Compound 56	B	−2.8627	5.52	3013.01	86.56	NA	NA
DMBI	C	−5.5592	5.83	1482.81	54.34	NA	NA
EGFR/ErbB-2 inhibitor	B	0.3668	<5	>1E4	67.43	NA	NA
GSK-3 inhibitor IX	C	−3.9610	5.95	1122.02	104.53	NA	NA
GSK-3 inhibitor X	C	−4.3149	<5	>1E4	106.06	NA	NA
IC261	C	−4.7642	<5	>1E4	78.81	NA	NA
Indirubin derivative E804	C	−6.0607	6.16	685.49	101.13	NA	NA
Indirubin-3′-monoxime	C	−5.0838	5.75	1786.49	85.53	NA	NA
JAK3 inhibitor II	B	0.2193	5.92	1202.26	63.02	NA	NA
JAK3 inhibitor VI	C	−5.6557	6.76	173.78	40.36	<5.7	>2000
Met kinase inhibitor	C	−4.7526	7.37	42.95	52.43	NA	NA
NF-KB activation inhibitor	B	0.7201	<5	>1E4	65.67	NA	NA
PD 174265	B	−3.4813	5.05	8871.56	67.74	NA	NA
PDGF receptor tyrosine kinase inhibitor III	B	−1.7337	<5	>1E4	72.83	NA	NA
PKR inhibitor	C	−5.8928	6.44	363.08	29	5.88	1312.00
PKR inhibitor. negative control	C	−5.9853	6.53	297.17	72.69	NA	NA
Purvalanol A	A	−5.8267	5.34	4570.88	121.26	NA	NA
Src kinase inhibitor I	B	1.8876	6.03	936.22	61.51	NA	NA
SU11652	C	−7.5028	7.96	10.89	14.19	6.50	316.23
SU6656	C	−7.3049	8.00	10.00	14.34	6.37	422.67
SU9516	C	−5.8674	5.68	2103.78	94.62	NA	NA
Syk inhibitor	C	−6.5548	6.20	632.41	57.29	NA	NA
VEGF receptor 2 kinase inhibitor I	C	−6.6034	7.61	24.55	60.83	NA	NA
VEGF receptor 2 kinase inhibitor II	C	−6.4887	7.44	36.31	37.26	<5.7	>2000
VEGF receptor 2 kinase inhibitor III	C	−5.4369	6.56	272.90	45.08	<5.7	>2000
VEGF receptor tyrosine kinase inhibitor III. KRN63	B	−0.9464	<5	>1E4	62.48	NA	NA
Cdk1/2 inhibitor III	NA	NA	9.04	0.91	13.15	8.93	1.18
Staurosporine	NA	NA	9.57	0.27	8.89	8.76	1.75
LRRK2 IN1	NA	NA	8.45	3.54	9.32	6.55	279.90
CZC25146	NA	NA	8.75	1.78	8.62	7.36	43.65

Compounds belong to one of three different structural classes (A, 9-methyl-N-phenylpurine-2,8-diamine, B, N-phenylquinazolin-4-amine, or C, 1,3-dihydroindol-2-one analogs, see second column) as well as selected reference compounds. In silico docking values given are GBVI/WSA dG. In vitro activities and cellular activities as measured by the LRRKtide assay and phosphoserine 935 assay, respectively, are given both as pIC50 as well as the corresponding IC50 expressed in nM. In vitro inhibition values at the single dose of 10 μM and cellular phosphorylation levels at 5 μM are given in ***Table 1*** *for all 160 compounds of the kinase inhibitor panel, cellular phosphorylation levels at 5 μM are also given here for all compounds in the table. See Results and Materials and Methods sections for more details. NA, not applicable.*

Table 3 | Overview of TKL kinases with a DFG in conformation available in the MOE "Kinase Database" and their sequence identity with the LRRK2 kinase domain.

PDB	Family	Kinase	Resolution (Å)	SEQ ID
3HMM	STKR	TGFβR1 (or ALK5)	1.70	23
3OOM		ALK2 (or ActR1)	2.00	24
3MDY		BMPR1B	2.05	23
3MY0		ALK1	2.65	21
3G2F		BMPR2	2.35	23
2QLU		ActR2	2.00	21
2NRU	IRAK	IRAK4	2.00	24
3OG7	RAF	B-RAF	2.45	29
3OMV		C-RAF (or RAF-1)	4.00	27
2EVA	MLK	TAK1	2.00	25
3DTC		MAPKKK9 (or MLK1)	2.60	34

The PDB code, resolution and the family each kinase belongs to as well as the sequence identity to the LRRK2 kinase domain are provided. The three TKL kinases chosen as templates are highlighted in red. Abbreviations: IRAK, interleukin-1 receptor–associated kinase; MLK, mixed lineage kinase; RAF, rapidly accelerated fibrosarcoma; SEQ ID, sequence identity to the LRRK2 kinase domain; STKR, S/T kinase receptors.

residues in the disallowed region and 2.1% residues outside generously allowed regions. 2.9% of the residues had unfavorable bond angles and 1.1% had unfavorable dihedrals. However, most of these residues were oriented away from the active site (**Figure 6C**). Assessment of model quality using Meta-MQAPII (Pawlowski et al., 2008) gave absolute global deviations, expressed as RMS deviation (3.47 Å) and Global Distance Test Total Score (65.98), for the model vs. the unknown true structure, indicating a medium quality model. The Meta-MQAPII score per residue is shown in **Figure 6C**. The only unfavorable regions were the loop regions, especially the activation loop, explained by the lack of a good template for these regions (**Figure 6A**). Overall, the activation loop is also rather flexible for kinases (un-crystallized region). Since these unfavorable modeled loops are not part of the ligand binding site, we proceeded with the LRRK2 kinase domain homology model. PyMOL and MOE were used as a visualization tool. The model is freely available upon request to the corresponding authors.

PREPARATION OF THE *IN SILICO* KINASE INHIBITOR DATABASE

The kinase inhibitor database was supplied as a two-dimensional structure data file by EMD4Biosciences (USA). Using the MOE Structure Database Tools (sdwash, sdcharges, and sdstereo commands), the database was curated. Based on the fact that different stereoisomers may have different activities, molecular docking simulations were carried out for both stereoisomers. Generation of 3D structures was done via the energy minimize command using default settings. We used the MMFF94x force field. All data were stored in a MOE molecular database file.

Common substructures, based on the analysis of PDB kinase ligands by Ghose and co-workers (Ghose et al., 2008), were found using Instant JChem (ChemAxon, Hungary).

PROTEIN-LIGAND DOCKING AND SCORING

After homology modeling with MODELLER (Sali and Blundell, 1993), the generated alternative conformations of the ligands were docked into the active site using MOE. A preliminary docking step, where staurosporine was docked in the LRRK2 active site, was applied to optimize the local environment to acquire the most optimal binding pose in the subsequent docking step. The crystal structures B-Raf (PDB 3OG7), MLK1 (PDB 3DTC), and IRAK-4 (PDB 2NRU) were protonated using Protonate3D module of MOE.

For docking simulations, initial binding conformations were generated for the purine, quinazoline, and oxindol derivatives (termed compound classes A–C) of the kinase inhibitor database. These initial binding conformations were refined using pharmacophore models for these three compound classes (**Figures 7A–C**). The pharmacophore models were based on structural elements, shared by all derivatives in one class that are essential for interaction with LRRK2. During the refinement step, ligands that fulfilled the pharmacophore hypothesis were allowed to advance and an optimized binding conformation was saved in a MOE molecular database file. Ligands that didn't satisfy the pharmacophore requirements were excluded from subsequent steps. The DOCK module was used and default settings were applied. A force field based scoring function was used: GBVI/WSA dG. After docking, the results were collected by receptor (i.e., docking values obtained with each of the four kinase structures tested). For each ligand the best scoring (e.g., lowest energy) docking pose was kept.

RECEIVER OPERATING CHARACTERISTICS PLOTS

Receiver operating characteristic plots are useful as a graphical illustration of the performance of the *in silico* docking strategy as they can evaluate the computed docking values together with measured activities (either *in vitro* or cellular). Here, we plotted the receiver operating characteristic plots as false positive rate (equivalent to the 1—*specificity)* vs. the true positive rate (equivalent to the sensitivity), therefore docking strategies which plotted on average above the diagonal can be considered to have predictive value (this is also reflected by the AUC which is >0.5 for predictive docking strategies). In more detail, for each ligand, the activity/inactivity was indicated by adding 1 and 0 respectively based on the *in vitro* LRRKtide assay or cellular pS935 dephosphorylation assay. Ligands with at least 50% inhibitory activity at 1 μM in the LRRKtide assay or at least 50% inhibitory activity at 5 μM in the cellular pS935 dephosphorylation assay were designated as active. A receiver operating characteristic plot was generated for each receptor (i.e., for each kinase model) using CROC v1.0 (Swamidass et al., 2010). Plots were made with Deltagraph V7 (2014 RedRock Software, Salt Lake City, USA).

CORRELATION ANALYSIS

To evaluate the correlation between the different activities obtained for tested kinase inhibitors, pairs of activity values were plotted against each other in GraphPad Prism 5.01 (GraphPad Software Inc.). Linear regression analysis was performed and a trendline was drawn as well as the 95% confidence band. Finally, Pearson's r-coefficient was calculated and a two tailed correlation

significance test performed (GraphPad). The level of statistical significance was set at $P < 0.05$.

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Interaction of LRRK2 with kinase and GTPase signaling cascades

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LRRK2 is a protein that interacts with a plethora of signaling molecules, but the complexity of LRRK2 function presents a challenge for understanding the role of LRRK2 in the pathophysiology of Parkinson's disease (PD). Studies of LRRK2 using over-expression in transgenic mice have been disappointing, however, studies using invertebrate systems have yielded a much clearer picture, with clear effects of LRRK2 expression, knockdown or deletion in *Caenorhabditis elegans* and *Drosophila* on modulation of survival of dopaminergic neurons. Recent studies have begun to focus attention on particular signaling cascades that are a target of LRRK2 function. LRRK2 interacts with members of the mitogen activated protein kinase (MAPK) pathway and might regulate the pathway action by acting as a scaffold that directs the location of MAPK pathway activity, without strongly affecting the amount of MAPK pathway activity. Binding to GTPases, GTPase-activating proteins and GTPase exchange factors are another strong theme in LRRK2 biology, with LRRK2 binding to rac1, cdc42, rab5, rab7L1, endoA, RGS2, ArfGAP1, and ArhGEF7. All of these molecules appear to feed into a function output for LRRK2 that modulates cytoskeletal outgrowth and vesicular dynamics, including autophagy. These functions likely impact modulation of α-synuclein aggregation and associated toxicity eliciting the disease processes that we term PD.

Keywords: GTPase, kinase, trafficking, cytoskeleton, actin, autophagy, cell death, dopamine

INTRODUCTION

Parkinson's disease (PD) is the most common age-related motor disorder (Hsu et al., 2010a; Ferree et al., 2012). Accumulation of aggregated α-synuclein to form Lewy bodies is a neuropathological hallmark for PD (Lees et al., 2009). Mutations in leucine-rich repeat kinase 2, LRRK2, gene are common genetic determinants of PD, with at least 20 different mutations identified to date causing late-onset, familial autosomal-dominant PD (Gasser et al., 2011; Greene, 2012). The most prevalent amino acid substitution mutation in LRRK2, G2019S, has been found in 1–2% of sporadic PD cases; with sporadic PD and LRRK2-associated PD being clinically and neurochemically indistinguishable (Healy et al., 2008). These all indicate that LRRK2 has an important role in the pathogenesis of PD.

LRRK2 is a large ubiquitous cytoplasmic protein consisting of 2527 amino acids with multiple functional domains (Cookson, 2010). LRRK2 has a homolog in mammals, termed LRRK1; LRRK1 and 2 expression appear to be complementary, with LRRK2 expression peaking in development and decreases rapidly after birth, while LRRK2 expression increases postnatally (Simon-Sanchez et al., 2006). The LRRK2 protein consists of kinase, Ras-of-complex proteins (ROCs) GTPase, C-terminal of ROC (COR), leucine-rich repeat, ankyrin and WD40 domains (Cookson, 2010). The two catalytic domains are the kinase and ROC GTPase domains. The kinase domain has the highest sequence homology to mitogen-activated protein kinase kinase kinase (MKKK)/MLKs (mixed lineage kinases) and receptor-interacting protein (RIP) kinase families. The most common mutation in LRRK2 associated with PD is the G2019S mutation located in the kinase domain of LRRK2 (Wszolek et al., 2004; Zimprich et al., 2004). Other common amino acid substitution include R1441C/G/H in the Roc GTPase domain and Y1699C in the COR domain (Taylor et al., 2006). The pathological mutations G2019S enhances the kinase activity without affecting the GTPase activity of LRRK2; whereas R1441C/G/H and Y1699C affect GTPase activity of LRRK2 by impairing GTP hydrolysis and has inconsistent effects on its kinase activity (West et al., 2005; Greggio et al., 2006; Ito et al., 2007; Lewis et al., 2007; Li et al., 2007). However, the underlying mechanisms linking such mutations to pathology still remain unclear.

LRRK2 MODULATES SURVIVAL OF DOPAMINERGIC NEURONS, AUTOPHAGY, AND NEURONAL OUTGROWTH

Studies of LRRK2 using over-expression in transgenic mice have been disappointing, with few studies showing consistent effects occurring upon over-expression of wildtype or mutant LRRK2 (Dawson et al., 2010). Knockout of LRRK2 in the mouse yielded a strong phenotype in the kidney pointing to a role for LRRK2 in autophagy, however, no clear differences in the brain; this suggests that mammalian LRRK2 *in vivo* models do a better job of modeling cellular function/dysfunction rather than providing a more general model of PD (Tong et al., 2010). In contrast, over-expression studies using invertebrate systems have yielded much clearer results. Orthologs of LRRK2 also exist in *Drosophila*

and Nematodes, although these species exhibit just one form of LRRK, referred to as lrk-1 (Marin, 2008). The invertebrate LRRK2 orthologs have kinase, COR and ROC domains that are homologous to mammalian LRRK2, and the lrk-1 protein appears to subsume the functions of both LRRK1 and LRRK2 (Marin, 2008; Saha et al., 2009). Studies from several groups examining the effects of LRRK2 expression, knockdown or deletion in *Caenorhabditis elegans* and *Drosophila* all show strong effects (Liu et al., 2008; Wang et al., 2008; Saha et al., 2009; Samann et al., 2009; Venderova et al., 2009; Yao et al., 2010; Yuan et al., 2011). The results appear to differ somewhat based on the system, but the effects are strong in all cases. Studies in our laboratory indicate that expressing WT LRRK2 promotes survival of dopaminergic neurons in response rotenone treatment, while mutant LRRK2 (G2019S or R1441C) enhances loss of dopaminergic neurons relative to WT (Saha et al., 2009). Another important aspect of our observations, which becomes important when considering studies of LRRK2 more generally, is that G2019S also potentiates loss of neuronal processes relative to WT LRRK2. Because nematodes lack endogenous α-synuclein, we recently crossed the LRRK2 lines to a line expressing α-synuclein in dopaminergic neurons. Interestingly, current studies with a new nematode model in our laboratory shows that both WT and G2019S LRRK2 potentiate age-related loss of dopaminergic neurons (and of their processes), suggesting that understanding the interaction between LRRK2 and α-synuclein is important for modeling the pathophysiology of PD. Studies from the Chen laboratory using a different *C. elegans* model (without incorporation of α-synuclein) show a deleterious effect of WT and G2019S LRRK2, while studies using *Drosophila* indicate that LRRK2 potentiates degeneration of retinal cells and loss of dopaminergic neurons (Liu et al., 2008; Yao et al., 2010). Thus, the specific results depend on the particular model used, but it is clear that invertebrate models are very sensitive to modulation of LRRK2 levels, and mutations in LRRK2 potentiate degeneration of dopaminergic neurons. While mouse models are not particularly sensitive to LRRK2 over-expression, knockout of LRRK2 appears to potentiate autophagic deficits in the kidney, which is a site that normally exhibits strong expression of LRRK2. This provides evidence that the effects observed with invertebrate systems have strong relevance to the mammalian system.

Studies using primary rodent neurons grown in culture have yielded much stronger results than the transgenic mouse models. These studies consistently indicate that expression of mutant LRRK2 potentiates neurodegeneration, and reduces neurite outgrowth (Smith et al., 2005; Chan et al., 2011; Skibinski et al., 2014). Interestingly, acute expression of WT LRRK2 appears to be beneficial, which parallels observations from our studies in *C. elegans*. One study, using in utero electroporation of LRRK2 in the rodent brain also observed detrimental effects of G2019S LRRK2 and beneficial effects of WT LRRK2, suggesting that acute expression of LRRK2 in the rodent brain might produce clearer results than chronic over-expression, such as occurs with transgenic mice (Macleod et al., 2006). Taken together, these results indicate that mutant LRRK2 is detrimental; the effects of WT LRRK2 are less clear, with some data suggesting that WT LRRK2 is beneficial, and other data suggesting that over-expression can be mildly detrimental. In general, most data suggest that loss of LRRK2 expression is detrimental.

LRRK2 REGULATES MAP KINASE SIGNALING PATHWAYS

Incorporating research from multiple different venues suggests roles for LRRK2 in two differing cellular networks. Prior studies from our laboratory, and subsequent studies from the Kahle laboratory indicate that LRRK2 interacts with the mitogen activated protein kinase (MAPK) pathways (Carballo-Carbajal et al., 2010; Hsu et al., 2010a,b). The MAPK pathway is a signaling network that begins with membrane signaling and extends through multiple successive kinases leading ultimately to phosphorylation of transcription factors that act on gene expression (Yang et al., 2013). Receptor signaling frequently begins with MAP Kinase kinase kinases (MAPKKKs, also termed mixed lineage kinases, MLKs), which are enzymes often act to initiate signaling cascades that ultimately lead to transcriptional regulation (Yang et al., 2013). The MLK family of kinases activates the c-jun NH2-terminal kinase (JNK) pathway by phosphorylating MKK4 and -7 and activates the p38 pathway by phosphorylating MKK3 and -6 (Gallo and Johnson, 2002; Wang et al., 2004). These stress kinase complexes required scaffold proteins in the regulation of their subcellular localization. In particular for the MAP kinase signaling cascades, the JNK-interacting proteins (JIPs) are the group of scaffold proteins in such regulation (Kelkar et al., 2005; Whitmarsh, 2006). Hence, these MKKs are then recruited into a multi-protein complex by scaffold proteins JIPs (Habig et al., 2013). JIP1-3 regulates the specificity and localization of the JNK pathway and JIP4 is a specific scaffolding protein for the p38 pathway (Verhey et al., 2001; Kelkar et al., 2005; Whitmarsh, 2006).

Multiple studies show that LRRK2 interacts with the MAPK pathway and has a kinase domain that is homologous to MAPKKK/MLKs and RIPs (West et al., 2005; Greggio and Cookson, 2009; Carballo-Carbajal et al., 2010; Hsu et al., 2010a,b). However, these same studies suggest that LRRK2 is not a strong activator of the pathway. Our laboratory has shown that LRRK2 binds MKK6, -3, and -7 in HEK293FT (Gloeckner et al., 2009; Hsu et al., 2010a). Binding of LRRK2 to MKK6, -3, and -7, activates the p38 and JNK pathway, but the amount of activation is strikingly modest (Hsu et al., 2010a). Receptor activation of the MAPK pathway produces many fold increases in the activities of the downstream kinases, but over-expressing LRRK2 increases activities of the kinases and downstream transcription factors by 60% or less (Hsu et al., 2010a). Studies from the Kahle laboratory showed similarly low levels of kinase activation upon LRRK2 over-expression (Carballo-Carbajal et al., 2010). These studies showed that LRRK2 upregulates alpha-synuclein transcription in parallel with MEK/ERK activation. This induction of transcriptional upregulation of alpha-synuclein was suppressed by treatment with the selective MAPK/ERK kinase inhibitor, U0126 (Carballo-Carbajal et al., 2010). Disease linked mutations increased activation by only an additional 25–30%. Similar results were observed upon analysis of JIP proteins, which act as scaffolds for MAPKs, and function in transport of MAPKs (Hsu et al., 2010b). We have also shown that LRRK2 binds to JIP1-4 and is associated with increased levels of total JIP1, -3, -4, oligomeric JIP, and ubiquitinated JIP. In addition,

G2019S, R1441C and I2020T (but not Y1699C) mutations in LRRK2 increased binding with MKK6 and also levels of JIP4 (Hsu et al., 2010b). The stimulation of JIP oligomerization was particularly striking effect observed upon co-expression of LRRK2 with JIPs.

Despite the relatively modest effect of LRRK2 on the MAPK cascades, LRRK2 produces a robust effect *in vivo*, and the MAPKs appear to be necessary for this function. Our *in vivo* studies in *C. elegans* showed robust protection against mitochondrial stress that was induced by LRRK2 expression. This protection was dependent on the action of MKK6 or p38 because RNAi knockdown of endogenous orthologs of MKK6 or P38 (sek-1 and pmk-1) or deletion of sek-1 in *C. elegans* abolish neuroprotection by LRRK2 (Hsu et al., 2010a). The MAPK cascade also appears to modulate the effects of LRRK2 on autophagy (Plowey et al., 2008). Using redox proteomics, we also demonstrated that LRRK2 regulates proteins associated with the lysosome, including ATPVA6 (Di Domenico et al., 2012). Application of a MAPKK (MEK) inhibitor blocked the effects of LRRK2 on autophagy and on neurite shortening (Plowey et al., 2008). Thus, interactions between LRRK2 and members of the MAPK pathway cause significant physiological effects despite causing only modest changes in phosphorylation.

The discordance between the modest effects of LRRK2 on activation of the MAPK pathway and the strong effects of LRRK2 in protecting dopaminergic neurons in the nematode, or the strong effect of LRRK2 on JIP oligomerization demands a novel model to explain LRRK2 function. One possibility is that LRRK2 acts as a scaffold that directs the location of MAPK pathway activity, without strongly affecting the amount of MAPK pathway activity. Location is as important to enzymatic action as amount of activity. JIPs for instance, are thought to function in vesicular trafficking. If LRRK2 also exhibited a role in vesicular trafficking, then it could impact on neuronal function by directing particular enzymes toward trafficked vesicles, rather than explicitly activating the MAPK pathway. Such a role would also be consistent with other studies suggesting a function for LRRK2 in regulating small GTPases associated with vesicles.

LRRK2 REGULATES MANY OTHER SIGNALING CASCADES

Recent transcriptome and proteome studies emphasize the diverse number of pathways regulated by LRRK2. Studies by the Dawson group recently highlighted regulation of translational functions by LRRK2, showing that LRRK2 binds to and phosphorylates the ribosomal protein RPS15 (Martin et al., 2014). This observation fits well with other studies showing roles for LRRK2 as a negative mediator of miR mediated translational repression (Gehrke et al., 2010). The regulatory network that we developed also identifies RPS15 as a primary member of the LRRK2 regulatory network, interacting with LRRK2 in a pathway that includes the ADP-ribose polymerase TNKS (Dusonchet et al., 2014). The Cookson group also recently published a proteomic study of LRRK2, which identified cyclin-G associated kinase (GAK) as a strong binding protein (Beilina et al., 2014). GAK is the third strongest genetic risk factor for PD, after synuclein and tau (Pankratz et al., 2009; Dumitriu et al., 2011).

Other studies identify LRRK2 as a negative regulator of PKA signaling, acting through direct interaction with PKAR11B, modulating neuronal development and function, by influencing dopamine signaling and synaptogenesis (Parisiadou et al., 2014). The R1441C LRRK2 mutation exhibits similar neuronal effects on LRRK2 knockout mice, causing dopamine signaling impairments as well as neurite retraction via enhanced PKA activation. PKA also regulates LRRK2 by phosphorylating S1444 in the ROC domain; 14-3-3 then binds to the phospho-serine 1444 and inhibits LRRK2 kinase activity (Muda et al., 2014). Binding of 14-3-3 to different phospho-epitopes (Ser-910/935) appears to stabilize LRRK2, while reduced binding destabilizes LRRK2 and causes aggregation of LRRK2 to form cytoplasmic inclusions (Dzamko et al., 2010). Identification of targets of LRRK2 kinase activity remains unclear. Studies have identified numerous putative substrates, including MAPK, 4E-BP, and Tau (Imai et al., 2008; Berwick and Harvey, 2011; Bailey et al., 2013).

Although LRRK2 has gained the most attention for its putative role in modulating dopamine function, LRRK2 also exerts regulation over immune responses. LRRK2 inhibits the transcription factor NFAT, which plays important roles in immune function as well as inflammatory bowel disease (Liu et al., 2011). Upon overexpression of LRRK2, NFAT remains cytosolic, and is unable to translocate into the nucleus, which suppresses its transcriptional activity (Liu et al., 2011). The MyD88 pathway is an additional pathway that is associated with the role of LRRK2 in immune function. Upon inflammation, TLR signaling via the MyD88 pathway leads to phosphorylation of LRRK2, implicating LRRK2 in macrophage biology (Dzamko et al., 2012).

LRRK2 REGULATES SMALL GTPases

A repeating theme in LRRK2 biology is its interactions with small GTPases. The ROC domain in LRRK2 is striking because it forms homo- and hetero- dimers with LRRK2 and LRRK2, respectively, *in vitro* (Shin et al., 2008; Hsu et al., 2010a; Kumar et al., 2010). The tendency of the LRRK2 ROC domain to dimerize appears to belie a broader biological characteristic, which is an ability to bind multiple small GTPases. As described below, we have shown that LRRK2 binds the small GTPase, rac1 (Chan et al., 2011). This observation parallels other studies showing that LRRK2 binds to or is functionally dependent on other small GTPases, such as rab5, rab7L1 and endoA (Matta et al., 2012; Beilina et al., 2014). Additionally, LRRK2 binds multiple GTP modulating proteins (described in following sections). In the case of rac1, the interactions of LRRK2 appears to regulate the site of action of the small GTPase, and leads to strong effects on the cytoskeleton, and for arfGEF7, they regulate the growth cone (Habig et al., 2013).

The interaction between LRRK2 and rac1 was observed by co-immunoprecipitation. LRRK2 could immunoprecipitate with rac1 upon over-expression in cell lines (HEK293FT cells), as well as from endogenous human brain striatum; in contrast binding to Cdc42 was weak and binding to RhoA was not apparent (Chan et al., 2011). Specificity of the interaction was shown by selective precipitation of rac1, without precipitation of the other classic Rho GTPases – Cdc42 and RhoA, although another study did find evidence for interaction of LRRK2 with cdc42 (Chan et al., 2011; Habig et al., 2013). The interaction between LRRK2 and rac1 occurs through the ROC–COR kinase domains. Co-expressing LRRK2 and rac1 enhanced rac1 activity by increasing binding

of rac1 to p21-activated kinase, which in turn modulates actin cytoskeletal dynamics. LRRK2 with inactivated kinase or GTPase domains does not activate rac1 (Chan et al., 2011). LRRK2 does not increase membrane-bound rac1, but it significantly changes the cellular localization of rac1, causing polarization, which is further augmented when LRRK2 is co-expressed with constitutively active rac1. G2019S and R1441C LRRK2 mutations decrease rac1 binding; whereas Y1699C and I2020T increase rac1 binding.

Rac1 is known to play an important role in actin cytoskeleton remodeling that is required for the maintenance of neurite morphology (Chan et al., 2011). The interaction between LRRK2 and rac1 results in distinct effects associated with changes in the actin cytoskeleton. Previous studies have shown that G2019S induces neurite retraction in both *in vitro* and *in vivo* studies through the MAPK signaling pathway and such pathology precedes dopaminergic neuronal death by apoptosis (Liou et al., 2008; Plowey et al., 2008; Parisiadou et al., 2009; Carballo-Carbajal et al., 2010; Hsu et al., 2010a,b). In SH-SY5Y cells, co-expression of rac1 and G2019S has shown to rescue neurite retraction induced by G2019S. These studies suggest that mutations in LRRK2 can lead to a decrease in activation of rac1, which causes disassembly of actin filaments leading to neurite retraction (Chan et al., 2011).

SYSTEMS BIOLOGY PROVIDES A COMPREHENSIVE ASSESSMENT OF CELLULAR PATHWAYS INTERACTING WITH LRRK2

The studies showing LRRK2 interacting with many varied cellular proteins present a challenge for understanding its function. LRRK2 has been suggested to bind to many different proteins, including moesin, tubulin, MKK3, 6 and 7, JIP1, 3, and 4, arfGAP1, arhGEF7, endoA, cyclin-GAK, rab5, rab7L1, 14-3-3, (Imai et al., 2008; Shin et al., 2008; Ko et al., 2009; Sancho et al., 2009; Gehrke et al., 2010; Hsu et al., 2010a,b; Kumar et al., 2010; Nichols et al., 2010; Chan et al., 2011; Matta et al., 2012; Stafa et al., 2012; Habig et al., 2013; Beilina et al., 2014). LRRK2 has a regulatory role in a wide variety of biological processes; such as, protein translations, cytoskeletal processes, vesicular dynamics, neurite extension, mitochondrial function, endoplasmic reticulum function, and autophagy. Broad proteomics studies point to interactions with multiple new proteins, as well as regulation of many mitochondrial and lysosomal proteins (Di Domenico et al., 2012; Beilina et al., 2014). The multiple functions and pathways associated with LRRK2 suggest a complex role for LRRK2 in neuronal biology. We have approached this question by using systems biology to create a regulatory network that outlines the multiplicity of functional interactions of LRRK2 with its partners.

We employed an *in silico* approach to elucidate the gene regulatory network linked to LRRK2 (Dusonchet et al., 2014). This approach used a network algorithm, termed the context likelihood of relatedness (CLR; Faith et al., 2007). This algorithm is designed to analyze state-dependent genome-wide expression data based on the degree of synchrony in variation of transcript levels among samples. Thus, transcripts whose expression varies in concert with LRRK2 transcripts are deemed neighbors. We analyzed both brain and white blood cells; use of white blood cells diversified the pathway representation away from a predominance of genes linked to cell death pathways, presumably because white blood cells are not post-mortem tissues. The LRRK2-centered association subnetwork identified many known interactors, as well as many novel linked pathways (Dusonchet et al., 2014). For instance, network proteins that have been previously associated with LRRK2 are present in this network, including DJ-1, PINK1, MKK7, and JIP1 (Ho et al., 2009; Sancho et al., 2009; Carballo-Carbajal et al., 2010; Hsu et al., 2010a,b; Ferree et al., 2012; Dusonchet et al., 2014). The network also identified many novel networks linked to LRRK2. Subsequent studies validated the putative roles of these interacting proteins using knockdown studies in *C. elegans*.

Previous work from the laboratory established that LRRK2 expression protects dopaminergic neurons against degeneration induced by the mitochondrial toxin, rotenone. We tested the action of the putative network partners in LRRK2 mediated protection of dopaminergic neurons, and identified about 280 genes whose knockdown modified the effects of LRRK2 on dopaminergic neurons. Genes whose knockdown impaired LRRK2 action included genes known to be part of the LRRK2 network (DJ-1, PINK1, MKK7, and JIP1), as well as multiple other genes, including HDAC6, vps34 and unc51, each of which is associated with autophagy (Dusonchet et al., 2014). The connection between LRRK2 and autophagy is consistent with several other prior studies, as well as with studies suggesting a role for LRRK2 in vesicular biology (Biskup et al., 2006; Plowey et al., 2008; Alegre-Abarrategui et al., 2009).

One particularly interesting proteins identified through the network analysis was the signaling gene RGS2 (regulator of G protein signaling 2), which encodes for a GTPase-activating protein (GAP), as a statistically significant regulatory "hub" in the pathways linking LRRK2 with DJ-1, PINK1, and Parkin (Dusonchet et al., 2014). RGS2 is also of particular interest because prior studies indicate strong expression in dopaminergic neurons. This positioning of RGS2 as a "hub" for multiple genes linked to PD suggests a key role for RGS2 as a regulator of LRRK2 activity, function and neuronal toxicity (Dusonchet et al., 2014). RGS proteins are a family of proteins characterized by a GAP domain of ∼130 amino acids, the RGS domain. Other GAP proteins have also been associated with LRRK2 function, including ArfGAP1 and ArhGEF7 (Stafa et al., 2012; Xiong et al., 2012; Habig et al., 2013). Although GAPs and GTPase exchange factors (GEFs) are classically considered to function in regulating signaling of G-protein coupled receptors, these GAPs exhibit a strong role in regulating LRRK2 GTPase activity. Recombinant RGS2 and ArhGEF7 increase the GTPase activity of immunopurified full-length LRRK2 in a dose-dependent manner *in vitro*. Recombinant RGS2 also inhibits the LRRK2 kinase activity in a dose-dependent manner. However, the concentration of RGS2 required to achieve maximal inhibition of LRRK2 kinase activity, occurs at one tenth of the concentration of RGS2 that is required to stimulate GTPase activity maximally. It is increasingly clear that the output of LRRK2 is modulated by the particular GAP or GEF bound to it. All of the GAPs and GEFs appear to increase LRRK2 GTPase activity, but RGS2 and ArhGEF7 reduce LRRK2 kinase activity, while ArfGAP1 increases LRRK2 autophosphorylation (Stafa et al., 2012; Habig et al., 2013; Dusonchet et al., 2014). This suggests that the output from the GTPase domain is determine, in

part, by the particular GAP or GEF with which it is associated (**Figure 1**).

GAPs AND GEFs MODULATE CYTOSKELETAL EFFECTS OF LRRK2

A putative role for LRRK2 in regulating cytoskeletal function consistently appears in the literature. LRRK2 has been observed to regulate neurite outgrowth, tubulin and microtubules, trafficking proteins and actin (Hsu et al., 2010b; Chan et al., 2011; Caesar et al., 2013; Law et al., 2014). Our studies suggest that RGS2 inhibits the tendency of LRRK2 (WT and G2019S) to reduce length and complexity of neuronal processes in primary neurons (**Figure 1**; Dusonchet et al., 2014). Co-expression of RGS2 with LRRK2 significantly protects against G2019S LRRK2-induced neurite shortening, although RGS2 alone has no effect on axonal length. ArhGEF7 also increases LRRK2 GTPase activity but induces LRRK2 to increase neuronal arborization, length and growth cone formation (**Figure 1**; Habig et al., 2013). In contrast, knockdown of ArfGAP1 protects against G2019S LRRK2-induced neurite shortening (**Figure 1**; Stafa et al., 2012). ArhGEF7 acts through the actin cytoskeleton, which raises the possibility that it might act in tandem with rac1 and cdc42, both of which bind LRRK2 (Chan et al., 2011; Habig et al., 2013). These data suggest that small GTPases and their regulatory proteins act to regulate the actions of LRRK2 on the cytoskeleton and neurite outgrowth (**Figure 1**). Redundant actions of RGS2 and ArhGEF7 toward neurite cytoskeletal activity seems unlikely, which raises the possibility that RGS2 and ArhGEF7 exhibit divergent effects on LRRK2 when examining other functions, such as vesicular biology.

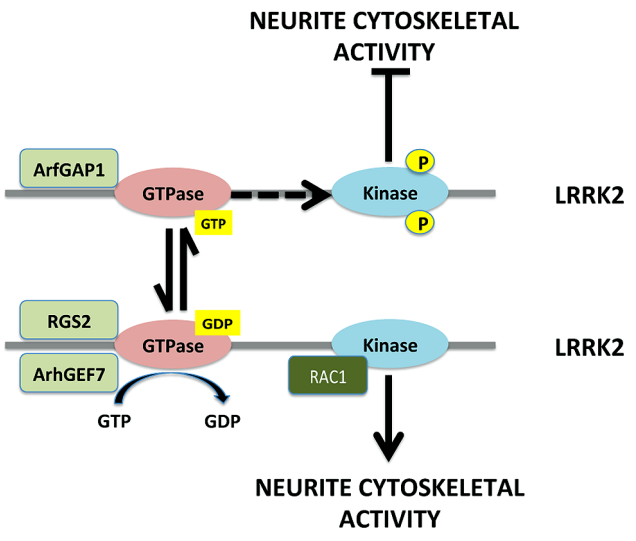

FIGURE 1 | Regulation of LRRK2 output by GTPase activity, GAPs and GEFs. RGS2 and ArhGEF7 both bind to LRRK2 and inhibit GTPase activity, which also inhibits LRRK2 kinase activity and appears to direct LRRK2 toward increasing neuronal cytoskeletal activity. Rac1 is a small GTPase known to regulate actin polymerase. Rac1 binds to LRRK2 and might act in concert with LRRK2 to increase cytoskeletal activity. In contrast, binding of ArfGAP1 to LRRK2 increases its autophosphorylation, which appears to inhibit cytoskeletal activity.

CONCLUSION

LRRK2 is a large molecule with many molecular interactions. Multiple studies have focused on its kinase activity, with the resulting identification of LRRK2 inhibitors that might have therapeutic benefit. The function of LRRK2 kinase activity remains unclear, with LRRK2 itself being the most robust substrate identified to date. Meanwhile, LRRK2 has been shown to interact with many different proteins, suggesting that important function of LRRK2 might lie in domains linked indirectly or not at all to its kinase function. A recent study suggests that an important action of phosphorylation is regulation of LRRK2 degradation, which would imply that one must look beyond kinase function to understand the role of LRRK2 in the neuron (Skibinski et al., 2014). The need to look beyond kinase function is emphasized by examination of the actions of GAPs and GEFs on LRRK2, where proteins that exhibit seemingly similar actions toward LRRK2 kinase activity elicit opposite actions on regulation of neurite outgrowth and cytoskeletal function (**Figure 1**).

Studies from our laboratory and other laboratories have identified numerous other signaling molecules that interact with LRRK2. The interactions of LRRK2 are clearly pleiotropic, and vary depending on the cell type and the function being investigated. Despite this complexity, several distinct themes are evolving, and these themes potentially have important implications for our understanding of the pathophysiology of PD. One theme that arises is the role of LRRK2 in regulating signaling cascades, such as the MAPK cascade. It is clear that LRRK2 acts in a manner that is different than classic modulators of the MAPK cascade, because it does not directly stimulate phosphorylation of MAP Kinases. The large size of LRRK2 enables it to bind multiple different proteins, and potentially bring them together as a signaling scaffold, analogous to JIPs.

A general theme occurring throughout the field of PD is regulation of vesicular transport. The vast majority of genes associated with PD have some function related to membranes and vesicles. α-Synuclein is thought to regulate production of small vesicles by promoting membrane curvature (Perlmutter et al., 2009; Varkey et al., 2010; Ducas and Rhoades, 2012). LRRK2 also shows strong interactions with membranes and is thought to modulate autophagy, which also involves vesicular dynamics. PINK1, parkin, and HTRA2 are all proteins that regulate mitochondrial function, possibly including mitophagy (Plun-Favreau et al., 2007; Narendra et al., 2010). ATP13A2 and GBA are both associated with lysosomes (Mazzulli et al., 2011; Usenovic et al., 2012). The consistent appearance of vesicular biology in the pathophysiology of PD suggests that interactions of LRRK2 with vesicles are likely to contribute to its mechanism of disease pathogenesis. Future studies will need to specifically investigate how signaling pathways regulate the interactions of LRRK2 with membranous organelles, such as autophagosomes. In this context, the preliminary data showing roles for the MAPK cascade in regulating the interactions of LRRK2 with the autophagic system are intriguing (Plowey et al., 2008).

The complexity of LRRK2 signaling provide insight into the nature of pathology associated with LRRK2-mediated disease. Most cases with LRRK2 mutations exhibit α-synuclein pathology, such as Lewy bodies, but some cases exhibit tau pathology

(Devine and Lewis, 2008). This pathological heterogeneity might derive from the impact of different disease processes on LRRK2 biology, with some signaling cascades (such as MAPKs) promoting tau pathology and other signaling cascades (such as vesicular biology) promoting α-synuclein pathology.

AUTHOR CONTRIBUTIONS

Joon Y. Boon drafted the manuscript, Chelsea Trengrove helped with revisions, and Benjamin Wolozin edited the manuscript. Julien Dusonchet provided important concepts for the manuscript.

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LRRK2 kinase activity regulates synaptic vesicle trafficking and neurotransmitter release through modulation of LRRK2 macro-molecular complex

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Mutations in Leucine-rich repeat kinase 2 gene (*LRRK2*) are associated with familial and sporadic Parkinson's disease (PD). LRRK2 is a complex protein that consists of multiple domains executing several functions, including GTP hydrolysis, kinase activity, and protein binding. Robust evidence suggests that LRRK2 acts at the synaptic site as a molecular hub connecting synaptic vesicles to cytoskeletal elements *via* a complex panel of protein-protein interactions. Here we investigated the impact of pharmacological inhibition of LRRK2 kinase activity on synaptic function. Acute treatment with LRRK2 inhibitors reduced the frequency of spontaneous currents, the rate of synaptic vesicle trafficking and the release of neurotransmitter from isolated synaptosomes. The investigation of complementary models lacking LRRK2 expression allowed us to exclude potential off-side effects of kinase inhibitors on synaptic functions. Next we studied whether kinase inhibition affects LRRK2 heterologous interactions. We found that the binding among LRRK2, presynaptic proteins and synaptic vesicles is affected by kinase inhibition. Our results suggest that LRRK2 kinase activity influences synaptic vesicle release *via* modulation of LRRK2 macro-molecular complex.

Keywords: LRRK2, kinase, presynaptic vesicle, synaptic activity, protein interaction

INTRODUCTION

Parkinson's disease (PD) is an age-related neurodegenerative disease affecting 2% of the population above 65-years and is clinically characterized by bradykinesia, rigidity, and resting tremor. The neuropathological hallmark of the disease is the progressive loss of dopaminergic neurons in the *substantia nigra* (Moore et al., 2005; Hardy et al., 2006). Although the majority of cases are idiopathic, mutations in the Leucine-rich repeat kinase 2 (LRRK2) gene (PARK8; OMIM 609007) cause late-onset PD. LRRK2 mutations account for up to 13% of familial PD cases compatible with dominant inheritance (Paisan-Ruiz et al., 2004; Zimprich et al., 2004) and have been identified in 1–2% of sporadic PD patients (Aasly et al., 2005; Berg et al., 2005). LRRK2 is a large protein encompassing several functional domains including a kinase domain with feature similar to mitogen activated protein kinase kinase kinases (MAPKKK) and receptor-interacting protein kinases (RIPK) (Bosgraaf and Van Haastert, 2003; Guo et al., 2006). Several single nucleotide variants have been identified in LRRK2 (Brice, 2005). While only the common G2019S mutation, located in the kinase domain, has been consistently associated with increased kinase activity *in vitro* (West et al., 2005; Gloeckner et al., 2006; Greggio et al., 2006), a recent study monitoring LRRK2 autophosphorylation at Ser 1292 suggested that other pathogenic mutants possess augmented activity in the cellular context (Sheng et al., 2012). Up to now few LRRK2 substrates have been identified in *in vitro* studies, but none has been convincingly proved *in vivo*, leaving the pathophysiological relevance of the kinase activity unclear. Instead, several lines of evidence suggest that kinase activity is linked to LRRK2 dimerization (Greggio et al., 2008; Sen et al., 2009; Civiero et al., 2012) as well as subcellular distribution (Berger et al., 2010) and regulates binding to 14-3-3 proteins (Nichols et al., 2010). Accumulating data correlate LRRK2 to synaptic functions. Several studies suggested that LRRK2 is part of a protein complex that influences the trafficking of synaptic vesicles belonging to the recycling pool (Shin et al., 2008; Piccoli et al., 2011; Matta et al., 2012). The description of synaptic phenotype in LRRK2 mutant models (Tong et al., 2009; Migheli et al., 2013; Yun et al., 2013) further underlines the tight link among LRRK2, synaptic vesicle trafficking and neurotransmitter release. In this study we investigated the functional

impact of LRRK2 kinase activity on presynaptic function and we determined functional properties of neurons upon LRRK2 pharmacological inhibition. A combination of electrophysiological, biochemical and imaging analyses suggested that LRRK2 inhibition impacts synaptic transmission acting on the organization of LRRK2 macro-molecular complex at the presynaptic site.

MATERIALS AND METHODS

ANIMALS, NEURON CULTURES, AND DRUGS

Non-transgenic wild-type (WT) and LRRK2 knock-out (KO) mice, back-crossed on a C57BL/6J strain, were obtained from Mayo Clinic (Jacksonville, FL, USA) through a collaboration with Prof. Matthew Farrer and Dr. Heather Melrose (Hinkle et al., 2012). Animals were kept following guidelines of Ministry of Education, Universities and Research (MIUR). Neuron cultures were prepared from either mouse cortexes or hippocampi obtained from embryonic day 15.5–16.5 mice (C57BL/6J). High-density (750–1000 cells/mm^2) and medium-density (150–200 cells/mm^2) neuron cultures were plated and grown as described on 12-well plastic tissue culture plates (Iwaki; Bibby Sterilin Staffordshire, UK) or on 12 mm diameter coverslips put into 24-well plastic tissue culture plates (Iwaki) (Piccoli et al., 2007). IN-1 and GSK-2578215A compounds (Tocris Bioscience, Bristol, UK) or DMSO were added to culture media at the concentrations indicated through the text.

PLASMIDS AND PROTEIN PURIFICATION

N-terminal 3xFLAG and myc hLRRK2 full length (hereinafter FLAG-LRRK2 and myc-LRRK2), N-terminal FLAG hLRRK2 A2106T (a kind gift of Prof. Dario Alessi, MRC, University of Dundee), LRRK2 silencing and control viral constructs vectors have been already described (Bauer et al., 2009; Nichols et al., 2009; Civiero et al., 2012). FLAG-LRRK2 was purified via affinity chromatography using FLAG-M2 agarose beads (Sigma Aldrich) as previously described (Civiero et al., 2012) from HEK293T cells transfected by lipofection using Lipofectamine 2000 (Life Technologies Carlsbad, CA, USA) according to manufacturer's instructions. Viral particles were produced as in (Bauer et al., 2009). Neurons were infected at DIV4 and processed when indicated.

IMMUNO-PRECIPITATION AND ANTIBODIES

Immunoprecipitation was performed as described previously (Onofri et al., 2007) using 25 µl of settled prewashed protein G-Sepharose beads (GE-Healthcare, Freiburg, Germany) to precipitate the immunocomplexes. NaCl 150 mM, Tris 50 mM (pH 7.4), NP-40 (1% v/v), SDS (0.1% v/v) and protease and phosphatase inhibitors extracts of Percoll-purified synaptosomes obtained from rat cerebral cortex were incubated for 2 h at RT in absence or in presence of IN- 1 (1 µM) with anti-LRRK2 antibodies (10 µg/sample; MJFF C41-2 Abcam, Cambridge UK) or a control rabbit IGg (10 µg/sample; Sigma-Aldrich, St. Louis, MO, USA). The eluted proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane (GE-Healthcare) and analyzed by western-blotting. Antibodies list includes rabbit anti LRRK2 1:500 MJFF C41-2, rabbit anti LRRK2 P-Ser 935 UDD2 10(12) (Abcam), rabbit anti synapsin I 1:500 (Synaptic System, Goettingen, Germany), mouse anti actin 1:1000, mouse anti FLAG 1:1000, mouse anti myc 1:1000, mouse anti synaptophysin 1:1000 (Sigma-Aldrich St. Louis, MO, USA). The secondary antibodies (HRP-conjugated anti-mouse, anti-rabbit) (BIORAD, Hercules, CA, USA) were used in a ratio of 1:5000 coupled with the ECL chemiluminescence detection system. Immunoblots were quantified by densitometric analysis of the fluorograms (Quantity One software, Bio-Rad) obtained in the linear range of the emulsion response.

IN VITRO KINASE ASSAY

GST-LRRK2$^{970-2527}$ (Life technologies) at the concentration of 30 nM were incubated with 500 µM LRRKtide, 100 µM ^{33}P-ATP (0.5 µCi) in kinase reaction buffer consisting of 25 mM Tris-HCl (pH7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$ and increasing concentrations of inhibitors at 30°C for 1 h. Reactions were carried out in triplicate and spotted onto P81 phosphocellulose. Following different washing of phosphocellulose membranes with 75 mM phosphoric acid, ^{33}P incorporation into LRRKtide was quantified with Cyclone (Perkin Elmer, Alameda, CA, USA).

SIZE EXCLUSION CHROMATOGRAPHY

Cells transiently transfected with FLAG-LRRK2 wild-type were solubilized in lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM $NaVO_4$, protease inhibitor cocktail (Sigma-Aldrich) and lysates were cleared for 30 min at 14,000 xg. When appropriate, proteins were further purified via FLAG immunoprecipitation as described above. Cleared lysates (0.5 ml; 5 mg total proteins) or purified proteins (0.5 ml; 1.3 µg of purified protein) were injected and separated on a Superose 6 10/300 column (GE Healthcare). The column was preequilibrated with buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.07% Triton X-100) and used at a flow rate of 0.5 ml/min. Elution volumes of standards were 7.5 ml for Blue Dextran (V0), 11.5 ml for hemocyanin from Carcinus aestuarii (900 kDa), 12 ml for thyreoglobin (669 kDa), 14 ml for ferritin (440 kDa). When appropriate, inhibitors (1 µM IN-1 and 1 µM GSK-2578215A) were applied for 90 min before lysis and kept throughout the following purification steps, including equilibration of chromatographic mobile phase. Chromatographic fractions were analyzed by dot blot. One microliter of each fraction from SEC was applied onto a nitrocellulose membrane. The membrane was blocked with 10% (w/v) milk in TBS plus 0.1% Triton (TBS-T) for 1 h and subsequently incubated with mouse monoclonal anti-Flag M2-peroxidase (Sigma-Aldrich). Immunoreactive proteins were visualized using enhanced chemiluminescence plus (ECL plus, GE Healthcare).

SYNAPTIC VESICLE PURIFICATION AND LRRK2 BINDING ASSAYS

Synaptic vesicles (SV) were obtained from rats by homogenization of the isolated forebrains and finally purified through the step of controlled-pore glass (CPG) chromatography (Huttner et al., 1983). After elution, purified SV were centrifuged for 2 h at 175,000 × *g* and resuspended at a protein concentration of 1–2 mg/ml in 0.3 M glycine, 5 mM HEPES, 0.02% sodium azide, pH

7.4 (glycine buffer). Protein concentrations were determined by the Bradford or BCA assays. SDS-PAGE was performed according to Laemmli (1970). For the dissociation of endogenously bound LRRK2 purified SV (40 μg/sample) were incubated for 1 h at 30°C with or without IN-1 (1 μM) in glycine buffer plus 30 mM NaCl, 25 mM Tris/HCl, 2 mM DTT, 10 mM $MgCl_2$ protease and phosphatase inhibitors. After the incubation, LRRK2 bound to SV were separated by soluble LRRK2 by high-speed centrifugation (400,000 × g for 45 min) (Messa et al., 2010). Aliquots of the resuspended pellets were subjected to SDS–PAGE and subsequent Western blotting with anti LRRK2 MJFF C41-2 (Abcam) antibody. The recovery of SV, used to correct the amounts of LRRK2 bound to SV, was determined by Western blotting with anti-synaptophysin antibody (kind gift of Prof. Paul Greengard The Rockefeller University New York USA). The binding of purified FLAG-LRRK2 to native SV was performed like below. SV (10 μg/sample) were incubated for 1 h at 0°C with FLAG-LRRK2 (50 nM) in glycine buffer plus 30 mM NaCl, 25 mM Tris/HCl, 2 mM DTT, 10 mM MgCl2 protease and phosphatase inhibitors and 1.0 μg/ml bovine serum albumin in absence or in presence of IN-1 (1 μM). After incubation, SV-bound FLAG-LRRK2 was separated by high-speed centrifugation (400,000 g for 45 min). Aliquots of the resuspended pellets were subjected to immunoblotting with anti-FLAG (Sigma-Aldrich) antibodies. The recovery of SV was determinated like above.

EXO-ENDOCYTOTIC ASSAY

The endocytosis assay to monitor SV recycling was performed using rabbit polyclonal antibodies directed against the intravesicular domain of synaptotagmin1 (Synaptic System), applied for 5 min if not indicated otherwise at RT on the cultures, as described previously (Matteoli et al., 1992). Incubations with the antibody (1:400) were performed in Tyrode solution containing 124 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 30 mM glucose, 25 mM HEPES, pH 7.4 and 2 mM $CaCl_2$. After fixation and permeabilization, a synaptophysin counter staining with mouse anti synaptophysin, 1:400 (Sigma-Aldrich) visualized the totality of SV. Acquired images were processed and quantitatively analyzed with ImageJ software as previously described (Verderio et al., 1999). Briefly, GFP positive processes were manually tracked and the number of synaptotagmin and synaptophysin positive clusters and synaptophysin positive clusters present in the region of interest were automatically counted.

NEUROTRANSMITTER RELEASE

Synaptosome were isolated from cerebral cortex (fronto-temporal areas) as described previously (Marti et al., 2003; Mela et al., 2004). The synaptosomal pellet was resuspended in oxygenated (95% O2, 5% CO2) Krebs solution (mM: NaCl 118.5, KCl 4.7, CaCl2 1.2, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, glucose 10). One millilitre aliquot of the suspension (∼0.35 mg protein) was slowly injected into nylon syringe filters (outer diameter 13 mm, 0.45 μM pore size, internal volume of about 100 μl; Teknokroma, Barcelona, Spain) connected to a peristaltic pump. Filters were maintained at 36.5°C in a thermostatic bath and superfused at a flow rate of 0.4 ml/min with a preoxygenated Krebs solution. Under the superfusion conditions adopted in the present study, the fast and continuous removal of endogenous substances released by nerve terminals rules out that endogenous glutamate is uptaken by glutamate transporters, or even activates autoreceptors. Sample collection (every 3 min) was initiated after a 20 min period of filter washout. The effect of IN-1 was evaluated on both spontaneous efflux and K+-stimulated neurotransmitter outflow. IN-1 (3 μM) was added to the perfusion medium 9 min before a 90 s pulse of 15 mM KCl, and maintained until the end of the experiment. In other experiments purified synaptosomes were prepared on Percoll gradients (Sigma-Aldrich) and incubated at 37°C for 15 min in presence of 0.03 μM [^{3}H]D-aspartate (Marte et al., 2010). A 90 s period of depolarization was applied at $t = 39$ min of superfusion with 15 mM KCl, substituting for an equimolar concentration of NaCl. IN-1 1 μM was added 9 min before depolarization. Fractions collected and superfused synaptosomes were counted for radioactivity by liquid scintillation counting. The efflux of radioactivity in each fraction has been expressed as a percentage of the total radioactivity present in synaptosomes at the onset of the fraction collected (fractional rate). Depolarization-evoked neurotransmitter overflow was calculated by subtracting the transmitter content of the two 3-min fractions, representing the basal release, from that in the two 3-min fractions collected during and after the depolarization pulse.

SLICE ELECTROPHYSIOLOGY

C57Bl/6J mice were anesthetized in a chamber saturated with chloroform and then decapitated. The brain was rapidly removed and placed in an ice-cold solution containing 220 mM sucrose, 2 mM KCl, 1.3 mM NaH_2PO_4, 12 mM $MgSO_4$, 0.2 mM $CaCl_2$, 10 mM glucose, 2.6 mM $NaHCO_3$ (pH 7.3, equilibrated with 95% O2 and 5% CO2). Coronal hippocampal slices (thickness, 250–300 μm) were prepared with a vibratome VT1000 S (Leica, Wetzlar Germany) and then incubated first for 40 min at 36°C and then for 30 min at room temperature in artificial CSF (aCSF), consisting of (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM glucose, and 26 mM $NaHCO_3$ (pH 7.3, equilibrated with 95% O_2 and 5% CO_2). Slices were then divided into 2 experimental groups: the first one was the control group and the second one was the group of slices incubated with the inhibitor 1 at concentration of 2 μM for at least 2 h. Slices were transferred to a recording chamber perfused with aCSF, where the concentration of $CaCl_2$ was increased to 4 mM and MgCl2 decreased to 0.5 mM, due to the low frequency of miniature excitatory post-synaptic currents (mEPSCs) in CA1 hippocampus, at a rate of ∼2 ml/min and at 38°C. Whole-cell patch-clamp electrophysiological recordings were performed with an Axon Multiclamp 700 B amplifier (Molecular devices, Sunnyvale, CA USA) and using an infrared-differential interference contrast microscope. Patch microelectrodes (borosilicate capillaries with a filament and an outer diameter of 1.5 μm; Sutter Instruments, Novato, CA USA) were prepared with a four-step horizontal puller (Sutter Instruments) and had a resistance of 3–5 MΩ.

mEPSCs were recorded at a holding potential of −65 mV with an internal solution containing: 126 mM K-gluconate, 4 mM NaCl, 1 mM EGTA, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 3 mM ATP (magnesium salt), 0.1 mM GTP mM (sodium salt), 10 mM

glucose, 10 mM HEPES (pH adjusted to 7.3 with KOH). Access resistance was between 10 and 20 MΩ; if it changed by >20% during the recording, the recording was discarded. All glutamatergic currents were recorded in the presence of bicuculline (20 μM) in the external solution, to block the GABAergic transmission, and lidocaine (500 μM), to block the action potentials onset. Currents through the patch-clamp amplifier were filtered at 2 kHz and digitized at 20 kHz using Clampex 10.1 Software (Molecular Devices). Analysis was performed offline with Clampfit 10.1 software (Molecular Devices).

ELECTROPHYSIOLOGICAL RECORDINGS OF CULTURED NEURONS

Whole-cell voltage clamp recordings were performed using a MultiClamp 700 A amplifier (Molecular devices) coupled to a pCLAMP 10 Software (Molecular Devices), and using an inverted Axiovert 200 microscope (Zeiss, Oberkochen Germany). Patch electrodes, fabricated from thick borosilicate glasses (Sutter Instruments) were pulled and fire-polished to a final resistance of 3–5 MΩ using a two-stage puller (Narishige, Japan). Experiments were performed at room temperature (20–25°C) in the external control solution KRH (125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO, 2 mM $CaCl_2$, 6 mM D-glucose, and 25 mM HEPES/NaOH, pH 7.4). The age of the patched neurons ranged between 13 and 16 DIV. Recordings were performed keeping neurons at holding potential of -70 mV, in the presence of 1 μM TTX and using the following internal solution (Potassium Gluconate—KGluc): 130 mM KGluc, 10 mM KCl, 1 mM EGTA, 10 mM HEPES, 2 mM $MgCl_2$, 4 mM MgATP, 0.3 mM Tris-GTP (pH 7.4, adjusted with KOH). Traces were acquired at 10 kHz and lowpass filtered at 4 kHz. Recordings with either leak currents >300 pA or series resistance >20 MΩ were discarded. Series resistance was monitored during experiments and recordings with changes over 20% control during experiments were also discarded. mEPSC traces were analyzed using MiniAnalysis Program (Synaptosoft Decatur, GA USA) with a threshold of 10 pA. Only events exceeding the baseline noise by >2 SDs were considered. The mean mEPSC frequency for CTRL neurons was 1.23646 ± 0.13746 Hz (mean ± *SE*).

STATISTICAL ANALYSIS

All data are expressed as mean ± standard error of the mean (SE). Data were analyzed with an unpaired Student's *t*-test (two groups) or ANOVA followed by Dunn's *post-hoc* test (more than two groups). The indication of number of experiment (n) and level of significance (p) are indicated throughout the text.

RESULTS

LRRK2 KINASE INHIBITION IMPAIRS SYNAPTIC TRANSMISSION

We previously demonstrated that LRRK2 controls synaptic transmission acting as a presynaptic scaffold (Piccoli et al., 2011). Given that LRRK2 possesses an active kinase domain, we investigated the impact of LRRK2 kinase inhibition on synaptic activity. We modulated LRRK2 kinase activity taking advantage of two potent LRRK2 inhibitors, IN-1 (Deng et al., 2011) and GSK-2578215A (Reith et al., 2012) (hereinafter GSK). These two molecules proved to inhibit LRRK2 kinase activity when tested by *in vitro* assays where GST-LRRKtide was offered to recombinant LRRK2 (**Figures A1A–C**). We confirmed IN-1 and GSK efficacy on acute hippocampal slices treated with IN-1 and GSK (both 1 μM, 2 h) before solubilization and western-blotting. LRRK2 kinase activity, indirectly monitored by P-Ser 935 level, was clearly impaired upon incubation of slices with IN-1 and GSK (**Figures 1A,B**). Thus, we monitored the effect of kinase inhibition on presynaptic SV recycling by exposing living culture to rabbit polyclonal antibodies directed against the intravesicular domain of synaptotagmin1, which are internalized inside the vesicle lumen upon SV recycling (Matteoli et al., 1992). Cortical cultures were infected at DIV4 with control viruses co-expressing GFP to track neuronal processes and assayed at DIV14. Prior to these assays, primary cultures were treated with vehicle (DMSO) or IN-1 or GSK (both 2 μM for 2 h). Vesicles within GFP positive processes were then monitored via laser confocal microscopy. The vesicles appeared as clusters either synaptotagmin and synaptophysin positive (i.e., cycling vesicles) or only synaptophysin positive (**Figure 2A**). The analysis showed that LRRK2 inhibition via either IN-1 or GSK induced a significant decrease in the number of synaptotagmin and synaptophysin positive clusters (**Figure 2B**). The total number of synaptic contacts, however, remained unaltered despite any pharmacological treatments (**Figure 2C**). A number of off-targets has been described for LRRK2 IN-1, including ERK5 (Luerman et al., 2014). Thus, in order to determine if the effect reported was specifically related to LRRK2 kinase inhibition, we quantified SV recycling rate in cultures infected at DIV4 with viruses expressing LRRK2 silencing constructs, siRNA LRRK2 (Bauer et al., 2009; Piccoli et al., 2011). As previously reported, LRRK2 silencing was associated to an increase in synaptotagmin uptake (Piccoli et al., 2011). Interestingly, we did not measure any significant alteration of SV recycling rate or number of synaptic contact upon IN-1 or GSK treatment in LRRK2 silenced culture (**Figures 2D,E**). These results likely exclude that the effect seen on SV trafficking arises from substantial off target effect of IN-1 or GSK. Furthermore, our data suggest that IN-1 and GSK have similar efficacy in terms of kinase inhibition and impact on SV trafficking. Based on the equivalent behavior of the two inhibitors, in the subsequent functional assays we focused mainly

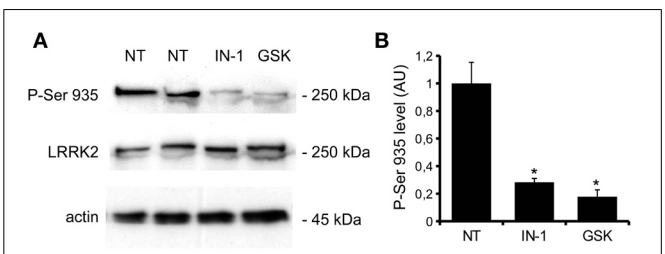

FIGURE 1 | IN-1 and GSK inhibit LRRK2 activity *ex-vivo*. (A) Acute hippocampal slices were incubated in regular aCSF (NT) or in aCSF + IN-1 (IN-1) or GSK (GSK) both 1 μM for 2 h at RT. Slices were then solubilized and assayed by western blotting for P-Ser 935, LRRK2 and actin level. **(B)** The graph reports P-Ser 935 level, normalized on LRRK2 level and calculated as fold over not treated sample. Data are expressed as mean ± s.e.m; $*p < 0.01$ vs. not treated sample, $n = 4$, ANOVA followed by Dunn's *post-hoc* test.

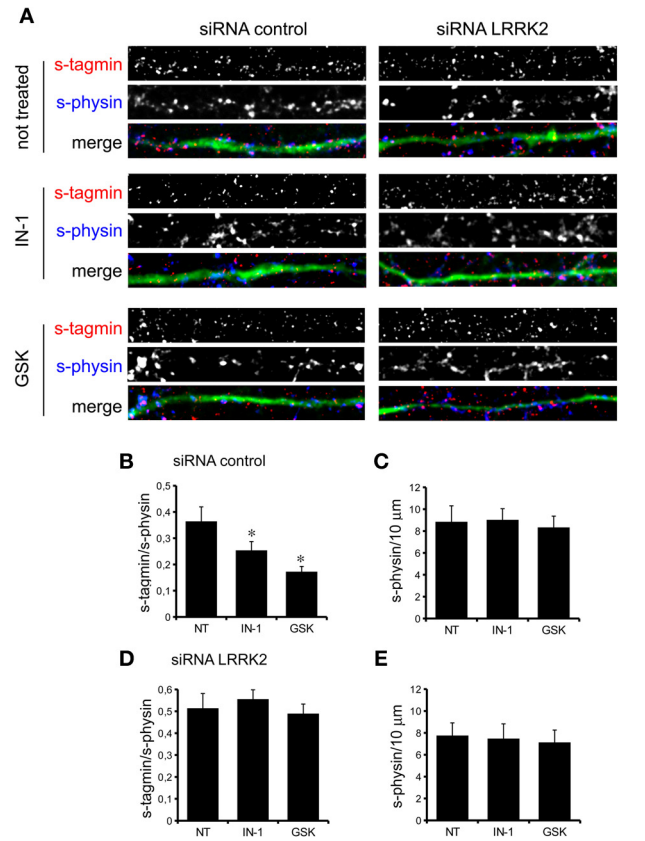

FIGURE 2 | LRRK2 kinase inhibition impairs SV trafficking. (A) The exo-endocytotic assay was performed on cortical neurons infected at DIV4 with virus expressing control siRNA and GFP and left untreated (NT) or incubated with IN-1 or GKS compound (both 2 µM for 2 h) before being tested at DIV14. Cycling SV appear as synaptotagmin (s-tagmin) positive clusters along neuron processes. Total SV pool was revealed by staining with anti-synaptophysin antibodies upon fixation and permeabilization. Images show signals acquired for synaptotagmin, synaptophysin and their superimposition plus GFP (merge). **(B)** The percentage of s-tagmin and s-physin positive clusters within the totality of s-physin positive clusters reflects the pool of cycling vesicles. **(C)** Total number of SV pools was not altered by treatment with IN-1 and GSK compound. The graph reports number of synaptophysin-positive clusters per 10 µm of GFP-positive process. **(D)** Similar experiments were performed on cortical neurons infected on DIV4 with viruses expressing LRRK2 siRNA and GFP. In LRRK2 down-regulated culture SV cycling is not affected upon treatment with IN-1 and GSK compound (both 2 µM for 2 h). **(E)** Total number of SV pools was not altered by treatment with IN-1 and GSK compound. The graph reports number of synaptophysin-positive clusters per 10 µm of GFP-positive process. Data are expressed as mean ± s.e.m.; $*p < 0.05$ vs. not treated, $n = 20$, ANOVA followed by Dunn's *post-hoc* test. Panel size is 35 × 5 µm.

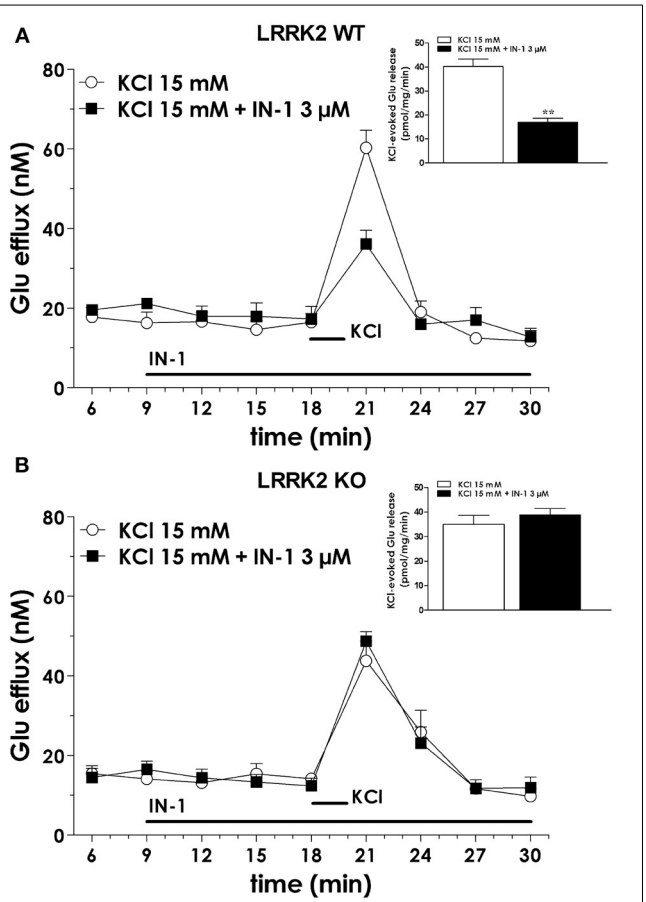

FIGURE 3 | IN-1 impairs neurotransmitter release from isolated synaptosome. (A) Synaptosomes obtained from the cerebral (fronto-temporal) cortex of LRRK2 WT mice were perfused with Krebs solution, and stimulated with a 90 s pulse of 15 mM KCl. IN-1 (3 µM) was perfused 9 min before KCl and maintained until the end of experiment. IN-1 reduced K^+-evoked glutamate overflow. Data are means ± s.e.m. of 5–6 determinations per group, and are expressed as absolute glutamate concentrations in the superfusate (in nM) or K^+-evoked glutamate overflow (in pmol/mg protein/min; insets). **(B)** Similar experiments were executed on cortical synaptosome obtained from LRRK2 KO mice. IN-1 (3 µM) failed to impair K^+-evoked glutamate overflow. Statistical analysis was performed on overflow values by the Student *t*-test for unpaired data. $**p < 0.01$ different from KCl alone.

on IN-1. Given the impact of LRRK2 inhibition on SV trafficking, we next investigated the effect on IN-1 on neurotransmitter release. To this aim we measured glutamate release from isolated synaptosomes upon IN-1 treatment in either basal or stimulated condition (**Figure 3A**). A pulse of 15 mM K^+ caused an approximate three-fold, transient elevation of glutamate levels. IN-1 (3 µM) did not affect spontaneous glutamate efflux, but inhibited the K^+-evoked glutamate overflow by about 60%. In a complementary approach, we measured the basal and evoked (15 mM K^+) release of [3H]D-aspartate in presence or not of IN-1 (1 µM). Also in this model, IN-1 impaired the K^+ evoked release by about 35% (calculated as fraction of overflow and expressed as mean ± s.e.m.: K^+ alone = 1.4 ± 0.01 K^+ + IN-1 = 0.9 ± 0.01, $p < 0.01$, $n = 7$, Student's *t*-test). To exclude potential off target effect of IN-1, we studied glutamate release in synaptosomes obtained from LRRK2 KO mice (**Figure 3B**). Spontaneous and K^+-evoked glutamate efflux was not different between the two genotypes. However, IN-1 (3 µM) did not significantly influence the K^+-evoked glutamate release in LRRK2 KO mice. Robust evidence correlates synapsin I to the mobilization of SV and release of neurotransmitter (Orenbuch et al., 2012).

Thus, we verified whether the lack of effect of IN-1 on glutamate release we reported in LRRK2 KO mice could arise from disturbed synapsin I level. Western blotting analysis of synaptosome from wild-type and LRRK2 KO mice did not shown any significant difference (**Figure A1D**). This evidence indicates that the impairment in neurotransmitter release arises from a specific effect of IN-1 on LRRK2. Given the impact of LRRK2 kinase inhibition on presynaptic functions, we next evaluated the functional outcome of LRRK2 inhibition in terms of neuronal activity. To this aim, we studied the electrophysiological properties in two different neuronal models, namely acute hippocampal slices and hippocampal cultures. First we exposed acute hippocampal slices obtained from wild-type mice at P21-22 to IN-1 2 μM for 2 h before electrophysiological recording in CA1 region. We detected a clear reduction in miniature excitatory responses (mEPSCs) frequency in the absence of any change in amplitude, rise, or decay time upon acute IN-1 treatment (**Figures 4A–F**). In order to validate the impact of LRRK2 inhibition, we measured the electrophysiological properties of primary hippocampal neurons treated with IN-1, 2 μM for 2 h. Our experiments demonstrated that acute IN-1 treatment reduces mEPSC frequency but not amplitude, rise or decay time (**Figures 4G–K**). Similar results were obtained by treating cells with GSK (1 μM, 2 h; $NT = 1 \pm 0.21$, GSK $= 0.5 \pm 0.09$, $p = 0.057$, $n = 6$; values represent frequency normalized on

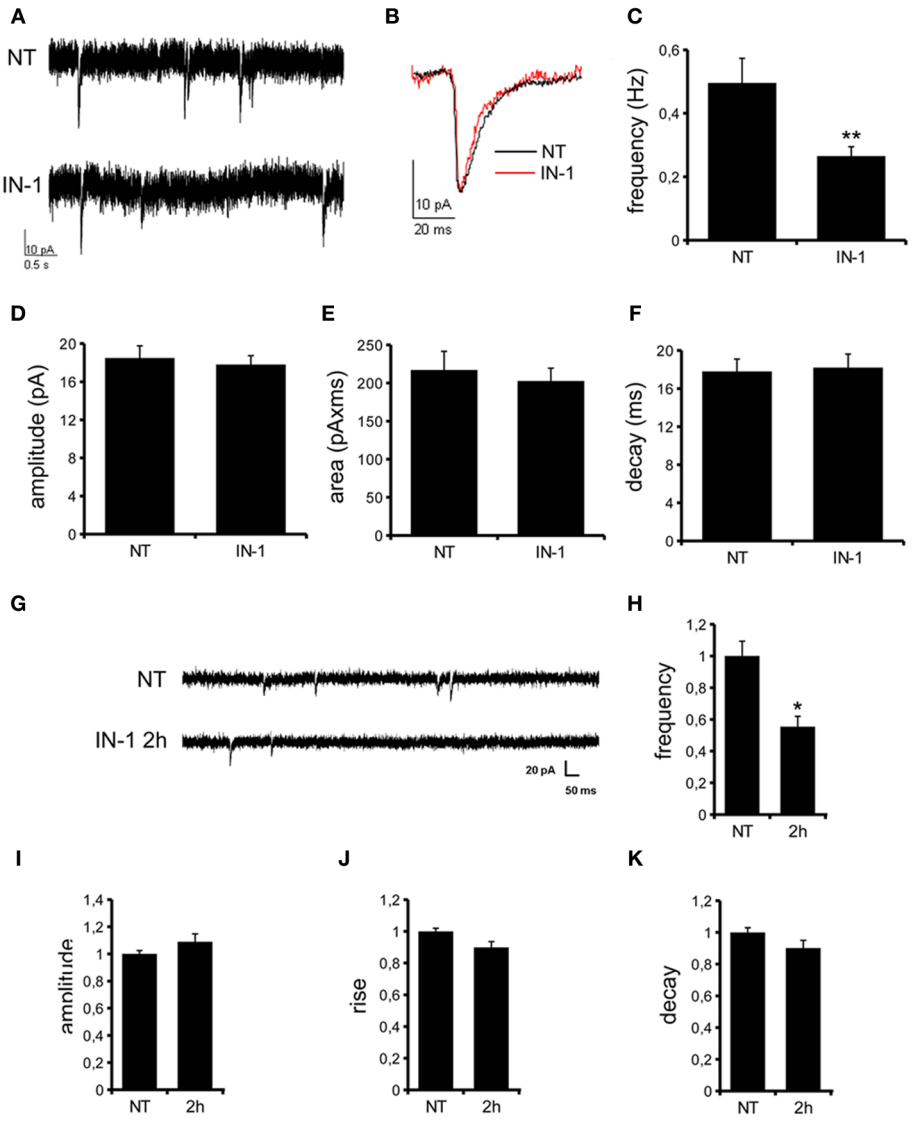

FIGURE 4 | IN-1 influences mEPSC frequency. (A) Representative traces of mEPSCs from CA1 pyramidal neurons of hippocampal slices incubated in regular aCSF (NT) or in aCSF + IN-1 (2 μM) for at least 2 h (IN-1). **(B)** A single mEPSC is shown. Quantification of mEPSCs basal properties reveal changes in current frequency **(C)** but not amplitude **(D)**, area **(E)** and decay **(F)**. Data are expressed as mean ± s.e.m.; $^{**}p < 0.01$ vs. NT, Student's *t*-test, $n = 15$. **(G)** Representative traces of mEPSCs from hippocampal culture at DIV12-13 under control condition (NT) or after incubation with IN-1 (2 μM) for 2 h. IN-1 pretreatment reduces mEPSC frequency **(H)**, but not amplitude **(I)**, rise **(J)** or decay **(K)** time. Data were normalized on not treated condition and expressed as mean ± s.e.m.; $^{*}p < 0.05$ vs. not treated, $n = 14$, ANOVA followed by Dunn's *post-hoc* test.

untreated cultures and expressed as mean ± s.e.m.). These experiments indicate that the pharmacological inhibition of LRRK2 kinase activity reduces synaptic transmission affecting SV recycling and thus neurotransmitter release.

KINASE ACTIVITY CONTROLS LRRK2 BINDING PROPERTIES

Independent studies demonstrated that LRRK2 exists in multiple oligomeric state: kinase-active dimer (Deng et al., 2008; Greggio et al., 2008; Klein et al., 2009) and monomers or oligomers mainly inactive (Sen et al., 2009). Thus, we asked whether LRRK2 kinase inhibition might influence LRRK2 oligomeric state. First we explored whether kinase inhibition affects LRRK2 homologous interaction by evaluating the extent of LRRK2 dimerization in presence of IN-1. To this aim we co-expressed FLAG-LRRK2 and myc-LRRK2 in HEK293T cells; we subsequently treated the cell with IN-1 (2 h, 1 μM) and eventually we immobilized LRRK2 on FLAG-M2 beads. After elution, we measured the recovery of FLAG and myc LRRK2 by immunoblotting with specific anti tag antibodies (**Figure 5A**). We found that IN-1 treatment does not significantly affect the amount of myc LRRK2 co-precipitating with FLAG-LRRK2 (**Figure 5B**). To further explore the impact of kinase inhibition on LRRK2 oligomerization, we performed size exclusion chromatography (SEC) experiments on FLAG-LRRK2 proteins purified from untreated cells and then incubated with IN-1 (1 μM, 90 min). As shown in **Figures 5C,D**, the elution profile of purified LRRK2 is only marginally affected by IN-1 inhibition. We obtained comparable results incubating FLAG-LRRK2 with GSK (1 μM, 90 min, data not shown). These data suggest that kinase inhibition minimally impacts LRRK2 oligomeric state. Next, we asked whether kinase inhibition engages LRRK2 in differential heterologous interactions. We have previously demonstrated that LRRK2 interacts with a panel of proteins, including actin (Piccoli et al., 2011). Thus, we analyzed by SEC the elution profile of FLAG-LRRK2 and actin in lysates extracted from cells treated with IN-1 (1 μM, 90 min). Interestingly, we observed that IN-1 shifted both LRRK2 and actin toward higher molecular weight forms and that the two elution profiles partially overlap (**Figures 6A,B**). This outcome might be consistent with the possibility that LRRK2 forms higher molecular weight complexes with actin upon IN-1 binding. To further substantiate this hypothesis we over-expressed FLAG-LRRK2 A2016T, an artificial variant unable to bind IN-1, in HEK293T cells (Nichols et al., 2009; Deng et al., 2011). When we analyzed by SEC the elution profile of FLAG-LRRK2 A2016T and actin in lysates extracted from cells treated with IN-1 (2 h, 1 μM), we observed that IN-1 failed to shift either LRRK2 or actin elution profiles (**Figures 6C,D**). All together these data strongly suggest that kinase inhibition induces the formation of high-molecular weight complexes including LRRK2 and its interacting partners. To further explore this hypothesis, we asked whether LRRK2 inhibition might affect LRRK2 affinity toward SV associated proteins such as synapsin I and actin. To this aim we immunoprecipitated LRRK2 with anti-LRRK2 antibodies [MJFF C41-2] using purified synaptosomes treated with IN-1 (1 μM) during the assay as protein source. We found that the binding of LRRK2 to synapsin I and actin increased in presence of IN-1 (**Figures 7A,B**). Given the effects of kinase inhibition on LRRK2 binding features, we first investigated if LRRK2 binds to SV and, next, if kinase inhibition disturbs LRRK2 and synapsin I binding to SV. To this aim we incubated native purified SV (in the range of 40 μg/sample) under phosphorylation permissive conditions or in the presence of IN-1 (1 μM, 1 h). After incubation, we recovered SV by high speed centrifugation and determined the amounts of bound LRRK2 and synapsin I by immunoblotting. The SV recovery in the pellet was evaluated based on synaptophysin immunoreactivity. We found that LRRK2 binds SV and that this interaction is significantly decreased in the presence of IN-1 while synapsin I binding to SV was unaffected by IN-1 (**Figures 7C,D**). As a complementary approach we analyzed the impact of IN-1 (1 μM, 1 h) on the interaction between SV and exogenous recombinant FLAG-LRRK2 (**Figure A1C**). After incubation, we separated SV-bound LRRK2 by high-speed centrifugation and evaluated the recovery of SV and bound LRRK2 and synapsin I in the pellet by immunoblotting. Our data showed that IN-1 significantly reduces exogenous LRRK2 binding to SV while the yield of SV-bound synapsin I remains unaltered (**Figures 7E,F**). This evidence suggests that kinase inhibition interferes with the macro-molecular complex bound to LRRK2 at the presynaptic site.

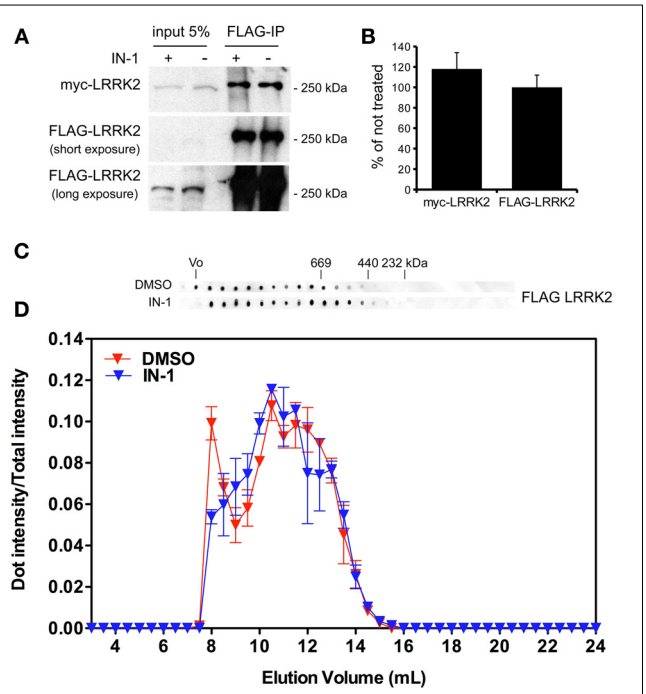

FIGURE 5 | IN-1 does not influence LRRK2 dimerization. (A) HEK293T cells expressing both myc and FLAG LRRK2 were treated or not with IN-1 (2 μM for 2 h), solubilized and processed for FLAG immunopurification. We evaluated the extent of LRRK2 homodimerization by measuring the amount of myc LRRK2 co-precipitating with FLAG LRRK2 **(B)** The graph reports the amount of FLAG and myc LRRK2 recovered in FLAG immunoprecipitates upon IN-1 incubation. Data were calculated as fraction of untreated sample and expressed as mean ± s.e.m. ($n = 4$). **(C)** Full-length LRRK2 purified by Flag immunoaffinity from untreated HEK293T cells was separated by size exclusion chromatography (SEC) and subsequently treated or not with IN-1 (1 μM, 90 min on ice). **(D)** The intensity of each dot (fraction) is normalized by the integrated intensities. Column void volume is 7.5 ml.

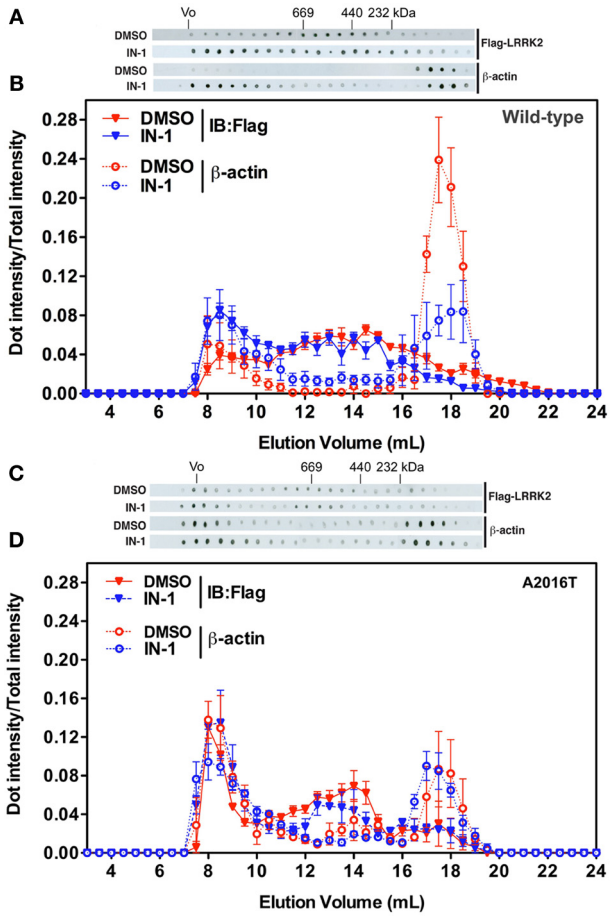

FIGURE 6 | IN-1 alters LRRK2 macromolecular complex. (A) HEK293T cells expressing FLAG-LRRK2 wild-type were treated or not with IN-1 (1 μM, 90 min), solubilized and then separated by SEC. FLAG-LRRK2 and actin were revealed by western-blotting of fractions spotted on nitrocellulose. **(B)** The intensity of each dot (fraction) is normalized by the integrated intensities. **(C)** HEK293T cells expressing FLAG-LRRK2 A2016T were treated or not with IN-1 (1 μM, 90 min), solubilized and then separated by SEC. LRRK2 and actin were revealed by western-blotting of fractions spotted on nitrocellulose. **(D)** The intensity of each dot (fraction) is normalized by the integrated intensities.

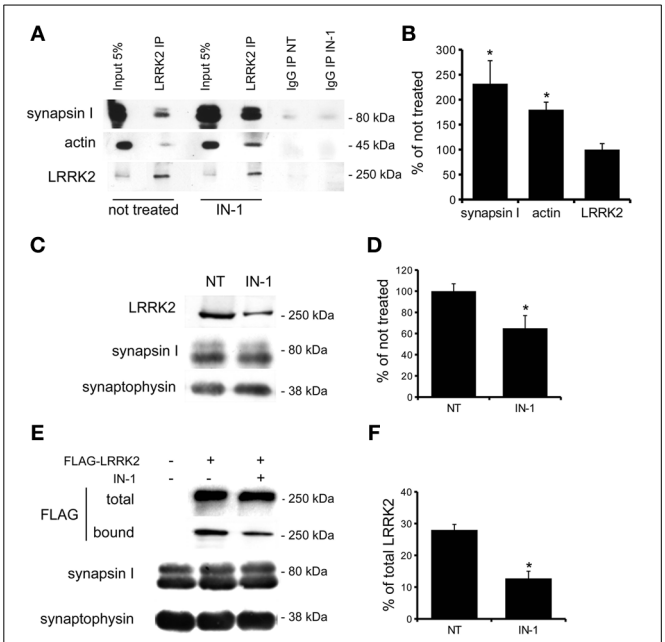

FIGURE 7 | IN-1 modifies LRRK2 binding properties. (A) Extracts of purified cortical synaptosomes were incubated with anti-LRRK2 antibodies or rabbit IgG in absence (not treated) or in presence of IN-1 (IN-1, 1 μM 2 h). The immunocomplexes were sedimented with protein G-Sepharose and the samples were resolved by SDS-PAGE and analyzed by immunoblotting with anti synapsin I, anti actin and anti LRRK2 antibodies. **(B)** Quantification of IN-1 effect on LRRK2 interaction with synapsin I, actin and LRRK2 itself. Results are calculated as percent of respective controls (not treated sample) and expressed as mean ± s.e.m. (*$P < 0.05$, $n = 4$, Student's *t*-test vs. not treated sample). **(C)** purified native synaptic vesicles (SV; 40 μg/sample) were incubated in the absence (NT) or presence of IN-1 (IN-1, 1 μM 2 h). After incubation, SV were recovered by high speed centrifugation and the residual amounts of endogenous LRRK2 bound to SV were determined by immunoblotting with anti-LRRK2 antibodies. The recovery of SV in the pellet was evaluated based on synaptophysin immunoreactivity. **(D)** LRRK2 recovery in the SV pellet was calculated as the percentage of the not treated sample and shown as mean ± s.e.m. (*$P < 0.05$, $n = 8$, Student's *t*-test vs. relative control). **(E)** Purified FLAG-LRRK2 was incubated with SV (10 μg protein/sample) in presence or absence (NT) of IN-1 (IN-1, 1 μM 2 h). SV-bound FLAG-LRRK2 was separated from free FLAG-LRRK2 by high-speed centrifugation and quantified by immunoblotting with anti-FLAG antibody. The recovery of SV in the pellet was evaluated based on synaptophysin immunoreactivity. **(F)** The binding of FLAG-LRRK2 to SV was calculated as the percentage of total FLAG-LRRK2 and expressed as mean ± s.e.m. *$p < 0.05$; Student's *t*-test vs. relative control.

DISCUSSION

Our previous observations provided evidence that LRRK2 executes critical functions at the presynaptic site; given its relative position as an integral part of a presynaptic protein network, LRRK2 may serve as a molecular hub coordinating both the storage and the mobilization of SV driven by activity (Piccoli et al., 2011, 2014). Recent work has clarified that LRRK2 controls SV in the ready releasable pool via inhibitory phosphorylation of the SNAP-25 interacting protein Snapin (Yun et al., 2013). The evidence reported here adds one more level of complexity: the implication of LRRK2 kinase activity within synaptic functions. As wild type LRRK2 is characterized by a low kinase activity (MacLeod et al., 2006), it might be argued that physiologically LRRK2 acts as a scaffold protein and its kinase activity mainly regulates its macro-molecular organization. In fact, several independent studies have revealed that LRRK2 exists in different forms in equilibrium, namely monomer, dimer and oligomer, being the dimer the predominant status under native conditions (Sen et al., 2009; but see Ito and Iwatsubo, 2012). Interestingly, PD associated LRRK2 mutations disturb both LRRK2 dimerization (Sen et al., 2009) and ternary complex formation (Nichols et al., 2010). Furthermore, acute treatment with IN-1 induces the aggregation of ectopic LRRK2 expressed in heterologous cell lines and interferes with 14-3-3 binding (Deng et al., 2011). Our hypothesis is that kinase inhibition triggers the formation of high molecular weight complexes encompassing LRRK2 and LRRK2 interacting

proteins. These phenomena might affect LRRK2 function at the presynaptic site. Accordingly, we described that kinase inhibition reduces LRRK2 binding to SV and at the same time increases LRRK2 affinity toward actin and synapsin I. As suggested by the SEC analysis of LRRK2 expression in heterologous lines, we can speculate that kinase inhibition induces the formation of LRRK2 high molecular weight complex also within neuronal cells. Such complex might act as dominant-negative on synaptic function. In fact it might not only sequester LRRK2 but also free actin and synapsin I, making them unavailable for physiological binding to SV (**Figure 8**). Indeed we did not report a clear reduction of synapsin I bound to SV upon IN-1 incubation or in presence of exogenous FLAG-LRRK2. This lack of an effect could arise from the design of our experimental setup. In fact we treated with IN-1 and/or FLAG-LRRK2 purified SV isolated from their cellular context and incubated in artificial buffer. LRRK2, synapsin I and actin regulate SV mobility from intrabouton pool to membrane where eventually SV fuse (Greengard et al., 1993; Piccoli et al., 2011; Orenbuch et al., 2012). Given the pivotal role of these three proteins during SV cycle and the biochemical consequences of kinase inhibition on the stability of LRRK2 binding with actin and synapsin I on one side and with SV on the other, not surprisingly kinase inhibition impairs proper SV cycling, reduces neurotransmitter release and, eventually, decreases synaptic activity. It remains unclear how kinase activity can interfere with LRRK2 biochemical features. In particular, kinase inhibition might modulate LRRK2: 1) by reducing cis or trans homo-phosphorylation thus affecting homodimerization and/or 2) by impairing binding of LRRK2 interacting proteins and/or 3) by abolishing phosphorylation of LRRK2 substrates themselves involved in regulating LRRK2 macro-molecular complex. While our studies do not address the third hypothesis, our present work favors the possibility that kinase activity drives the organization of LRRK2 macro-molecular complex. In fact, in our hands LRRK2 kinase inhibition does not have a major effect on LRRK2 dimerization but instead increases affinity toward interacting partners. Furthermore, while we executed a number of experiments in intact cells where potential LRRK2 interactors as well as signaling machinery up or down stream to LRRK2 kinase activity might be present and functional, we addressed the effect of kinase inhibitors also on isolated protein, i.e., purified LRRK2 extrapolated from its cellular context. In particular, we reported that upon IN-1 incubation purified LRRK2 binds less efficiently SV. Thus, a fourth intriguing hypothesis is that the binding itself of IN-1 affects LRRK2 properties. A recent observation suggested that IN-1 binding into the activation segment within kinase domain induces a wide-spread conformational change (Gillardon et al., 2013). Thus, it is possible that the structural stress consequent to IN-1 binding slightly modifies LRRK2 folding and thus impairs LRRK2 binding to SV. Given that our SEC analysis of isolated LRRK2 failed to reveal any significant effect of IN-1, we predict such effect to be minor or at least not able to robustly perturb LRRK2 oligomerization. Although further studies are indeed required to fully dissect these different hypothesis, overall our data indicate that LRRK2 function at the synaptic site depends on kinase regulation of LRRK2 macro-molecular organization. This might play a critical role also in PD pathogenesis. In fact severe synaptic defects have been reported in different models expressing kinase hyper-active LRRK2 (reviewed in Belluzzi et al., 2012). In particular, G2019S transgenic mice display an altered striatal DA release (Li et al., 2010; Melrose et al., 2010) and the overexpression of mutant G2019S influences SV trafficking rates (Shin et al., 2008; Yun et al., 2013). Thus, synaptic activity might arise as a key pathway affected by LRRK2 mutation. Being G2019S predicted as a gain-of-function mutation, huge effort has been spent to develop specific LRRK2 inhibitors. However, the side effect on kidney described upon chronic treatment with LRRK2 inhibitor (Herzig et al., 2011) together with the functional implication of LRRK2 endogenous kinase activity here described, suggest that other therapeutic strategies might result necessary. Our work suggests that the regulation of LRRK2 complex is a crucial molecular actor implicated in LRRK2 physiological function and demonstrate the necessity to tackle LRRK2 biology beyond its kinase activity.

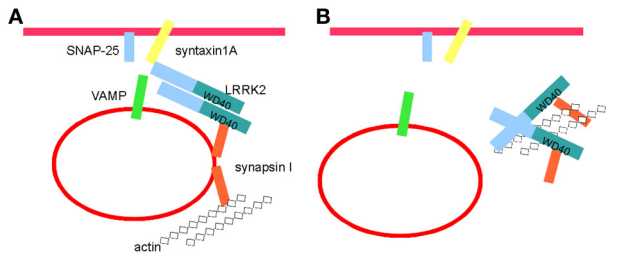

FIGURE 8 | Kinase activity controls LRRK2 molecular complex at the synaptic site. (A) LRRK2 binds SV and regulates thier trafficking via interaction with a panel of presynaptic proteins, including synapsin I, syntaxin 1, SNAP-25, VAMP, and actin (see also Piccoli et al., 2011, 2014). **(B)** Kinase inhibition detaches LRRK2 from SV and might induce the formation of high-molecular weight complex including LRRK2, synapsin I and actin. Such complex might sequester LRRK2, synapsin I and actin thus hampering their function within SV cycle.

AUTHOR CONTRIBUTIONS

Maria D. Cirnaru, Antonella Marte, Elisa Belluzzi, Isabella Russo, Martina Gabrielli, Francesco Longo, Ludovico Arcuri, Luca Murru performed experiments; Luigi Bubacco, Michela Matteoli, Ernesto Fedele, Carlo Sala, Maria Passafaro, Michele Morari, Elisa Greggio, Franco Onofri, and Giovanni Piccoli analyzed data; Michele Morari, Elisa Greggio, Franco Onofri, and Giovanni Piccoli designed experiments and wrote the paper.

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APPENDIX

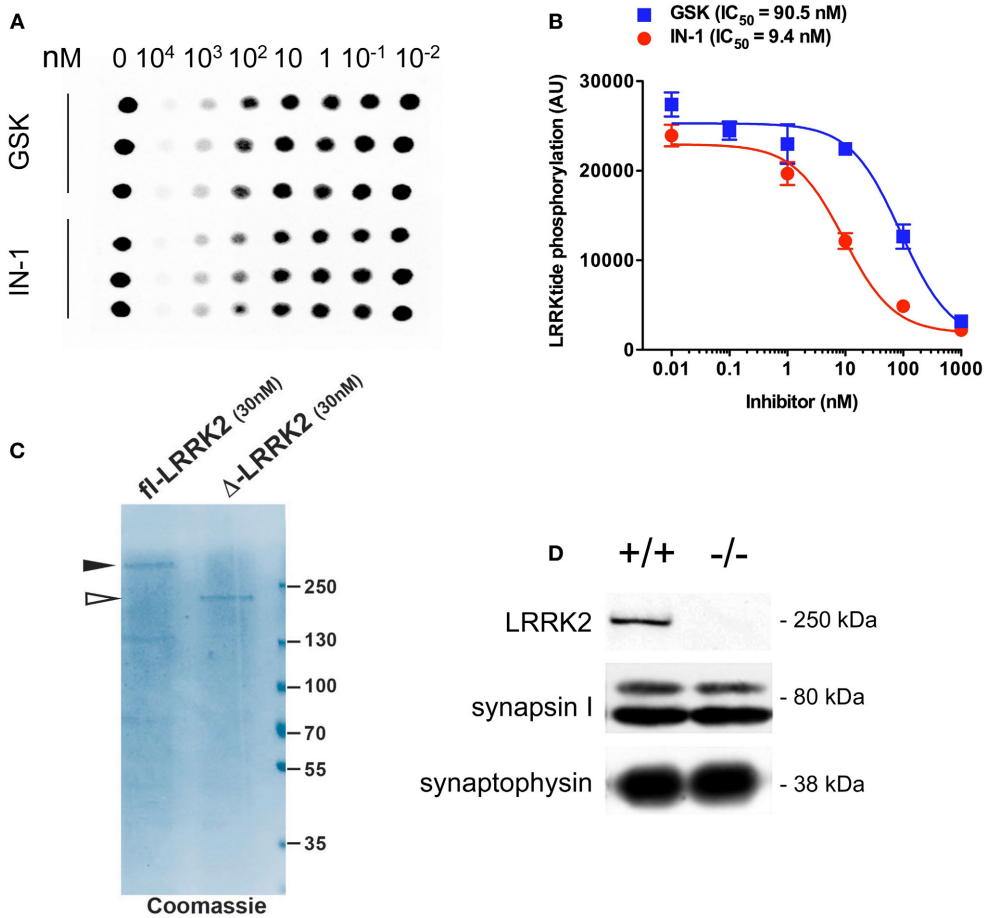

FIGURE A1 | IN-1 and GSK inhibit LRRK2 activity *in vitro*. **(A)** Recombinant GST-LRRK2$^{970-2527}$ was incubated with increasing concentrations of LRRK2 inhibitors IN-1 or GSK in the presence of 500 μM LRRKtide and 100 μM ATP (0.5 μCi ^{33}P-ATP). Reactions were spotted onto P81 phosphocellulose paper and LRRKtide radioactivity quantified by phosphoimaging scanner. **(B)** Dose-response curves and calculation of IC50 values indicates that both inhibitors are active against LRRK2 but at different potencies ($n = 6$ replicates, from 2 independent set of experiments). **(C)** Full length FLAG-LRRK2 purified from transfected HEK293T cells and GST-LRRK2$^{970-2527}$ were resolved on SDS-PAGE and visualized via coomassie staining. **(D)** Western-blotting analysis of synaptosome purified from wild-type and LRRK2 KO mice. Synapsin I level remains does not differ between the two genotypes.

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LRRK2 kinase activity and biology are not uniformly predicted by its autophosphorylation and cellular phosphorylation site status

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Missense mutations in the Leucine-Rich Repeat protein Kinase 2 (LRRK2) gene are the most common genetic predisposition to develop Parkinson's disease (PD) (Farrer et al., 2005; Skipper et al., 2005; Di Fonzo et al., 2006; Healy et al., 2008; Paisan-Ruiz et al., 2008; Lesage et al., 2010). LRRK2 is a large multi-domain phosphoprotein with a GTPase domain and a serine/threonine protein kinase domain whose activity is implicated in neuronal toxicity; however the precise mechanism is unknown. LRRK2 autophosphorylates on several serine/threonine residues across the enzyme and is found constitutively phosphorylated on Ser910, Ser935, Ser955, and Ser973, which are proposed to be regulated by upstream kinases. Here we investigate the phosphoregulation at these sites by analyzing the effects of disease-associated mutations Arg1441Cys, Arg1441Gly, Ala1442Pro, Tyr1699Cys, Ile2012Thr, Gly2019Ser, and Ile2020Thr. We also studied alanine substitutions of phosphosite serines 910, 935, 955, and 973 and specific LRRK2 inhibition on autophosphorylation of LRRK2 Ser1292, Thr1491, Thr2483 and phosphorylation at the cellular sites. We found that mutants in the Roc-COR domains, including Arg1441Cys, Arg1441His, Ala1442Pro, and Tyr1699Cys, can positively enhance LRRK2 kinase activity, while concomitantly inducing the dephosphorylation of the cellular sites. Mutation of the cellular sites individually did not affect LRRK2 intrinsic kinase activity; however, Ser910/935/955/973Ala mutations trended toward increased kinase activity of LRRK2. Increased cAMP levels did not lead to increased LRRK2 cellular site phosphorylation, 14-3-3 binding or kinase activity. In cells, inhibition of LRRK2 kinase activity leads to dephosphorylation of Ser1292 by Calyculin A and Okadaic acid sensitive phosphatases, while the cellular sites are dephosphorylated by Calyculin A sensitive phosphatases. These findings indicate that comparative analysis of both Ser1292 and Ser910/935/955/973 phosphorylation sites will provide important and distinct measures of LRRK2 kinase and biological activity *in vitro* and *in vivo*.

Keywords: LRRK2, Parkinson's disease, kinase, GTPase, phosphorylation, kinase inhibitor

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting 1–2% of the population over 65 years of age, with approximately 60,000 newly diagnosed patients per year. It is estimated that the prevalence of PD cases worldwide will double by the year 2030 (Dorsey et al., 2007). The increasing disability caused by the progression of disease burdens the patients, their caregivers, as well as society. Hallmark clinical features of PD include resting tremor, bradykinesia, postural instability and rigidity. PD also exhibits a wide variety of non-motor features such as autonomic dysfunction and dementia. Although the pattern of neuronal loss in PD is well-characterized, the molecular mechanisms of progressive cell death are still being elucidated. The majority of PD patients suffer from idiopathic disease with no clear etiology. However, approximately 5% of patients present with familial PD (Zimprich et al., 2004; Di Fonzo et al., 2005, 2006; Farrer et al., 2005; Healy et al., 2008; Paisan-Ruiz et al., 2008; Simon-Sanchez et al., 2009). Furthermore, exposure to a number of environmental toxicants has been shown to increase the risk of PD. Therefore, understanding the mechanisms of these known causes and risks will likely lead to the development of novel therapeutics, an unmet need.

Insights provided by understanding biochemical and cellular functions of the normal as well as mutated PD genes can provide insights into the pathogenesis of both inherited and idiopathic PD, because mutations in the leucine-rich repeat kinase 2 gene (*LRRK2*) are a common cause of inherited and idiopathic forms of PD. Genome-wide association studies have also identified LRRK2 as a risk factor for sporadic PD (Satake et al., 2009; Simon-Sanchez et al., 2009; Ross et al., 2011; Lill et al., 2012).

The informative nature of PD causing mutations on the molecular basis of disease is demonstrated by LRRK2, because clinical phenotypes of PD caused by LRRK2 mutation are largely indistinguishable from idiopathic disease (Ishihara et al., 2006; Ross et al., 2006; Haugarvoll et al., 2008; Haugarvoll and Wszolek, 2009), however ascertainment of larger LRRK2 Gly2019Ser patient populations will certainly define distinct clinical and pathologic features of LRRK2 parkinsonism (Zimprich et al., 2004; Adams et al., 2005; Whaley et al., 2006; Sossi et al., 2010).

LRRK2 encodes a large multi-domain protein with both a GTPase and a kinase domain. The amino terminus [1–1287 aa] is dispensable for kinase activity (Jaleel et al., 2007) but participates in regulation of LRRK2. This region of LRRK2 contains a phosphorylation cluster, an armadillo domain, an ankyrin domain, and a leucine-rich repeat domain. The remaining protein [1335–2527aa] consists of the minimal catalytic fragment, which includes the GTPase domain termed Ras of complex proteins (Roc), followed by the C-terminal-of-Roc domain (COR), which is contiguously connected to the N-terminus of the kinase domain. The kinase domain bears similarity to mixed lineage kinases, which are typically involved in kinase signaling cascades; however no upstream or downstream kinases have been validated yet. The carboxy terminus contains a WD40 domain and is essential for kinase activity. Deletion of the carboxy terminus or substitution of the risk factor mutation Gly2385Arg decreases kinase activity of LRRK2 (Jaleel et al., 2007; Greggio et al., 2008; Jorgensen et al., 2009; Rudenko et al., 2012). Many of the LRRK2 substitutions described to be pathogenic are concentrated in the catalytic tri-domain, including the Asn1437His, Arg1441Cys, Arg1441Gly, Arg1441His, and Tyr1699Cys in the Roc and COR domains, and Gly2019Ser and Ile2020Thr in the kinase domain (Biskup and West, 2009). The Arg1628Pro risk factor mutation falls within the COR domain (Ross et al., 2008; Tan et al., 2008). The most common mutation in inherited and idiopathic PD encodes Gly2019Ser, and is located in subdomain VII of the kinase domain and has consistently been shown to increase kinase activity 2–3 fold (Paisan-Ruiz et al., 2004; Zimprich et al., 2004; West et al., 2005; Jaleel et al., 2007). Other pathogenic substitutions, Arg1441Cys, Arg1441Gly, Arg1441His, Tyr1699Cys, and Ile2020Thr, modestly increase kinase activity and GTPase activity (West et al., 2005, 2007; Greggio and Cookson, 2009; Nichols et al., 2010; Webber et al., 2011).

LRRK2 kinase is itself a phosphoprotein that is regulated by upstream kinases and autophosphorylation. LRRK2 autophosphorylation has been observed on more than 20 threonine and serine residues with a preference for threonine (Kamikawaji et al., 2009; Gloeckner et al., 2010; Li et al., 2010; Pungaliya et al., 2010). Supplemental Table 1 gathers the reported autophosphorylation sites of LRRK2 from several publications. An Ala substitution of Thr1503 decreases GTPase activity and kinase activity (Webber et al., 2011). Autoregulation of Thr1348 or Thr1349 affects GTP binding and kinase activity (Kamikawaji et al., 2013). Autophosphorylation of Ser1292 has recently been observed within cells as an indicator of LRRK2 kinase activity through autophosphorylation (Sheng et al., 2012); however the comparative utility to other autophosphorylation sites has yet to be explored. In the absence of a validated downstream substrate for LRRK2, autophosphorylation is a potential tool to understand LRRK2 kinase activity in experimental or pathological conditions.

A cluster of serines, including Ser910, Ser935, Ser955, and Ser973, found preceding the namesake LRR domain, appears to be constitutively phosphorylated on LRRK2. It has been proposed that these sites are phosphorylated by kinases other than LRRK2 itself (West et al., 2007; Gloeckner et al., 2010; Nichols et al., 2010; Li et al., 2011; Doggett et al., 2012) and are referred to as the cellular phosphorylation sites herein. These sites are dynamically regulated, becoming rapidly dephosphorylated in cells and tissues after inhibition of LRRK2 with small molecule kinase inhibitors. This has been utilized as an indirect measure of LRRK2 activity in cells and tissues by multiple groups. The phosphorylation of LRRK2 is also implicated in mutation induced disease, as PD-associated mutations Asn1437His, Arg1441Cys, Arg1441Gly, Arg1441His, Tyr1699Cys, and Ile2020Thr all show decreased phosphorylation at the cellular phosphorylation sites Ser910/935/955/973. Dephosphorylation of the cellular sites links PD mutations and inhibition with similar molecular outcomes such as relocalization to cytoplasmic accumulations and filamentous skein like structures, loss of 14-3-3 binding and increased binding of PP1α (Greggio et al., 2006; Alegre-Abarrategui et al., 2009; Nichols et al., 2010; Kett et al., 2011; Lobbestael et al., 2013).

LRRK2 activity is regulated by inputs from other domains; these could be intramolecular or intermolecular via domain-domain interactions or oligomerization. The GTPase activity of Roc influences kinase activity of the kinase domain, while the carboxy terminus is also necessary for kinase activity. The PD-associated mutation Gly2385Arg or deletion of only the last seven amino acids in the C-terminus of LRRK2 leads to down-regulation of kinase activity. PD-associated mutations of the Roc domain [Arg1441Cys/Arg1441Gly/Arg1441His] or the COR domain [Tyr1699Cys], which showed reduced GTPase activity and/or impaired dimerization, also impact kinase activity of LRRK2. The kinase domain is thought to signal back to the amino terminus via a feedback phosphorylation mechanism of an intermediate kinase(s). Analysis of autophosphorylation mutants can help determine how each domain can regulate activity of LRRK2. Herein, we elucidate differential effects of PD mutations in the Roc-COR, kinase, and carboxy terminus of LRRK2 on the autophosphorylation at Ser1292, Thr1491, and Thr2483 with respect to cellular phosphorylation at Ser910/935/955/973. These outputs were also analyzed in phosphosite mutations of the amino terminus. We found that Ser1292 autophosphorylation is effective at detecting kinase activity in cells and reveals increased kinase activity of a heterozygous Gly2019Ser mutation in patient derived, Epstein-Barr virus (EBV) immortalized lymphoblasts. However, Ser1292 autophosphorylation is inversely regulated with the cellular site phosphorylation readouts in several PD associated mutations. We clearly show that Roc-COR PD mutations increase kinase activity and also decreased phosphorylation of the cellular sites. Alanine substitutions of Ser1292, Thr1491, Thr2483, Ser910/Ser935, Ser955, or Ser973 did not decrease LRRK2 kinase activity, nor did Ser1292, Thr1491, and Thr2483 to Ala substitutions decrease cellular site phosphorylation. We found that increased cyclic adenosine-monophosphate

(cAMP) does not lead to increased phosphorylation of Ser1292, Ser910, Ser935, Ser 955, or Ser973 in HEK293 cells. Additionally, we found that Ser1292 is regulated by Okadaic acid and Calyculin A sensitive phosphatases in contrast to the cellular sites which are only subject to regulation by Calyculin A sensitive phosphatases.

MATERIALS AND METHODS

REAGENTS AND GENERAL PROCEDURES

Tissue culture reagents were from Life Technologies or Thermo Scientific. The Flp-in T-REx system was from Invitrogen and stable cell lines were generated as per manufacturer instructions by selection with hygromycin as has been described previously (Nichols et al., 2010; Doggett et al., 2012). Restriction enzyme digests, DNA ligations, and other recombinant DNA procedures were performed using standard protocols with Fermentas enzymes. DNA constructs used for transfection were purified from Escherichia coli DH5a using Qiagen plasmid Maxi kits or Invitrogen Maxi pep kits according to the manufacturer's protocol. All DNA constructs and DNA transfections were performed using the polyethylenimine method according to Reed et al. (2006).

All DNA constructs used for transfections were provided by Dr. Dario Alessi (MRC-PPU, Dundee University, Dundee Scotland), except the autophosphorylation plasmids (FLAG-Thr1491, FLAG-Th2483, FLAG-S1292). These plasmids and the alanine substitution mutants of the autophosphorylation mutants were sub-cloned from the corresponding pCMV5-FLAG constructs. All mutagenesis experiments were carried out using the GeneArt site-directed mutagenesis kit (Life Technologies). All DNA constructs were verified by DNA sequencing, performed by Sequetech, Mountain View, CA. Full length amino terminal FLAG tagged LRRK2 was expressed by expressed by BacMam infection of HEK293 cells and purified by immunoaffinity chromatography with anti-FLAG M2. Inhibitor GNE1023 was described in Sheng et al. (2012) and synthesized at Genentech, LRRK2-IN1 was purchased from Tocris and Forskolin and IBMX were from Sigma.

BUFFERS

Lysis Buffer contained 50 mM Tris/HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM Benzamidine, and 1 mM phenylmethanesulphonylfluoride (PMSF) and was supplemented with 1% Triton X-100. Buffer A contained 50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.1 mM EGTA, and 0.27 M sucrose. Kinase buffer was 50 mM Tris 7.4, 0.1 mM EGTA.

CELL CULTURE, TREATMENTS AND CELL LYSIS

HEK-293 cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine and 1× antimycotic/antibiotic solution. HEK-293 T-REx cell lines were cultured in DMEM supplemented with 10% FBS and 2 mM glutamine, 1× antimycotic/antibiotic and 15 μg/ml Blasticidin and 100 μg/ml hygromycin. Cell transfections were performed by the polyethylenimine method (Reed et al., 2006). T-REx cultures were induced to express the indicated protein by inclusion of 1 μg/ml doxycycline in the culture medium for 24 h. Human lung alveolar epithelial A549 cells were cultured in Ham's F-12 (Kaighn's) with 10% FBS, 1× antimycotic/antibiotic. After the indicated culture conditions, cells were washed once with PBS and lysed *in situ* with 1.0 ml of lysis buffer per 15 cm dish on ice, then centrifuged at 15,000 × g at 4°C for 15 min. HEK-293 cells transfected with LRRK2 WT and mutant plasmids were lysed 48 h after transfection. Lymphoblastoid cell lines generated by EBV transformation of B lymphocytes were obtained from Coriell Institute for Medical Research. Cell line ND00075 (+/Gly2019Ser) is derived from a donor heterozygous for a G > A transition in exon 42 of LRRK2. Cell line ND03335 is an asymptomatic donor. Human lymphoblastoid cells were maintained in RPMI 1640 with 10% FBS, 2 mM glutamine, 1× antimycotic/antibiotic and were maintained at cell density of 0.3×10^6–2×10^6 cells per ml. Protein concentrations were determined using the Bradford method with BSA as the standard.

KINASE ASSAYS

Kinase assays were set up in a total volume of 50 μl with recombinant LRRK2 or immunoprecipitated LRRK2 as a source of kinase in 50 mM Tris/HCI, pH 7.5, 0.1 mM EGTA, 10 mM MgCl2, and 0.1 mM (γ-^{32}P) ATP (500–600 c.p.m/pmol) in the presence of 200 μM LRRKtide peptide substrate. Reactions were incubated at 30°C for the indicated times. Reactions were terminated by addition of LDS protein loading buffer or applying 40 μl of the reaction mixture on to P81 phosphocellulose paper and immersion in 50 mM phosphoric acid. After extensive washing, reaction products were quantified by Cerenkov counting. For autophosphorylation assays, 140 nm of FLAG-LRRK2 was incubated at 30°C for the indicated times in the presence of 10 mM $MgCl_2$ and 0.1 mM ATP and stopped by the addition of an equal volume of ice-cold 100 mM EDTA. Reaction products were spotted on nitrocellulose and immunoblotted with FLAG and autophosphorylation site antibodies.

LRRK2 IMMUNOPRECIPITATION ASSAYS

Cell lysates were prepared in lysis buffer (1.0 ml per 15 cm dish) and subjected to immunoprecipitation with anti-FLAG M2 agarose or GFP-Trap A beads (Chromotek) at for 1 h. Beads were washed twice with Lysis Buffer supplemented with 300 mM NaCl, then twice with Buffer A. Immune complexes were either used in kinase assays or incubated at 70°C for 10 min, passed through a Spin-X column (Corning) to separate the eluate from the beads, then boiled in LDS sample buffer. LRRK2 transfected HEK293 cell lysates were subjected to immunoprecipitation as with GFP-Trap beads. For endogenous immunoprecipitation assays, LRRK2 was immunoprecipitated using Anti-LRRK2 (UDD3, Abcam) noncovalently conjugated to protein-A sepharose (1 μg antibody: 1 μl bead) and analyzed by immunoblotting.

STATISTICAL ANALYSIS

For quantification of phosphorylation levels, LRRK2 protein levels were normalized for expression and to the control experimental condition. Statistical analysis was done using GraphPad Prism 6. One-sample *t*-tests using the hypothetical value of 1 for comparison and standard One-Way ANOVA tests were performed

with either the Dunnett correction for comparison with a single mean or the Tukey–Kramer correction if every mean is compared individually. *P*-values are designated as $^{*}p \leq 0.05$.

RESULTS

DIFFERENTIAL PHOSPHO-REGULATION OF LRRK2 IN PD ASSOCIATED MUTANTS

In vitro characterization of LRRK2 autophosphorylation in wild type and PD-associated LRRK2 mutants

LRRK2 phosphorylation is a heavily studied aspect of the enzyme's regulation. LRRK2 phosphosites have been identified from *in vitro* autophosphorylation kinase assays and from enzyme isolated from cells. LRRK2 contains sites of autophosphorylation and also sites that are modified by upstream kinases. We first wished to test the *in vitro* utility of the autophosphorylation site antibodies as indicators of LRRK2 kinase activity in an isolated system, to which we could compare the effects we observe in cellular studies. To detect LRRK2 phosphorylation, we utilized a series of rabbit monoclonal antibodies (anti-pSer910, anti-pSer935, anti-pSer955, anti-pSer973, anti-pThr1491, anti-pThr2483), generated by the Michael J. Fox Foundation and characterized by their Antibodies Working Group, and anti-pSer1292 (generously provided by Genentech), and a rabbit polyclonal anti-pThr1503 [kind gift of Dr. Andrew West, UAB (Webber et al., 2011)]. We verified specificity for the phosphorylation sites with Ser/Thr to alanine mutants, Supplemental Figure 1 and observed no *ab initio* phosphorylation of Thr1491 or Thr2483. We also observed that Ala substitutions at these sites did not negatively impact autophosphorylation at any of the other sites. We next employed full length recombinant LRRK2 [wild-type, Gly2019Ser, Arg1441Cys, and Asp1994Ala] (LifeTechnologies), which are approximately 80% pure as indicated by colloidal blue staining, **Figure 1A**. Immunoblot analysis of these preparations with anti phospho-Ser910, 935, 955, and 973 antibodies, revealed that phosphorylation of the cellular sites is maintained throughout the purification process and is similar between LRRK2 wild-type and Gly2019Ser. We observed no decrement in the cellular phosphorylation sites of the Arg1441Cys mutation, which has been previously described for Arg1441Gly, Arg1441His substitutions at this residue (Nichols et al., 2010; Doggett et al., 2012). Interestingly, only phosphorylation of the Ser1292 autophosphorylation site was detected in these recombinant protein preparations, as we could not detect *ab initio* phosphorylation of Thr1491 or Thr2483. The PD causing mutations Gly2019Ser and Arg1441Cys exhibited enhanced Ser1292 phosphorylation compared to wild-type, and kinase inactive LRRK2 is not modified at Ser1292 whereas the cellular sites are, **Figure 1B**.

We characterized the LRRKtide kinase activity of the full LRRK2 length proteins in order to correlate autophosphorylation activity with substrate phosphorylation, **Figure 1C**. We confirmed that Gly2019Ser mutation exhibited approximately a 2.5 fold increase in specific activity (determined from the linear time points of the reaction) and also observed that the Arg1441Cys mutation increases kinase activity against a peptide substrate approximately 20% over wild-type LRRK2 (Jaleel et al., 2007; Webber et al., 2011), but not at levels equal to Gly2019Ser.

We next compared LRRK2 autophosphorylation at Ser1292 in the amino terminal domain, at Thr1491 and Thr1503 in the central (Roc-COR) region and Thr2483 within the carboxy terminus. We performed *in vitro* autophosphorylation assays with full length LRRK2 in the absence of a substrate and analyzed the ability of the LRRK2 protein to phosphorylate itself over a time course of 60 min using immunoblot of the autophosphorylation sites as a readout. The reaction products were analyzed with anti-pSer1292, pThr1491, pThr1503, and pThr2483 antibodies and quantitated with Odyssey Software (LiCor).

The autophosphorylation sites on LRRK2 were fully modified and reached maximal detection by 20 min, showing similar *in vitro* kinetics of LRRKtide phosphorylation, **Figures 1C–G**. All antibodies detected a twofold increase in autophosphorylation in the Gly2019Ser mutant and the autophosphorylation activity of the Arg1441Cys mutant is increased relative to wild-type for each antibody tested. Since Ser1292 is phosphorylated at the beginning of the reaction, Gly2019Ser and Arg1441Cys time zero phosphorylation levels are not zero. In contrast to the autophosphorylation sites, the cellular LRRK2 signals did not increase throughout the kinase reaction (data not shown), suggesting that these sites have reached their limit of phosphorylation and further suggesting these are not autophosphorylation sites. Autophosphorylation of LRRK2 and LRRKtide phosphorylation are comparable indicators of LRRK2 kinase activity.

Reciprocal regulation of the cellular sites and LRRK2 autophosphorylation

Phosphorylation of the LRRK2 cellular sites is disrupted in the PD mutations Asn1437His, Arg1441Gly, Arg1441His, Tyr1699Cys, and Ile2020Thr (Nichols et al., 2010; Li et al., 2011; Doggett et al., 2012; Lobbestael et al., 2013), while at the same time exhibiting small or enhanced levels of kinase activity (Sheng et al., 2012). To characterize and further understand this dichotomy, we sought to directly compare phosphorylation of Serines910, 935, 955, and 973 with the three autophosphorylation sites Ser1292, Thr1491, and Thr2483 on LRRK2.

We compared the basal phosphorylation in cells to the *in vitro* autophosphorylation of PD mutated LRRK2 using anti-GFP immunoprecipitates from HEK293 T-REx cells expressing GFP-LRRK2 WT, Arg1441Cys, Arg1441Gly, Ala1442Pro, Tyr1699Cys, Ile2012Thr, Gly2019Ser, and Ile2020Thr in a tetracycline inducible manner, **Figure 2A**. Ala1442Pro is an uncommon mutation (Huang et al., 2007) and has been shown to illicit similar lack of phosphorylation of LRRK2 at the cellular sites and form cytoplasmic accumulations (Greene et al., 2014). Tyr1699Cys is located in the COR domain and also is dephosphorylated at the cellular sites and forms cytoplasmic inclusions, and may impact Roc GTPase activity via decreasing self-association or directly on GTPase activity (Daniels et al., 2011). This mutant will allow us to directly assess the *in cis* influence of Roc-COR domain mutations on the intrinsic kinase activity of LRRK2. The various LRRK2 PD associated mutations were incubated in the presence or absence of Mg^{2+}/ATP, where reactions performed in the absence of ATP are representative of the basal state of modification in cells. Reaction products were analyzed by immunoblot with cellular site antibodies and antibodies against

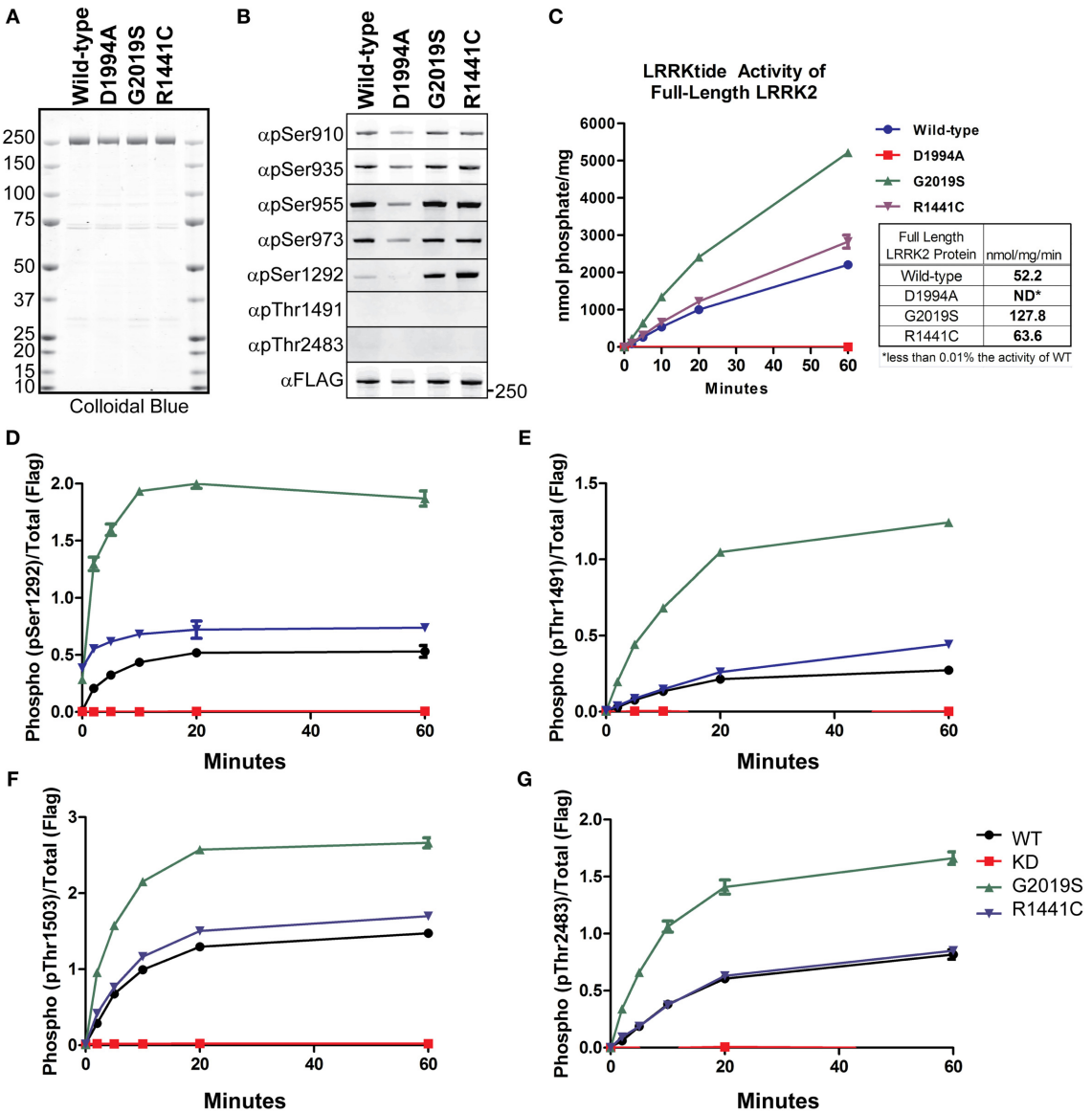

FIGURE 1 | Detection of autophosphorylation and cellular phosphorylation sites on recombinant LRRK2. The indicated full-length recombinant LRRK2 proteins were resolved by SDS-PAGE and stained with either **(A)** Colloidal Blue to quantify relative protein levels or **(B)** the indicated phospho-specific antibodies. **(C)** 14 nM of recombinant wild type LRRK2 (WT) or mutant LRRK2 [Asp1994Ala, Gly2019Ser, Arg1441Cys] *in vitro* kinase activity was measured against LRRKtide. Incorporated ^{32}P was assessed by Cerenkov counting and results are representative of three independent experiments. Error bars represent mean ± *SD*. **(D–G)** 140 nM recombinant LRRK2 was incubated with Mg^{2+}/ATP over a timecourse of 60 min where reaction aliquots were stopped by 1:1 (vol:vol) dilution in 200 mM EDTA and analyzed by dot-blot immunoblot with rabbit monoclonal anti-phospho-Ser1292 (αpS1292), anti-phospho-Thr2483 (αpThr2483), anti-phospho-Thr1491 (αpThr1491) and rabbit polyclonal anti-phospho-Thr1503 antibodies. Results are representative of three independent experiments performed in duplicate and blotted in duplicate. Error bars represent mean ± *SD*.

Thr1491, Thr2483, and Ser1292; data are presented as quantified LiCor Odyssey values of phospho-signal/total protein set to WT in Supplemental Figure 2. The basal phosphorylation state in the PD-associated mutants showed that Ser1292 and cellular sites (Ser935, 955, and 973) are not uniformly regulated. For mutants Arg1441Cys, Arg1441Gly, Ala1442Pro, and Tyr1699Cys we found that LRRK2 is dephosphorylated at the cellular sites, but is hyperphosphorylated at Ser1292. But like Arg1441Cys behaved *in vitro*, after incubation with ATP, Ser1292 phosphorylation was comparable with WT LRRK2, leaving Gly2019Ser and Y1699C as the only mutants that can incorporate significantly more phosphate at this site from all the mutants tested here. Threonines 1491 and 2483 were evaluated in the presence of ATP and we found that Thr1491 revealed significant increase in modification

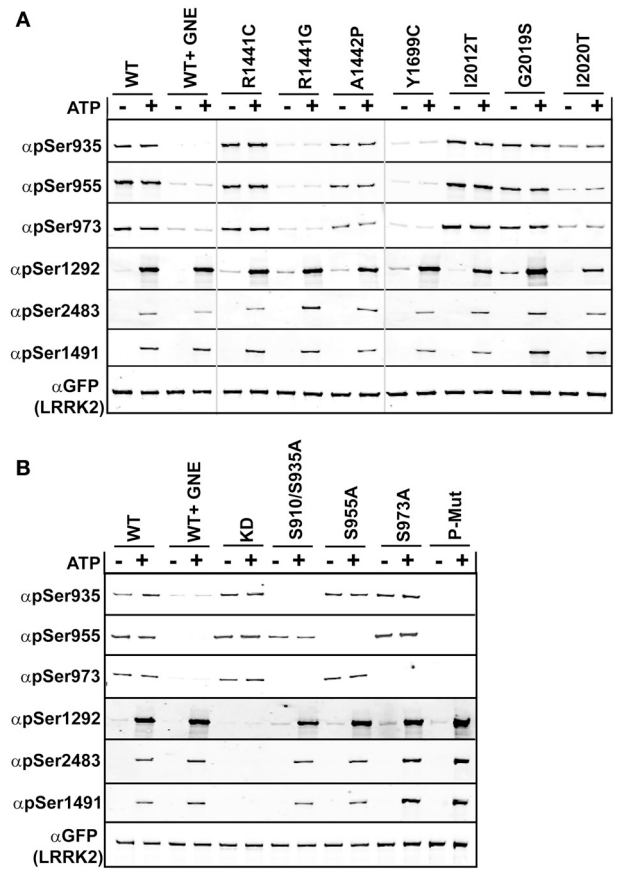

FIGURE 2 | Comparison of autophosphorylation and cellular phosphorylation sites in PD-associated mutants. Stable-inducible HEK 293 T-REx cell lines harboring the indicated forms of LRRK2 were incubated for 48 h with 1 μg/ml doxycycline to induce expression. The GFP-LRRK2 WT line was treated with 1 μM GNE1023 for 90 min, before immunoprecipitation-kinase assay using GFP beads, in the presence (+) or absence (−) of ATP. LiCor immunoblot analysis of **(A)** WT and PD associated mutants of LRRK2 and **(B)** WT and cellular phosphorylation mutants. Reaction products were probed with rabbit monoclonal anti-phospho-Ser935 (αpS935), anti-phospho-Ser955 (αpS955), anti-phospho-Ser973 (αp973), anti-phospho-Ser1292 (αpS1292), anti-phospho-Thr2483 (αpThr2483), anti-phospho-Thr1491 (αpThr1491). Blots were probed with anti-GFP (αGFP) for total protein control. Phosphorylation levels are quantitated in Supplemental Figure 2.

from the Gly2019Ser mutation, while Thr2483 revealed significant increased kinase activity from Arg1441Gly, and Gly2019Ser. Both Ile2020Thr and Ile2012Thr mutations reduced the basal level of Ser1292 and autophosphorylation activity similar to activity determinations using LRRKtide and Nictide (Jaleel et al., 2007; Nichols et al., 2009). The Ala1442Pro mutant revealed reduced levels of phosphorylation at Thr1491 and Ser1292.

Evidence that autophosphorylation and cellular phosphorylation sites are independently regulated, but dependent on LRRK2 kinase activity

Several LRRK2 PD mutations that have exhibited increased Ser1292 phosphorylation (Arg1441Cys, Arg1441Gly, Tyr1699Cys) were dephosphorylated at the cellular sites. Arg1441Gly, Arg1441Cys, and Tyr1699Cys mutants have been shown to form aggregates, which are also seen in the Ser910/935Ala mutant, due to the loss of phospho-dependent interaction with 14-3-3 proteins. We wanted to determine if the Roc-COR mutations effect on kinase activity could be correlated with the loss of cellular phosphorylation. Previous data and **Figure 2A** indicate that Roc-COR mutations act across the enzyme intramolecularly to the kinase domain to activate the kinase, but also intermolecularly inducing dephosphorylation of the amino terminal cellular sites. We hypothesized that the increase in 1292 phosphorylation in these mutants in cells could be due to intramolecular effects of the amino terminus on the kinase domain. An alternate explanation is there could be an increase in the local concentration or compaction of dephosphorylated LRRK2 in cytoplasmic inclusions that caused more autophosphorylation or less dephosphorylation. We tested these hypotheses on isolated GFP-LRRK2 from HEK293 T-REx cells with stable and inducible expression of LRRK2 cellular phosphosite to alanine mutations [Ser910/935Ala, Ser955Ala, Ser973Ala, and a combination mutant Ser910/935/955/973Ala], **Figure 2B**, which mimic dephosphorylation of the cellular sites by inhibition or mutation and analyzed LRRK2 phosphorylation after incubation with and without Mg^{2+}/ATP in an *in vitro* kinase assay.

As with the recombinant LRRK2 preparations, we found that autophosphorylation of Thr1491 and Thr2483 was only detected after incubation of LRRK2 with Mg^{2+}/ATP *in vitro*, in immune-complex kinase assays, **Figure 2B** and quantitated in Supplemental Figure 2. Mutation of cellular phosphorylation sites [Ser910/935Ala, Ser955Ala, and Ser973Ala] individually had no significant effect on the autophosphorylation of Thr1491 and Thr2483 in the presence of ATP, nor on Ser1292 in cells or *in vitro* in the presence of ATP, **Figure 2B**. However, in the combination mutant, all autophosphorylation sites trended toward increased kinase activity, (pSer1292 $p = 0.066$; pThr1491 $p = 0.097$; pThr2483 $p = 0.055$). Supplemental Figure 1 also indicates that reciprocal mutation of the autophosphorylation sites does not negatively affect phosphorylation at the cellular sites.

ANALYSIS OF SERINES 910/935/955/973 AND Ser1292 DEPHOSPHORYLATION

Serines 910/935/955/973 and Ser1292 are dephosphorylated after inhibition of LRRK2

In cells, phosphorylation of the cellular sites and autophosphorylation of Ser1292 is dependent on kinase activity of LRRK2. Inhibition of WT LRRK2 with a LRRK2 inhibitor induces the rapid dephosphorylation at the cellular phosphorylation sites (Ser910, 935, 955, 973) and the 1292 site (–ATP lane), **Figure 2**. To compare the sensitivity of dephosphorylation on serine 1292 vs. the various cellular sites, which can be used to indicate activity of LRRK2, we subjected cells expressing LRRK2 [Gly2019Ser] to a dose response of two structurally distinct LRRK2 inhibitors, LRRK2-IN1 (Deng et al., 2011) and GNE1023 (Estrada et al., 2012; Sheng et al., 2012) for 90 min. Cell lysates were analyzed by immunoblot with antibodies against Ser910, 935, 955, 973 in order to compare the phosphorylation status at each site, LiCor quantitation of immunoblots in Supplemental Figure 3. Both inhibitors induced a dose-dependent dephosphorylation of

LRRK2 at the cellular sites and 1292 site, Supplementary Table 1. We find that the shift in biochemical IC_{50} to cellular IC_{50} was on the same order of magnitude for Ser1292 compared to the cellular Ser935, Ser955, and Ser973 sites. We also found that LRRK2 Ala2016Thr mutation blocks the induced dephosphorylation at both types of sites showing that direct inhibition of LRRK2 is required for both dephosphorylation of the autophosphorylation site Ser1292 and the cellular phosphorylation sites, Supplemental Figure 3F. These data indicate that the four cellular phosphorylation sites are effective, though indirect measures of LRRK2 inhibition compared to the direct detection of *in vivo* LRRK2 kinase activity with pSer1292.

Comparison of Ser1292 and cellular site dephosphorylation in Parkinson's disease patient-derived lymphoblastoid cells

Since many mutations of LRRK2 increase the kinase activity of the enzyme, LRRK2 inhibitors could serve as a potential preventative or disease modifying drug. In the absence of reliable measures of drug-target engagement such as LRRK2 inhibitor PET ligands, peripheral markers of LRRK2 inhibition could serve as surrogate markers of LRRK2 inhibition in patients. LRRK2 is highly expressed in immune cells and we sought to compare the effectiveness of cellular site and autophosphorylation site antibodies as indicators of LRRK2 activity in EBV transformed lymphoblasts from a control and a PD affected patient heterozygous for Gly2019Ser missense mutation. The cells were treated for 90 min with 2 μM GNE1023 before endogenous LRRK2 was immunoprecipitated and equal amounts of protein were immunoblotted for pSer1292 and Ser935, Ser955, and Ser973 cellular phosphosite antibodies, **Figure 3B**. Similar levels of LRRK2 were found in the wild type and Gly2019Ser cell line. We were able to detect Ser1292 in the WT lymphoblasts (arrow in **Figure 3B**), with an increase in pSer1292 observed from the heterozygous Gly2019Ser cells, **Figure 3**. The increase in Ser1292 signal therefore reveals upregulated LRRK2 activity in Gly2019Ser lymphoblasts. Furthermore, the cells responded to treatment with GNE1023 in culture, showing dephosphorylation at both autophosphorylation and cellular sites, further validating the potential of these sites as potential pharmacodynamic markers of LRRK2 inhibition in patients.

cAMP does not increase LRRK2 phosphorylation in cells of kidney or lung origin

One model of LRRK2 regulation places the kinase domain downstream of the GTPase domain. In support of this, mutational analysis of the GTPase domain at Arg1441 appears to prolong the active state of LRRK2 by increasing its affinity for GTP and decreasing GTPase activity (Liao et al., 2014). Tyr1699Cys also decreases GTPase activity which could also lead to increased kinase activity (Daniels et al., 2011). We demonstrate in **Figures 1–3**, that these Roc-COR mutations increase kinase activity while strongly promoting dephosphorylation of the cellular sites. An alternate defect in LRRK2 regulation by Arg1441Gly was recently reported in Muda et al. (2014), where Ser1444 has been proposed as a new site of PKA phosphorylation that is required for 14-3-3 binding. *In vitro* Ser1444 phosphorylation and 14-3-3 binding are disrupted in the Arg1441Gly mutant, resulting in increased kinase activity in this model. LRRK2 was also found to intersect with PKA function by downregulating PKA activity in striatal projection neurons, where loss of LRRK2 or expression of a Arg1441Cys mutation leads to increased GluR1 and cofillin phosphorylation (Parisiadou et al., 2014). We therefore asked if activation of PKA, through modulating cAMP levels, changes LRRK2 phosphorylation at the cellular sites or activity in cells assayed through Ser1292. If PKA downregulates LRRK2 kinase activity and directly phosphorylates the cellular sites, then increasing PKA activity in cells would lead to decreased autophosphorylation of Ser1292 and increased phosphorylation at the cellular sites. We expressed GFP-LRRK2 WT, KD, Gly2019Ser, Arg1441Gly, Tyr1699Cys, Ser910/935Ala in HEK293 T-REx cells for 1 day, followed by 18 h of serum starvation then stimulation with Forskolin and IBMX for 30 min followed by immunoblot analysis **Figure 4**. These compounds increase cyclic AMP levels and block phosphodiesterase activity yielding increased PKA activity respectively, as indicated by increased PKA substrate antibody signal on whole cell lysates, **Figure 4** quantitation in **Figure 4A** and immunoblot in **Figure 4B**. We found that all the cellular phosphorylation sites were significantly diminished by approximately 20% in a PKA activated environment and pSer1292 was not significantly changed. These data are in agreement with a recent report by Hermanson et al. (2012), which analyzed pSer935 levels following treatments with various LRRK2 inhibitors. In that report, PKA inhibitor H89 was not effective or had high cellular IC_{50} values compared other LRRK2 inhibitors in unstimulated HEK293 and U20S and SHSY5Y cells.

According to the model presented in Muda et al. (2014), PKA stimulated 14-3-3 binding negatively regulates LRRK2 activity and in the absence of this interaction activity would be increased. Thus, when PKA is activated, LRRK2 activity would be repressed. With the ability to assess LRRK2 activity directly in cells, we next asked if LRRK2 mutated in the Roc [Arg1441Gly] and COR [Tyr1699Cys] mutations, which would be deficient in cellular site phosphorylation, could regain 14-3-3 binding after Forskolin/IBMX treatment. The Ser910/935Ala mutant was included to detect PKA induced 14-3-3 binding independent of these sites. We observed no significant change *in vivo* LRRK2 kinase activity as indicated by Ser1292 phosphorylation after PKA stimulation, **Figure 4B**. This is consistent with previous reports that demonstrate the 14-3-3 interaction with LRRK2 is abolished by Ala substitution of Serines 910 and 935 in unstimulated conditions (Nichols et al., 2010). FSK/IBMX treatment also decreased the amount of LRRK2-14-3-3 complex in cells as demonstrated by co-immunoprecipitation, **Figure 4C**.

We wanted to confirm the effects of IBMX/FSK treatment in an additional cell line. LRRK2 knockout rodents develop abnormalities of the kidney and lung, showing accumulation of membranous materials and disruption of autophagy (Herzig et al., 2011; Baptista et al., 2013). A549 lung alveolar epithelial cells express significant amounts of endogenous LRRK2 (Lobbestael et al., 2013), and we used this cell system as a pertinent model of endogenous LRRK2 regulation. LRRK2 was immunoprecipitated from A549 cells after stimulation with IBMX/FSK, **Figure 4D**. Similar to the GFP expression system, we also observed a decrease in LRRK2 phosphorylation at the cellular sites. We were not able to detect a distinct pSer1292 on the endogenous LRRK2. Based

A

IC50 values (μM)

P site	compound GNE	2IN1
910	0.10	0.08
935	0.10	0.06
955	0.17	0.12
973	0.16	0.17
1292	0.25	0.25

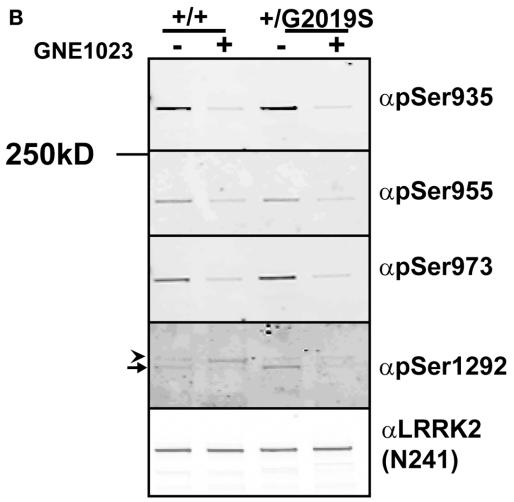

FIGURE 3 | Comparative analysis of inhibitor induced dephosphorylation for Ser1292 and cellular phosphorylation sites after kinase inhibition. (A) HEK 293 T-REx cells stably expressing GFP-LRRK2-Gly2019Ser were treated with DMSO or increasing concentrations of GNE1023 or LRRK2-IN1 for 90 min. Cell lysates were co-immunoblotted for GFP and anti-phospho-Ser935 (αpS935), anti-phospho-Ser955 (αpS955), anti-phospho-Ser973 (αp973), anti-phospho-Ser1292 (αpS1292). IC_{50} values were calculated using non-linear regression analysis using GraphPad Prism 6.0. **(B)** Detection of endogenous pSer1292 in human lymphocytes. Cultures of control donor and +/Gly2019Ser donor lymphoblasts were treated with GNE1023 for 90 min. Endogenous LRRK2 was immunoprecipitated and subjected to immunoblot analysis with the indicated phospho-antibodies. Blots were co-probed with N241 monoclonal antibody to detect total endogenous protein. Arrowhead indicates non-specific band and arrow indicates LRRK2.

on these results, the activation of LRRK2 by PKA may be specific to COS cells, which were used in Muda et al. Our experiments didn't demonstrate a role for PKA in LRRK2 phosphorylation or autophosphorylation. In fact cAMP stimulation downregulated LRRK2 phosphorylation and suggested that a phosphatase may be activated in HEK293 and A549 cells.

Chemical analysis of Ser910/935/955/973 and Ser1292 phosphatases

Phosphorylation of LRRK2 at the cellular sites and Ser1292 are efficient, although distinct indicators of LRRK2 kinase activity. The use of cellular phosphorylation sites as readouts of kinase activity in the Arg1441Gly, Arg1441Cys, Arg1441His, or Tyr1699Cys mutants is complicated by the increased interaction with phosphatase PP1, which leads to dephosphorylation of the cellular sites and reduced 14-3-3 interaction similar to treatment with LRRK2 inhibitors. Blocking PP1 activity with Calyculin A or expression of inhibitor proteins suppressed the induced dephosphorylation and inclusion body formation from kinase inhibition or PD mutations (Lobbestael et al., 2013). We assayed the sensitivity of Ser1292 to Calyculin A (CA) and Okadaic acid (OA), inhibitors of PP1 and PP2 respectively, in the context of LRRK2 inhibition and Arg1441Gly mutation, **Figure 5**. We expressed GFP-LRRK2 Gly2019Ser and Arg1441Gly in HEK293 T-REx cells in a doxycycline inducible manner for 1 day and treated cells with OA, CA, with and without GNE1023. We found that unlike Ser910/935/955/973, Ser1292 phosphorylation is sensitive to both CA and OA. Similar to previous reports, cellular site phosphorylation is rescued after inhibition of CA sensitive phosphatases, but not OA ones. Ser1292 phosphorylation is enhanced by both CA and OA treatment, indicating that LRRK2 autophosphorylation is regulated by both PP1 and PP2 type phosphatases. Unlike the cellular phosphorylation sites, Ser1292 phosphorylation is not restored by phosphatase inhibition after LRRK2 inhibition, confirming that Ser1292 is an autophosphorylation site and the cellular sites are regulated by upstream kinases.

DISCUSSION

Understanding how PD mutations in LRRK2 affect its biochemical properties and functional activities is essential to elucidation of how LRRK2 dysfunction causes neurodegenerative disease. The neurotoxicity of LRRK2 is connected with its kinase activity and mutants that increase kinase activity may be more pathogenic in patients. The GTPase and COR domains feed into the kinase domain and participate in LRRK2 regulation via intramolecular and intermolecular mechanisms (Biosa et al., 2013). By using mutations across the enzyme, we derive a better understanding of regulation of LRRK2 by itself and upstream enzymes. In this study, we used *in vitro* assays and expression systems to characterize the interplay of PD mutations and Ser910/935/955/973 phosphorylation and LRRK2 kinase activity that can be neurotoxic.

To gain insight into the effects of mutations in the Roc-COR and kinase domains on LRRK2 catalytic activity and regulation, we compared the detection of autophosphorylation sites in LRRK2 that fall between the amino terminal LRR and Roc domain [LRRK2-Ser1292], the Roc domain [LRRK2-Thr1491] and the carboxy terminal domain [LRRK2-Thr2483] to cellular site phosphorylation at Ser910, Ser935, Ser955, and Ser973. We employed a recombinant preparation of full length LRRK2 purified from mammalian cells, which provides an advantage over previous widely used preparations of recombinant forms

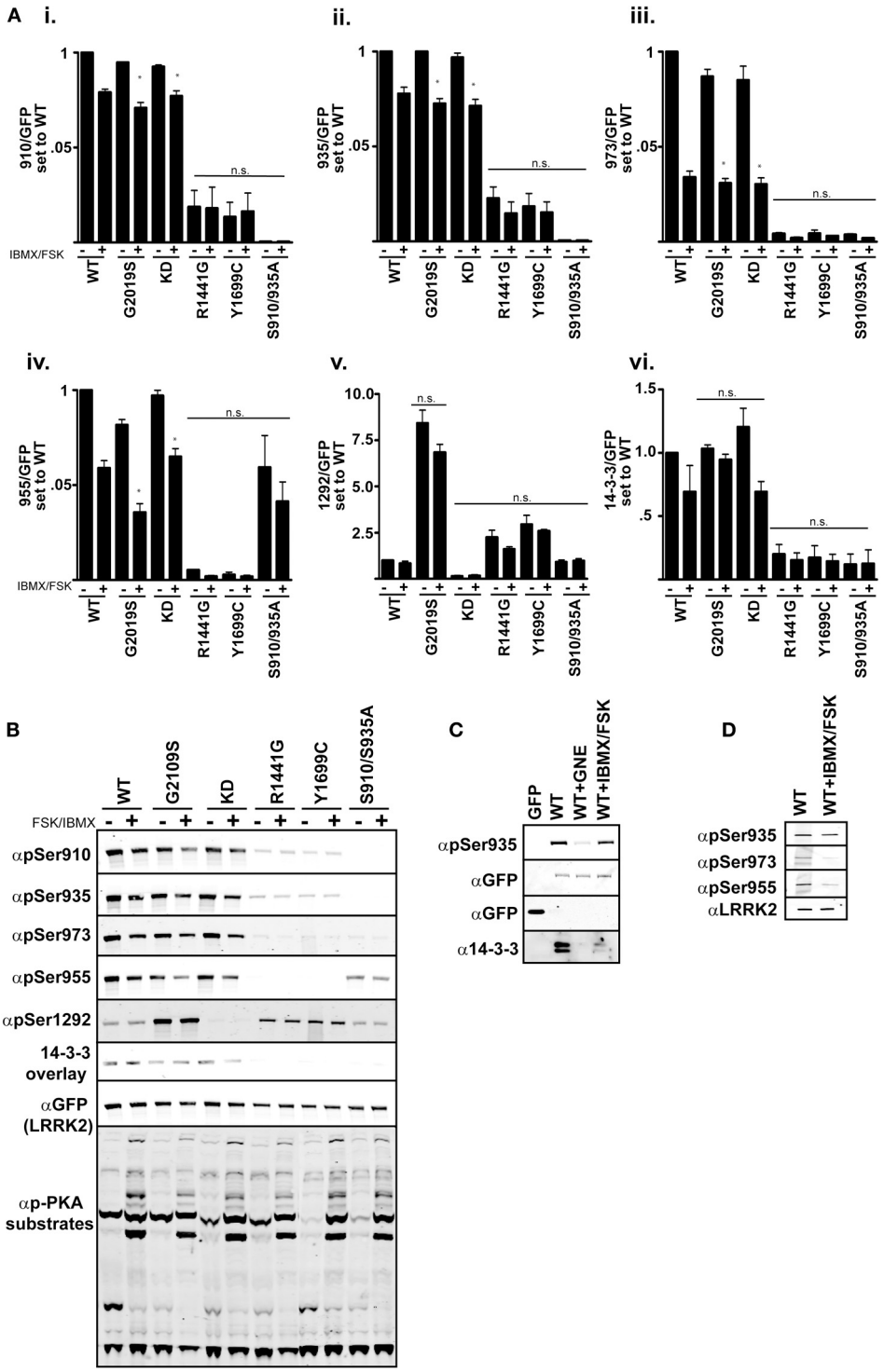

FIGURE 4 | cAMP stimulation does not increase 14-3-3 binding in HEK 293 cells. (Ai–vi) Quantification of the phosphorylation levels at the cellular sites (935, 955, 973), autophosphorylation site 1292, and 14-3-3 binding in IBMX/FSK treatment relative to the DMSO treated control from **(B)**. **(B)** Representative blots of cellular site phosphorylation levels and autophosphorylation at Ser1292 in the indicated GFP-LRRK2 mutants after 30 min treatment with 50 μM FSK/100 μM IBMX. Stimulation of PKA activity is verified by immunoblotting cell lysates with phospho-PKA substrate antibody (Cell Signaling). Results are representative of 5 independent experiments for WT, Gly2019Ser, and KD mutants, and 2 independent experiments for the Arg1441Gly, Tyr1699Cys, and S910/935A mutants. The ratio of phosphorylation over total GFP signal was normalized to WT DMSO, error bars represent s.e.m. Statistical significance was assessed using the

(Continued)

FIGURE 4 | Continued
One-Way ANOVA test combined with Tukey's correction. $*p \leq 0.05$. Quantification of WT samples treated with DMSO and IBMX/FSK are included in the graphs for reference, but were excluded in the statistical analysis. **(C)** LRRK2 interaction with 14-3-3 in cells is diminished by IBMX/FSK treatment. GFP or GFP-LRRK2 WT expressing HEK 293 T-REx cells were treated with GNE1023 for 90 min or 50 μM FSK/100 μM IBMX for 30 min, or in combination, then subjected to GFP Trap immunoprecipitation. Immunoprecipitates were immunoblotted for 14-3-3 co-immunoprecipitation. **(D)** Lung epithelial alveolar A549 cells were treated with 50 μM FSK/100 μM IBMX for 30 min and endogenous LRRK2 was immunoprecipitated and analyzed by immunoblot with anti-LRRK2 (N241), anti-phospho-Ser935, anti-phospho-955, anti-phospho-973 antibodies.

lacking the amino terminus (LRRK2 aa970–2527) and purified from eukaryotic cells. This full length protein allows characterization of LRRK2 with all domains and their potential regulatory action in place. We compared phosphorylation of LRRKtide with autophosphorylation activity using pSer1292, pThr1491, pThr1503, and pThr2484 antibodies in wild-type, Arg1441Cys and Gly2019Ser mutants. LRRKtide and autophosphorylation activity saturate *in vitro* and are predictive for outcomes in cells. Out of these sites, only Ser1292 faithfully revealed active LRRK2 *in vitro* and in cells as an autophosphorylation site. Ile2012Thr suppressed kinase activity against Ser1292 and Thr1491 and also diminished the phosphorylation at the cellular sites.

Mutations Arg1441Cys, Arg1441Gly, Ala1442Pro, and Tyr1699Cys in the Roc-COR domain could activate LRRK2 kinase through structural changes that limit GTPase activity, prolonging the active state, leading to intramolecular stimulation of kinase activity (Taymans et al., 2011; Tsika and Moore, 2013; Liao et al., 2014). Recombinant Arg1441Cys exhibited increased kinase activity against LRRKtide, 20% above wild-type levels. Arg1441Cys, Arg1441Gly, Ala1442Pro, and Tyr1699Cys were dephosphorylated at the cellular sites, but are hyperphosphorylated at Ser1292 in cells. We did observe a larger effect of Tyr1699Cys on Ser1292 than previously described in Sheng et al. Threonine 1491 was evaluated in the presence of ATP and we found that this site revealed significant increase in kinase activity from the Gly2019Ser mutation. Interestingly, *in vitro* autophosphorylation at Thr2483 also revealed significant increased kinase activity from Arg1441Gly, and Gly2019Ser. The Tyr1699Cys mutation suppressed autophosphorylation of Thr1491 similar to what was observed for Thr1357 in Kamikawaji et al. (2013). Interrogation of individual autophosphorylation sites reveals different levels of mutation induced kinase activation, indicating that several sites should be analyzed simultaneously because these differences could be missed or misinterpreted by analysis of a single autophosphorylation site or total phosphate incorporation.

Protective LRRK2 haplotypes have been reported for Asn551Lys-Arg1398His-Leu1423Lys (Ross et al., 2011) and Arg1398His (Heckman et al., 2014), which further supports the notion that the amino terminus and the Roc-COR domain could feed into the kinase domain activity. When the cellular phosphorylation sites are mutated to unphosphorylatable Ala residues, we observed that mutation of Ser910/935, Ser955, and Ser973 to Ala did not affect intrinsic kinase activity in cells or on isolated LRRK2. However, mutation of all of the cellular sites to Ala trended toward slight increases in autophosphorylation activity of LRRK2 at Ser1292, and 2483, which could reflect regulatory activities of the amino terminal domain to the kinase domain through structural changes in the enzyme or through changes in subcellular localization. Increases in LRRK2 kinase activity from Arg1441Cys, Arg1441Gly, Ala1442Pro, and Tyr1699Cys PD mutations is not linked to the induced dephosphorylation of the cellular sites because alanine substitutions at serines 910/935/955/973 do not also increase LRRK2 kinase activity.

The increased phosphorylation of Ser1292 compared to the decreased phosphorylation of the cellular sites in Arg1441Cys, Arg1441Gly, Ala1442Pro, and Tyr1699Cys mutants indicated that there may be different phosphatases acting on these sites in addition to increasing the kinase activity of LRRK2. To understand the class of phosphatase that act on Ser1292 compared to the cellular sites, we utilized Calyculin A and Okadaic acid, pharmacological inhibitors of PP1 and PP2, respectively. Ser1292 dephosphorylation is mediated by both CA and OA sensitive phosphatases, while the cellular sites are sensitive to only CA sensitive phosphatases. This supports the notion that indeed Ser1292 is dephosphorylated by a different phosphatase class than the cellular sites. Ser1292 is a bona fide autophosphorylation site *in vivo* because it does not become re-phosphorylated after inhibition of both LRRK2 kinase and the phosphatase. This side-by-side comparison also validates that Ser910/935/955/973 are not phosphorylated by LRRK2 like the direct autophosphorylation site Ser1292, Thr1491, and Thr2483. Though the Ser1292 site is detected in LRRK2 isolated from cells it is thought to be of low stoichiometry with the total amount of LRRK2. The fact that Thr1491 or Thr2483 were not detectable in cells may be due to low activity against these sites in cells or increased phosphatase activity against these sites in cells. Alanine substitution of Ser1292 causes a decrease in the cytoplasmic filaments and an increase in the cytoplasmic puncta caused by Arg1441Gly mutations (Sheng et al., 2012). The different phosphatases that target both Ser1292 and serines 910/935/955/973 are presumably more active on LRRK2 that has been inactivated through inhibition. In the future it would be important to elucidate the Ser1292 phosphatase and/or the phosphatase regulatory subunits that regulate the phosphatase activity on LRRK2.

Phosphorylation of Ser1292 is a biochemical readout of LRRK2 activity in cells and *in vitro*. LRRK2 inhibitor induced dephosphorylation of LRRK2 cellular sites Ser910/935/955/973 and Ser1292 are observed at similar IC50s, indicating that both are measures of activity. We were also able to detect increased Ser1292 activity in a Gly2019Ser heterozygous patient derived lymphoblastoid culture, and that Ser935/955/973 as well as Ser1292 were efficiently dephosphorylated in these cells upon GNE treatment, suggesting that Ser1292 can be employed as a measure of LRRK2 activity in patients. High levels of LRRK2 expression in the periphery and responsiveness of the cellular sites and Ser1292 to specific LRRK2 inhibitors provides a tractable patient sample source to ascertain and analyze LRRK2

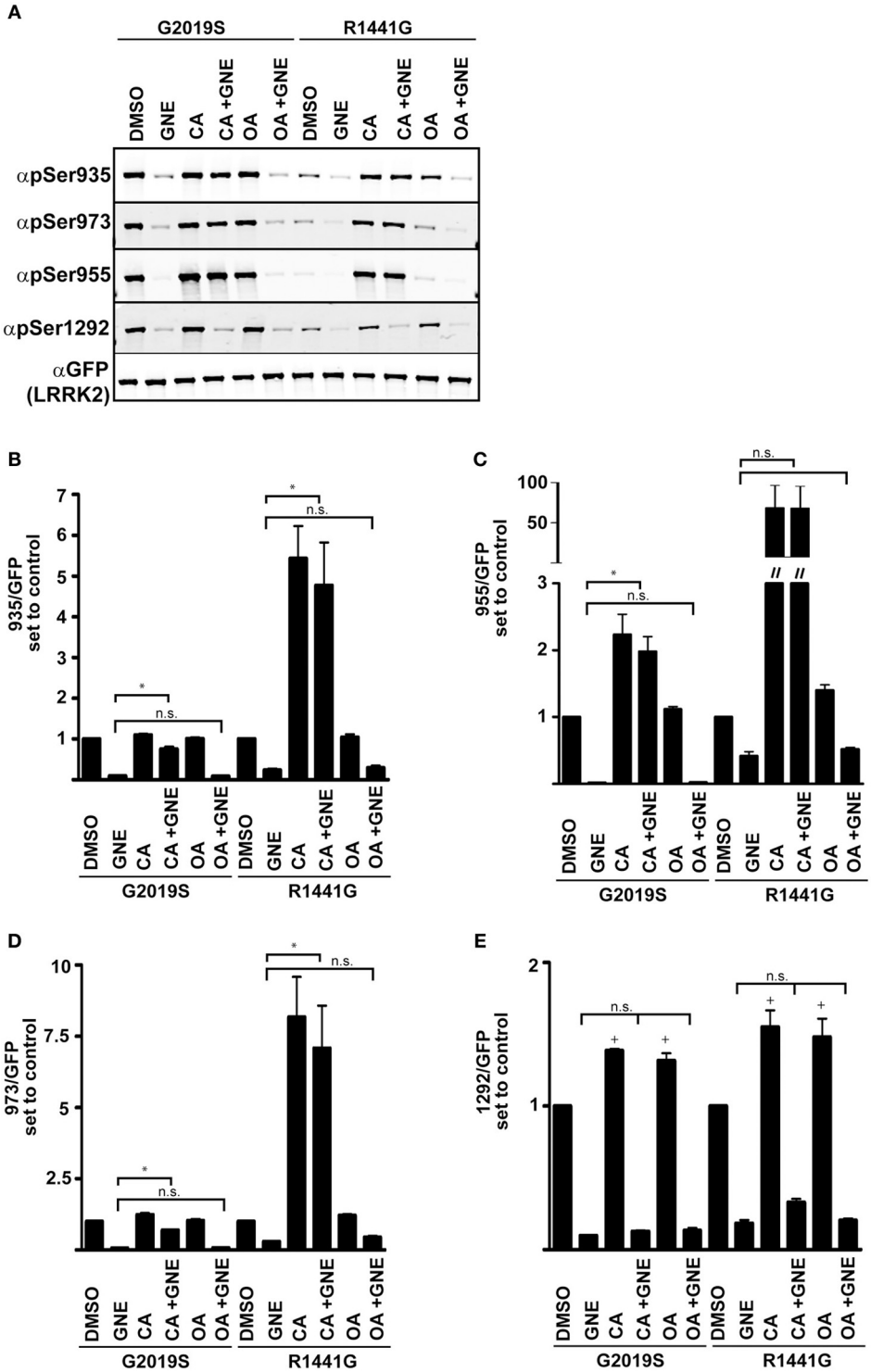

FIGURE 5 | Serine 1292 is regulated by Calyculin A and Okadaic acid sensitive phosphatases. Stable-inducible HEK 293 T-REx cell lines overexpressing Gly2019Ser or Arg1441Gly LRRK2 mutation were treated with Okadaic acid or Calyculin A alone, or combined with GNE1023. **(A)** Representative blots of phosphorylation levels in Gly2019Ser and Arg1441Gly LRRK2 mutation line. **(B–E)** Quantification of anti-phospho-Ser935, anti-phospho-955, anti-phospho-973, and anti-phospho-1292 phosphorylation levels relative to the DMSO treated control of each mutant. The immunoblots shown are representative of three independent experiments. Statistical significance of Ser1292 stimulation with CA and OA was assessed using a one sample *t*-test set to the hypothetical value of 1, $^{+}p \leq 0.05$. An ordinary One-Way ANOVA test combined with Dunnett's correction for multiple testing was used to assess the ability of CA and OA to overcome GNE inhibition $^{*}p \leq 0.05$ on connecting bars, n.s., not significant. DMSO control treated samples were not included in the ANOVA test and treatments were compared to GNE (alone) treated samples.

modification in longitudinal studies or as a surrogate marker for targeting LRRK2 during inhibitor based trials.

We next explored the effects of modulating cAMP induced PKA activity on LRRK2 autocatalytic activity and cellular phosphorylation. We found that Forskolin/IBMX treatment resulted in a consistent diminution of both the cellular sites and Ser1292, and no change in 14-3-3 binding. Though contradictory to the report in Muda et al. (2014), these data are consistent with previous reports that loss of either Ser910 or Ser935 phosphorylation does not change kinase activity but does result is loss of 14-3-3 binding in cells and in overlay far-western assays. Furthermore, treatment of cells with H89, a PKA inhibitor does not reduce LRRK2 phosphorylation at Ser935 (Hermanson et al., 2012).

We characterized three autoregulatory phosphosites [Ser1292, Thr1491, and Thr2483] alongside the cellular phosphorylation sites [Ser910/935/955/973] to reveal true differences in how pathogenic PD mutations affect the activity and phosphorylation status of LRRK2. The kinase domain of LRRK2 is peculiarly packed amongst several structural and enzymatic domains that influence the kinase activity of LRRK2. **Figure 6A** illustrates the domain structure of LRRK2, along with the position of PD mutations, autophosphorylation sites and the cellular phosphorylation sites. Roc-COR mutations can increase the kinase activity through intramolecular effects (**Figure 6A1**) as indicated by autophosphorylation sites **Figure 6A2**. **Figure 6B** depicts the proposed regulation of LRRK2 phosphorylation at the cellular phosphorylation sites and Ser1292 by upstream kinases (**Figure 6B3**) and phosphatases (**Figure 6B4**), which could be up and down-regulated by LRRK2, respectively. At the same time, Roc-COR mutations enhance the dephosphorylation of LRRK2 at the cellular sites through increased interaction with phosphatases, **Figure 6B5**. Ser1292 is dephosphorylated by Okadaic acid and Calyculin A sensitive phosphatases, **Figure 6B6**. LRRK2 autophosphorylates each site differently dependent on mutation and these autophosphorylation sites indicate LRRK2 activity different from

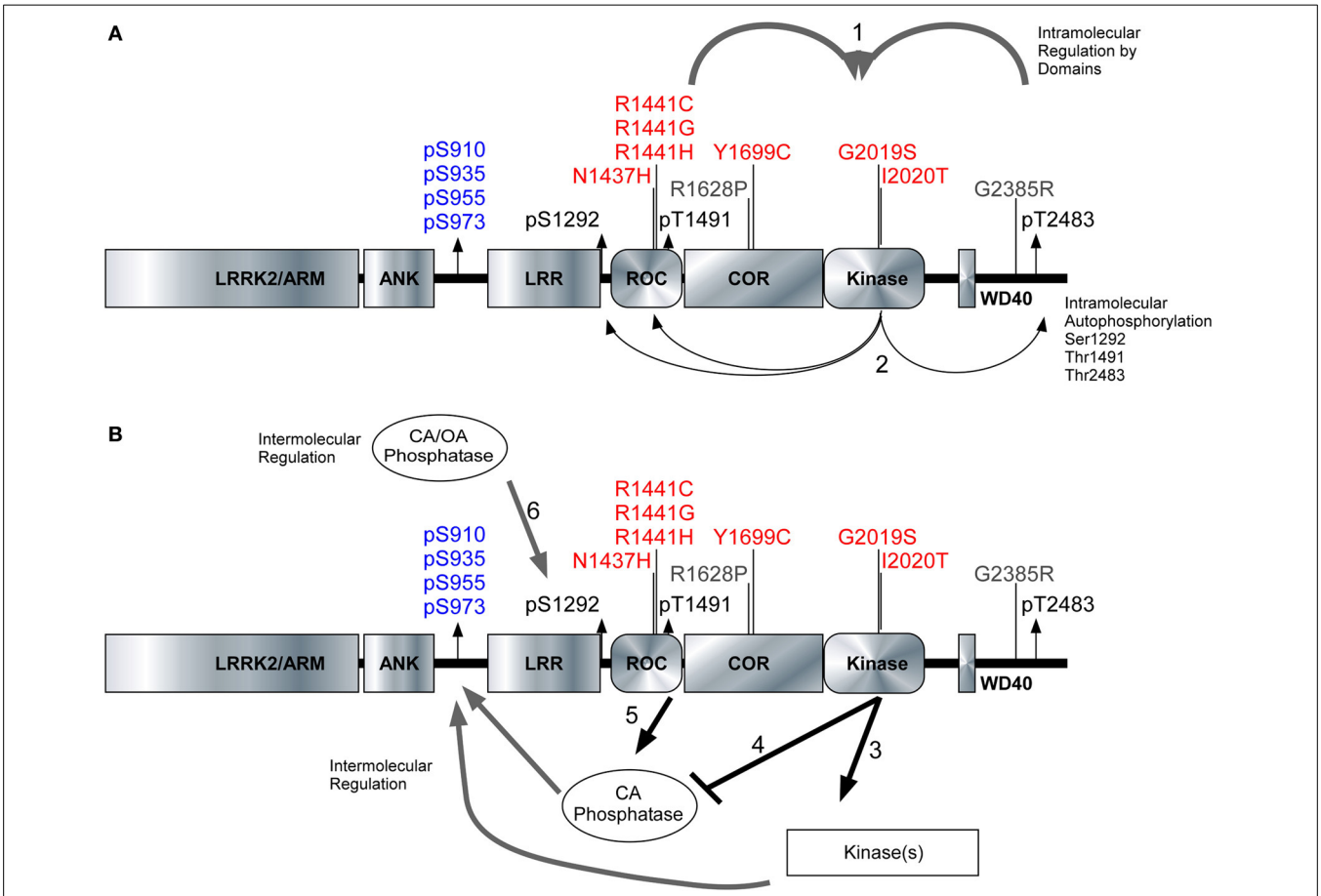

FIGURE 6 | Model of intramolecular and intermolecular LRRK2 regulation. The LRRK2 structure with PD associated mutants highlighted in red (pathogenic) and risk factors in gray. Cellular phosphorylation sites are highlighted in blue and autophosphorylation sites are in black. **(A1)** The intramolecular regulation of the PD associated mutants in the Roc-COR domain and the carboxy terminus on LRRK2 kinase activity. **(A2)** Differential effects of the PD mutations can be detected through the autophosphorylation sites Ser1292, Thr1491, and Ser2483. **(B3,B4)** Kinases and phosphatases contribute to the intermolecular regulation of LRRK2 and their effects can be demonstrated through cellular phosphorylation sites and autophosphorylation sites. **(B5)** Roc-COR mutations enhance the dephosphorylation of LRRK2 at the cellular sites through increased interaction with phosphatases. Ser1292 is dephosphorylated by Okadaic acid and Calyculin A sensitive phosphatases, **(B6)** Mutations within the Roc-COR allow for increased interactions with phosphatases and this decreases the cellular phosphorylation levels.

the cellular sites. Further investigation of the phosphoregulation of LRRK2 will continue to provide novel insight into the normal activities of the enzyme as well as how PD mutations may disrupt enzyme function.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnmol.2014.00054/abstract

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Targeting GTPases in Parkinson's disease: comparison to the historic path of kinase drug discovery and perspectives

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Neurological diseases have placed heavy social and financial burdens on modern society. As the life expectancy of humans is extended, neurological diseases, such as Parkinson's disease, have become increasingly common among senior populations. Although the enigmas of Parkinson's diseases await resolution, more vivid pictures on the cause, progression, and control of the illness are emerging after years of research. On the molecular level, GTPases are implicated in the etiology of Parkinson's disease and are rational pharmaceutical targets for their control. However, targeting individual GTPases, which belong to a superfamily of proteins containing multiple members with a conserved guanine nucleotide binding domain, has proven to be challenging. In contrast, pharmaceutical pursuit of inhibition of kinases, which constitute another superfamily of proteins with more than 500 members, has been fairly successful. We reviewed the breakthroughs in the history of kinase drug discovery to provide guidance for the GTPase field. We summarize recent progress made in the regulation of GTPase activity. We also present an efficient and cost effective approach to drug screening, which uses multiplex flow cytometry and mixture-based positional scanning libraries. These methods allow simultaneous measurements of both the activity and the selectivity of the screened library. Several GTPase activator clusters were identified which showed selectivity against different GTPase subfamilies. While the clusters need to be further deconvoluted to identify individual active compounds, the method described here and the structure information gathered create a foundation for further developments to build upon.

Keywords: Parkinson's, GTPase, kinase, drug, multiplex

INTRODUCTION

Parkinson's disease is a degenerative disorder occurring in the central nervous system (Shulman et al., 2011). Early symptoms are mostly movement related which include shaking, rigidity and slowness. As the disease progresses, thinking and behavior problems may arise with dementia common at the advanced stage. Diagnosis is usually based on symptoms, and neuroimaging is used for confirmation (Jankovic, 2008). Parkinson's disease is associated with loss of neurons in the substantia nigra of the midbrain and accumulation of Lewy bodies, which are aggregates of the protein α-synuclein, in the remaining neurons that generate insufficient dopamine. Parkinson's disease affects 1% of the population above age 60 and 4% of the population over 80 (de Lau and Breteler, 2006). The disease has put a huge financial burden on society costing around 23 billion dollars in the US each year (Findley, 2007). Epidemiological studies have linked exposure to pesticides as a provocative environmental factor (Freire and Koifman, 2012). However, the detailed cause and mechanism of the disease are still ill-defined. With the accumulated genetic information available, familial causes of the disease have emerged although representing a lesser percentage (Davie, 2008).

While exploring the origins of the Parkinson's disease, proteins from various families with different functions have been suggested to contribute to its occurrence at the molecular level. One of them is the GTPase family. Multiple GTPases have been identified through genetic studies to be causal factors. GTPases are guanine nucleotide binding proteins which switch between GTP and GDP binding states. These proteins play important roles in various cellular processes, such as division, signal transduction, protein synthesis, and vesicle transport. Based on their functions, GTPases are grouped as small Ras superfamily GTPases, large GTPases, heterotrimeric G proteins, and translation factor family GTPases. In spite of size differences, these proteins have a conserved and globular guanine nucleotide binding domain which constitutes α-helixes, β-sheets, and switch I and II regions (**Figure 1**). GTPases can intrinsically hydrolyze GTP to GDP. However, the conversion between GTP and GDP is most often regulated by guanine nucleotide exchange factors (GEFs) which facilitate GDP dissociation and GTP binding, and GTPase activating proteins (GAPs) which increase the hydrolytic activity of GTPases and convert GTPases to the GDP bound state. For large GTPases, the hydrolysis of GTP powers organelle reorganization, while for small GTPases, the hydrolysis inactivates the proteins since the GDP binding conformation cannot interact with downstream effectors.

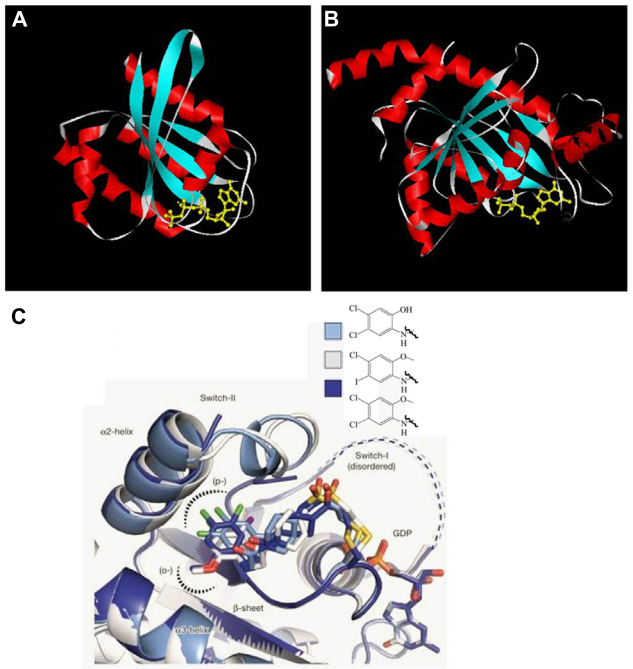

FIGURE 1 | GTPase domain core structure. (A) Human Ras small GTPase with GTP bound. Six stranded β-sheets are surrounded by five α-helices (PDB: 121p). **(B)** *Dictyostelium* dynamin large GTPase with GDP bound. Eight stranded β-sheets are surrounded by nine α-helices (PDB: 1jwy). Helices are shown in red, β-sheets in blue, switch region in white, and the guanine nucleotide in yellow. Though the nucleotide identities are different for the two structures, the compact and globular arrangement is conserved. Both Tiff files were taken from www.endocytosis.org/Dynamin/GTPbinding-motifs.htm. **(C)** Overlaid structures of three inhibitors with GDP bound K-Ras G12C. These allosteric inhibitors bind to switch-II region and induce an inactive GDP binding conformation (Ostrem et al., 2013).

In this review, we discuss the association of GTPases with Parkinson's disease and the potential for them to be the pharmaceutical targets. To shed light on GTPase drug discovery in more general terms, the milestones in the productive kinase field are summarized. We also describe recent progress in the search for GTPase activity regulators, including a pilot combinatorial library approach against multiple small GTPases that have been implicated in Parkinson's disease and other disorders.

PHYSIOLOGICAL PROCESSES ASSOCIATED WITH PARKINSON'S DISEASE AND GTPASE INVOLVEMENT

Years of study have yielded some clues to the etiology of Parkinson's disease. Several physiological processes are speculated to be linked to the cause and progression of the disease at the cellular level. These include organelle homeostasis and traffic, mitochondria fission and fusion, axon growth, neuron cell morphogenesis and survival, oxidative damage repair, and etc. GTPases, including large motor GTPases and small Ras superfamily GTPases, have been found to be involved in these processes. The important roles that they play are described (**Figure 2**).

GOLGI AND α-SYNUCLEIN AGGREGATE CLEARANCE

Genetic linkage studies have linked gene *PTEN induced putative kinase 1 (PINK1)* and *PARK2* to Parkinson's disease (Wang et al., 2011) while both the genetic linkage studies (Paisan-Ruiz et al., 2006) and genome wide association studies (GWAS; Simon-Sanchez et al., 2009) have identified leucine-rich repeat kinase 2 (LRRK2) to be genetically linked and associated with the disease. Among them, LRRK2 encodes a large multi-domain protein containing a Ras-of-complex (ROC) GTPase domain, a C-terminal of Roc (COR) domain and a serine/threonine kinase domain. The COR domain connects the GTPase and the kinase domain (Tsika and Moore, 2013). It has been suggested that the kinase and GTPase activity mutually affect each other, so that the GTP or GDP binding capacity of ROC induces kinase activation (Taymans et al., 2011) and the activated kinase phosphorylates the GTPase domain which alters conformation to further promote kinase activity (Gloeckner et al., 2010). Nonetheless, the detailed mechanism is still unresolved (Taymans, 2012). The most common mutation of LRRK2 found in Parkinson's disease is G2019S in the kinase domain (Tsika and Moore, 2013). This mutation increases kinase activity. Another common mutation is R1441C which is in the GTPase domain (Tsika and Moore, 2013). There have been conflicting results regarding the effects of the R1441C mutation on GTP binding. However, it has been consistently demonstrated that GTPase hydrolysis activity was reduced with the mutation (Lewis et al., 2007; Daniels et al., 2011). Parkinson's disease with mutations in the GTPase domain shows pure nigral neuron degeneration without severe Lewy body formation. Though most LRRK2 studies have been directed to control the kinase activity, it has been found that long term inhibition of LRRK kinase activity through genetic knockout has unwanted side effects including susceptibility to inflammatory bowel syndrome and kidney dysfunction (Herzig et al., 2011; Liu et al., 2011; Baptista et al., 2013). Considering the mutual regulation between the kinase and the GTPase domain, an alternative solution is to control the activity of the GTPase domain. We anticipate attempts to decrease GTP binding through minimizing the interactions between the GTPase domain and its GEFs, or efforts to increase the GTPase hydrolysis activity through controlling its GAPs could be explored (Anand and Braithwaite, 2009; Gandhi et al., 2009; Cookson, 2010; Tsika and Moore, 2013).

As for the mechanism of LRRK2 involvement in the pathogenesis of Parkinson's disease, studies have shown that LRRK2 regulates the degradation of defective Golgi, an organelle that contributes to synaptic vesicle formation (Beilina et al., 2014). Mutations in LRRK2 therefore lead to errors in vesicular endocytosis and recycling. By screening against protein interaction arrays, LRRK2 was found to complex with several proteins (Beilina et al., 2014). The complex is important for the clearance of Golgi-derived vesicles through the autophagy-lysosome system. Both Golgi clearance and autophagy have been implicated in neuron loss in Parkinson's disease (Fujita et al., 2006). Each protein of the complex, which is likely formed using LRRK2 as a scaffold, contributes to the Golgi clearance function. One of them, the GTPase Rab7L1 (MacLeod et al., 2013; Beilina et al., 2014), is a cytosolic GTPase belonging to the Rab family of GTPases. This GTPase has been shown to localize at the Golgi and regulate the vesicular sorting (MacLeod et al.,

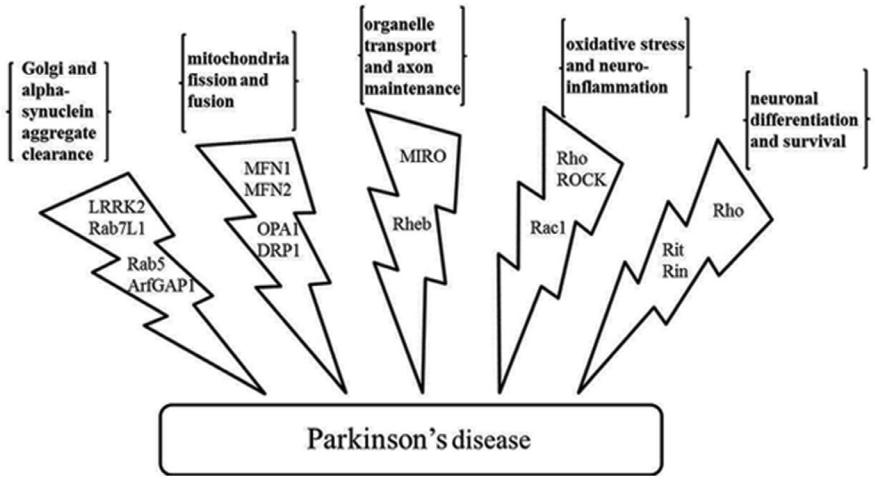

FIGURE 2 | Physiological processes related to the Parkinson's disease and the GTPases and the effectors involved. GTPases LRRK2, Rab7L1, Rab5, and GTPase effector ArfGAP1 regulate Golgi and α-synuclein aggregate clearance; large GTPases MFN1, MFN2, OPA1, and DRP1 regulate mitochondria fission and fusion; MIRO and Rheb have a role in organelle transport and axon maintenance; GTPase Rho, Rac1, and kinase effector ROCK are involved in oxidative stress management and neuroinflammation; Rho, Rit, and Rin are involved in neuronal differentiation and survival. LRRK2, leucine-rich repeat kinase 2; ArfGAP1, ADP-ribosylation factor GTPase-activating protein 1; MFN1 and MFN2, mitofusin-1 and mitofusin-2; OPA1, optic atrophy 1; DRP1, dynamin-related protein 1; MIRO, mitochondrial Rho-GTPase; Rheb, Ras homolog enriched in brain; ROCK, Rho-associated protein kinase.

2013). Constitutively active Rab7L1 effectively suppressed neurite shortening induced by LRRK2 mutations. Moreover, genetic studies showed that alterations at the Rab7L1 gene promoter are likely to enhance the expression of the gene locus and are associated with reduced Parkinson's disease risk (Gan-Or et al., 2012).

In another study, LRRK2 was also found to interact with GTPase Rab5b at the endosome in neurons which has established roles in regulating endocytosis (Shin et al., 2008). Moreover, LRRK2 interacts and phosphorylates a GAP, ADP-ribosylation factor GTPase-activating protein 1 (ArfGAP1). Silencing ArfGAP1 expression was shown to protect against mutant LRRK2 induced neurite shortening, suggesting that ArfGAP1 might also be a potential target in the Parkinson's disease (Stafa et al., 2012; Xiong et al., 2012).

Maintaining normal homeostasis is important for cell health. Not only defective organelles but also protein aggregates need to be removed over time. Aggregates of the α-synuclein protein can form Lewy bodies that are commonly found in neurons with Parkinson's disease. Biochemical studies have demonstrated that α-synuclein and Rab3a form a complex at the presynaptic membrane (Chen et al., 2013). Rab3a mutants with deficient GTPase activity blocked α-synuclein dissociation from the membrane. Although it is still controversial whether the sequestration of α-synuclein contributes directly to Parkinson's disease or is associated with the disease phenotype, there appears to be a link between the intracellular level of α-synuclein and Rab3a.

Moreover, age-dependent α-synuclein accumulation could interfere with how cells cope with stress by blocking protein interactions that regulate GTPase activities. α-Synuclein in Lewy bodies was phosphorylated at Ser129 by Polo-like kinase (Plk2) both *in vitro* and *in vivo* (Wang et al., 2012a). Plk2 also interacts with and phosphorylates the GEF and/or GAPs of small GTPase Rho1. By activating downstream protein kinase C, Rho1 is an import signaling node in the mitogen-activated protein kinase (MAPK) cascade response to cell stress. When the concentration of α-synuclein is high, α-synuclein occupies Plk2 and inhibits the interaction between Plk2 and Rho1 GEFs and/GAPs. Therefore the intracellular GTP-bound and active Rho1 decreases to a level insufficient to respond to cell stress. Accumulation of such cell defects eventually lead to cell death and neuron loss is characteristic of Parkinson's disease.

Therefore, both the cause and the consequence of α-synuclein aggregation have GTPase involvement. It is possible that by regulating GTPase activity, the formation of the α-synuclein aggregate could be reduced and its effect minimized.

MITOCHONDRIA FISSION AND FUSION

Mitochondria are crucial for cell energy production and oxidation control. It has been recognized that the organelle undergoes dynamic fission and fusion changes. In neurons, mitochondrial fission can provide energy at sites of demand, while mitochondria fusion can regenerate mitochondrial DNA and protein after neurotoxic insult. Mounting evidence shows that dysregulated mitochondria fission and fusion contributes to Parkinson's disease (Knott et al., 2008).

The dynamic fission and fusion of mitochondria is regulated by a group of large GTPases. The initial studies conducted in yeast have provided insight into mammalian cells (Knott et al., 2008). Mitofusin-1 and mitofusin-2 (MFN1 and MFN2) are large mammalian GTPases localized at the outer membrane of the mitochondria and direct the organelle fusion by interacting with another GTPase mitochondrial Rho-GTPase (MIRO). The amino-terminus of MFN1 and MFN2 contains the conserved GTPase

domain while the carboxy-terminus contains a coiled-coil structure. Mutations in the GTPase domain of their yeast orthologs inhibit mitochondrial fusion. Also in yeast, the inner mitochondrial membrane fusion is regulated by the large GTPase Mgm1, likely through *trans* interactions between the GTPase domain and the GTPase effector domain (GED). The mammalian ortholog OPA1 may play a similar role. As for the fission of the mitochondria, large GTPase dynamin-related protein 1 (DRP1) is likely involved. Inferred from studies on the yeast ortholog dynamin 1 (Dnm1), DRP1 can form oligomers at the fission site while hydrolysis of GTP induces DRP1 to assume a conformation that helps enhances fission. Both Dnm1 and DRP1 have three conserved domains including an N-terminal GTPase, a central helical domain and a GED domain. A direct interaction between LRRK2 and DRP1 has also been demonstrated. Parkinson's disease related mutations in the GTPase domain of LRRK2 enhanced the interaction and was shown to increase mitochondria fragmentation (Wang et al., 2012c). It has also been shown that inhibition of the large GTPase DRP1 protected neuron death both *in vitro* and *in vivo* (Grohm et al., 2012; Qi et al., 2013).

ORGANELLE TRANSPORT ALONG AXONS AND AXON MAINTENANCE

Intracellular transport of organelles such as Golgi and mitochondria is important for maintaining healthy neurons by providing necessary substances and energy at remote synapses as well as clearing unwanted waste (Sheng and Cai, 2012; Millecamps and Julien, 2013). Errors in mitochondria movement have been associated with Parkinson's disease (Liu et al., 2012). The characteristic α-synuclein aggregation of Parkinson's disease was shown to impede the movement of organelles along the axons by directly interacting with axon motor proteins. The stalling of vesicles triggered a cascade of signals that lead to neuron death (Li et al., 2004). Several GTPases have been linked to the regulation of organelle transport. Besides its role in mitochondria fusion, GTPase MIRO and its adaptor proteins have also been shown to control the movement of the mitochondria (Fransson et al., 2003; Frederick et al., 2004). The GTPase anchors kinesin to the mitochondria surface to facilitate its traffic along the axons. The small GTPase Rab7 has also been shown to control endosomal and lysosomal retrograde transport (Millecamps and Julien, 2013).

Moreover, two genes, *PINK1* and *PARK2,* found to link to Parkinson's disease from genetic linkage studies encode protein *PINK1* and Parkin. Mutations of the two proteins are often found in early onset familial forms of the Parkinson's disease (Wang et al., 2011, 2012b). *PINK1* is a serine/threonine kinase while Parkin is an E3 ubiquitin ligase. The two proteins have been shown to cooperatively promote the ubiquitination and degradation of MIRO at the mitochondria outer membrane (Liu et al., 2012). The fusion and transport of mitochondria need to be checked since defective mitochondria might help to spread protein aggregates and be neurotoxic. *PINK1* and Parkin therefore serve as mitochondria quality control checkpoints. Mutations in *PINK1* and Parkin can cause aberrant mitochondrial homeostasis allowing the dysfunctional mitochondria to evade being engulfed by mitophagy and survive to be neurotoxic (Liu et al., 2012).

In Parkinson's disease, it is suspected that the axon of the dopamine producing neuron plays a more important role than the cell body at the beginning stage, and it is the loss of the axon, rather than the cell body, that determines disease progression (Luo, 2000). The mammalian target of rapamycin (mTor) is a mediator of PI3K/Akt signaling. The PI3K-Akt-mTor pathway has been shown to participate in the neuron axon growth and maintenance by controlling axon number, branching, and growth cone dynamics (Kim et al., 2012). The GTPase Ras homolog enriched in brain (Rheb) is an upstream regulator which can activate mTor. By applying adeno-associated virus vector transduction, it was shown that the constitutively active GTPase Rheb could protect neurons in both normal adult mice and mice treated with neurotoxin through inducing axon sprouting and regrowth (Luo, 2000; Kim et al., 2012).

OXIDATIVE STRESS AND NEUROINFLAMMATION

It has been shown that oxidative stress and neuroinflammation play a role in the pathogenesis and progression of Parkinson's disease (Hirsch et al., 2012). Although the downstream signaling has not yet been elucidated, the Rho GTPase has been shown to upregulate the molecules that enhance inflammation and oxidative stress. When dopaminergic cells were treated with neurotoxins, Rho and Rho-associated protein kinase (ROCK) were activated, either directly or indirectly via the NADPH oxidase, and caused cell death. A ROCK inhibitor has been shown to alleviate the neurotoxin effect (Villar-Cheda et al., 2012). However, the effects of Rho GTPase might be multifaceted. In another study, Rho and ROCK were activated by a merlin-iso2-dependent complex and induced neurofilament heavy chain phosphorylation (Whitehead et al., 2009; Schulz et al., 2013). Down regulation of merlin-iso2 in an animal model caused symptoms of neurological disorder.

Reactive oxygen species (ROS) produced during neurotoxic stress could cause oxidative DNA damage and dopaminergic neuronal degeneration. NADPH oxidase 1 (Nox1) and small GTPase Rac1, an important regulator in the Nox1 system, were found to accumulate in the dopaminergic neurons of patients with the Parkinson's disease (Choi et al., 2012). Moreover, in both cellular and animal models, the expression levels of both proteins were increased by neurotoxin treatment accompanied by the accumulation of damaged DNAs. Pharmaceutical or genetic intervention toward Nox1 or Rac1 attenuated the neurodegeneration.

NEURONAL DIFFERENTIATION AND SURVIVAL

Rit and Rin are members of a novel branch of Ras superfamily GTPases and have been implicated in Parkinson's disease by GWAS (Latourelle et al., 2012; Pankratz et al., 2012). Rit and Rin are involved in multiple cell survival signaling pathways including ERK/MAPK and p38/MAPK. Cell culture studies have suggested that Rit regulates neuron morphogenesis through the MEK/ERK pathway, and dendritic remodeling possibly through the p38/MAPK pathway. Rit knockout flies showed reduced resistance toward neurotoxic insults. Moreover, as regulators of the cell actin cytoskeleton, the Rho GTPases have also been shown to play important roles in neuronal morphogenesis. Mutations in the signaling pathway of Rho GTPases have been associated with neurological disorders (Luo, 2000).

SUMMARY FOR PHARMACEUTICAL POTENTIAL

As has been discussed, multiple GTPases regulate various physiological processes and their misbehavior has been associated with the commencement and phenotype of Parkinson's disease. These GTPases belong to either large motor GTPases, which contain GED and other domains besides the guanine nucleotide binding domain, or small Ras superfamily GTPases, which typically have the small or globular guanine nucleotide binding domain. A direct link of some of the GTPases to Parkinson's disease has been explored by genetically controlling their expression at either the transcriptional or translational level. After many studies, the pathogenesis of the Parkinson's disease is still poorly defined and the available treatments only slow down disease progression at best. Additional therapies are obviously needed. Considering the implications of the GTPases in the disease, these proteins are legitimate molecular targets. In addition, small molecule drugs have the potential advantage of ease of use and simplicity of pharmacokinetics. Therefore, searching for small molecule GTPases activity modulators is a reasonable approach. However, the hunt has appeared quite challenging, especially in comparison to kinases, another superfamily of proteins, members of which have also been implicated in many human diseases. As a whole, kinase drug discovery has been fairly productive. We summarize here the milestones in that process in an effort to provide guidance for the quest in the GTPase field.

KINASE DRUG DISCOVERY RETROSPECTIVE

Kinases are superfamily proteins that catalyze the phosphorylation of substrates and are important for cell signal transduction, proliferation, differentiation, cell cycle, growth, and survival. With more than 500 kinases encoded in the human genome, literally every signal transduction circuit has to involve at least one phosphotransfer step (Zhang et al., 2009). A number of diseases including cancer, inflammation, and neurological disorders are caused by the misfiring of kinases. Kinases therefore have been drug targets with a long history. The kinases can be grouped into several subclasses (Noble et al., 2004): tyrosine kinases, serine threonine kinases, phosphatidylinositol 3′-kinases (PI3K), and cell cycle regulation kinases. Most success has been achieved with tyrosine kinases which can be further divided into receptor tyrosine kinases and non-receptor tyrosine kinases. Although catalysis by the different groups of kinases can be differentially regulated, the catalytic domains are well conserved in sequence and structure. The amino and carboxy termini form two separate lobe structures while the Mg-ATP complex sits in a deep cleft created by the bilobe.

It is often the case that activating mutations and overexpression of kinases lead to uncontrolled proliferation. Therefore kinase inhibitors are more commonly pursued than activators. Typically, kinase inhibitors are divided into several groups (Zhang et al., 2009): type I inhibitors bind to the ATP binding site and are competitive toward ATP; type II inhibitors bind both ATP binding site and a hydrophobic site created by the activation loop, usually in a DFG (a motif in the activation loop) out conformation; type III inhibitors bind to allosteric sites close to the ATP binding site; type IV inhibitors covalently and irreversibly bind to the active site cysteine residue of kinases.

From initial natural products that were found to have kinase inhibition activity to the designed US Food and Drug Administration (FDA)-approved drugs, the history of kinase inhibitor development represents a productive example for pharmaceutical intervention.

Staurosporine was originally isolated from the bacterium *Streptomyces staurosporeus* in 1977 and found to have antifungal and apoptosis-inducing activity (Omura et al., 1977). Earlier studies showed that staurosporine inhibited protein kinase C and bound to the ATP binding site in a competitive mode (Okazaki et al., 1988). Staurosporine is thus a prototypical type I inhibitor. However, additional studies found that staurosporine was not selective and inhibited multiple kinases (Ruegg and Burgess, 1989). This promiscuity precluded the compound from getting into clinical trials. Nonetheless, the elucidated structure of kinase CDK2 with staurosporine bound has contributed to the understanding of the interactions between the amino acids at the ATP binding site and a competitive inhibitor. Fragments of the staurosporine scaffold also helped to construct new compound libraries. Midostaurin, a derivative of staurosporine, has been studied for treatment of acute myeloid leukemia (Fischer et al., 2010).

The first FDA-approved and the most successful kinase inhibitor to date is imatinib. The break point cluster–Abelson tyrosine kinase (BCR–ABL) oncogene is formed in patients with chronic myeloid leukemia and acute lymphoblastic leukemia by fusing the BCR gene on chromosome 22 and the ABL tyrosine kinase on chromosome 9. A compound was initially discovered by screening chemical libraries to inhibit the overactive BCR–ABL kinase (Druker et al., 1996). The lead was later modified to enhance its binding affinity and given the name imatinib which receive FDA approval in 2001 (Druker et al., 2001). Imatinib was also found to inhibit both wild type c-KIT and mutant c-KIT that is often found in gastrointestinal stromal tumor (GIST) and was approved to treat the disease (Blay, 2011). The crystal structure showed that imatinib-bound BCR–ABL assumes a DFG-out inactive conformation and therefore represents the first generation type II inhibitor.

GNF2 is an allosteric type III kinase inhibitor targeting the BCL–ABL oncogene (Adrian et al., 2006). The compound did not inhibit the full length or catalytic domain of c-ABL in biochemical assays, but was as potent as imatinib in cellular proliferation assays. The crystal structure and molecule modeling showed that GNF2 bound to the myristate binding site of BCL–ABL. Therefore the compound inhibits the oncogene through a non-ATP competitive mechanism.

HKI-272 is a covalent inhibitor against epidermal growth factor receptor kinase (EGFR) which is a biomarker for many diseases including breast cancer, lung cancer, and brain tumor glioblastoma multiforme (Rabindran et al., 2004; Minami et al., 2007). HKI-272 reacts with a nucleophilic cysteine residue in EGFR through a Michael addition reaction and inhibits the autophosphorylation as well as the activation of the receptor kinase.

The above compounds represent milestones in the productive area of kinase drug discovery. By early 2009, eleven kinase inhibitors had received FDA approval for cancer treatment, and tests for treating illness other than the approved indications were

ongoing in late clinical trials. At the same time, ∼30 kinase targets were developed to be ready for Phase I clinical trials (Janne et al., 2009; Zhang et al., 2009). The success in kinase drug discovery undeniably surpasses what has been achieved when targeting other superfamily proteins including histone acetyltransferases and GTPases. In retrospect, the early discovery of kinase inhibitors had serendipitous elements such as finding staurosporine. As high throughput screening became available, the discovery process entered a productive phase. A number of type I ATP-competitive kinase inhibitors were identified. Lead compound optimization resulted in drugs in clinical trials, some of which were eventually approved. However, after the peak period, the movement slowed down due to the exhaustion of the available ATP-binding scaffolds. Also, the drugs obtained often lost their effectiveness due to drug resistance. Therefore, novel methods to inhibit kinases were explored. First, inhibitors with different mechanisms other than the competitive inhibition were pursued. These included type II inhibitors which could lock the kinase in an inactive conformation, type III allosteric inhibitors, and type IV covalently bound irreversible inhibitors. Drug selectivity is a common issue when the target is a member of a large superfamily of proteins. Type II and type III inhibitors are likely to have an improved selectivity profile since the binding site is not the conserved ATP binding site. As for the type IV inhibitors, although the potency has been increased, the toxicity issue still provides reservations for drug developers. Moreover, as more crystal structures of kinases with inhibitors bound are resolved, kinase drug discovery has increasingly relied on structure-based rational design. This includes lead optimization which improves upon the existing compound, structure-based design which generates novel compounds based on the knowledge obtained from the available structures, and fragment-based design where discrete fragments that bind to different parts of a target are combined to generate a new compound intended to have improved potency and selectivity. Notably, compound libraries generated by combinatorial chemical synthesis have facilitated the discovery of new kinase inhibitors where the library members can be individual compounds or compound mixtures (Liu and Gray, 2006).

STATUS OF GTPASE DRUG DISCOVERY AND PERSPECTIVES

Like kinases, GTPases are not only implicated in neurological disorders, they have also been associated with various other human diseases including cancer and inflammation (Luo, 2000; Shaw and Cantley, 2006; Tybulewicz and Henderson, 2009). Therefore, GTPases as drug targets have been studied for several decades. Initial studies discovered GTPase regulators that obstructed GTPase membrane localization and subsequent activation through essentially inhibiting the lipid transferases that prenylate or geranylate the GTPases. However, the unselective inhibition of the lipid transferases caused severe toxicity (Konstantinopoulos et al., 2007). Compared with the success achieved in the field of kinase drug discovery, there have not yet been drugs directly targeting GTPases proceeding into late clinical trials. The scenario is likely caused by several factors. First, the GTP binding domain of GTPases is relatively small and assumes a smooth and globular structure (Milburn et al., 1990; Niemann et al., 2001; Golen, 2010). This makes it difficult to find a drug binding pocket. Second, the binding affinity of the guanine nucleotide toward the GTPases is high making other molecules difficult to compete against (John et al., 1990; Bourne et al., 1991). Third, there have been multiple established biochemical and cellular assay methods, and animal models for kinase inhibitor discovery and characterization. However, these have been relatively sparse for GTPases (Milligan, 2003; Labrecque et al., 2009). Moreover, testing GTPase activity *in vitro* normally requires high nanomolar to low micromolar enzymes, while kinase biochemical assays usually only need low nanomolar enzymes due to their high enzymatic activity (Taymans, 2012). Fourth, the activity of the GTPases is regulated by separate proteins like GEF and GAPs, instead of other domains in the same protein as in the case of kinases (Vigil et al., 2010). Finally, in comparison to kinases whose functions are mainly involved in signal transduction, GTPases appear to play more diverse roles in cell physiology ranging from cytoskeletal changes to protein translation. Therefore, toxicity from unwanted side effects can be severe (de Boer et al., 2007). Also, while overactive kinases may be problematic, both overactive and deficient GTPases have been implicated in human diseases (Sahai and Marshall, 2002). Therefore, both GTPase inhibitors and activators should be considered for different circumstances. The differences in the drug discovery process targeting kinases and GTPases are summarized in **Table 1**.

Nonetheless, powered by accumulating structural knowledge and the lessons learned from kinase drug discovery, much progress

Table 1 | Comparison of the drug discovery process between kinases and GTPases.

	Kinase	GTPase
Nucleotide binding domain structure	Potential binding pockets	Smooth and globular
Nucleotide dissociation constant	Nanomolar to micromolar	Picomolar to nanomolar
Biochemical and cellular assay availability	Ample	In development
Protein concentration in biochemical assays	Low nanomolar	High nanomolar to low micromolar
Nucleotide binding and hydrolysis regulation	Different domains on the same protein	Separate GEF and GAPs
Function range	Mostly signal transduction	Including signal transduction, cytoskeleton organization, macromolecule transport
Misregulation	Hyperactive	Hyperactive and hypoactive

has been made in the GTPase field in recent years. This has been demonstrated in several ways. First, structure-based rational design and *in silico* screening have provided significant momentum in small molecule discovery and development (Gao et al., 2004; Shima et al., 2013). Second, in analogy to the search for the type II and type III inhibitors for the kinases, efforts have been directed to search for molecules that can either modulate the interactions between a GTPase and its effectors or that can directly inhibit the effector proteins. For example, virtual screening has identified Rho and Rac inhibitors that block the interactions between the GTPase and its GEF (Gao et al., 2004; Shang et al., 2012). From an *in silico* docking study, a Ras inhibitor was developed to inhibit the interactions between Ras and its downstream effector proteins (Shima et al., 2013). Compounds that directly inhibit the catalytic activities of the GEFs of Rho and Rac have also been developed (Nishikimi et al., 2012; Shang et al., 2013). Third, screening methods have evolved which are automated and cost efficient. A small molecule that inhibits the interactions between the farnesylated K-Ras and the prenyl-binding protein PDEδ was discovered from screening and shown to inhibit oncogenic Ras signaling (Zimmermann et al., 2013).

In our laboratory, we have developed a flow cytometry based multiplex screening and assay format (Surviladze et al., 2012) where different GTPases were linked to microsphere bead sets which had distinct fluorescence intensities and could be separated in the red fluorescence channel with excitation/emission of 635/750LP nm on a flow cytometer. The extent of fluorescent GTP binding to the individual GTPases in the presence of test compounds was analyzed by another channel with excitation/emission of 480/530 nm. This method allowed the potency and selectivity of a compound toward several GTPases to be revealed simultaneously. Also smaller quantities of GTPases were used compared to plate based homogeneous assays. Through screening the molecular library small molecule repository (MLSMR), we have identified a pan-GTPases inhibitor (Hong et al., 2010), Rho family GTPases inhibitors (Surviladze et al., 2010c, 2012), as well as a selective inhibitor for individual GTPase Cdc42 (Surviladze et al., 2010b; Hong et al., 2013). In addition, activators of GTP binding were also pursued along with the inhibitor search. Three molecules CID888706, CID7345532, and CID2160985 have been found to increase fluorescent GTP binding to multiple GTPases (Surviladze et al., 2010a). Using CID888706 as an example, biochemical studies confirmed that the compound had an EC50 in the low micromolar range and suggested an allosteric binding mechanism. Cellular assays also demonstrated that the compound could enhance the activity of Rho GTPases in regulating cytoskeleton reorganization.

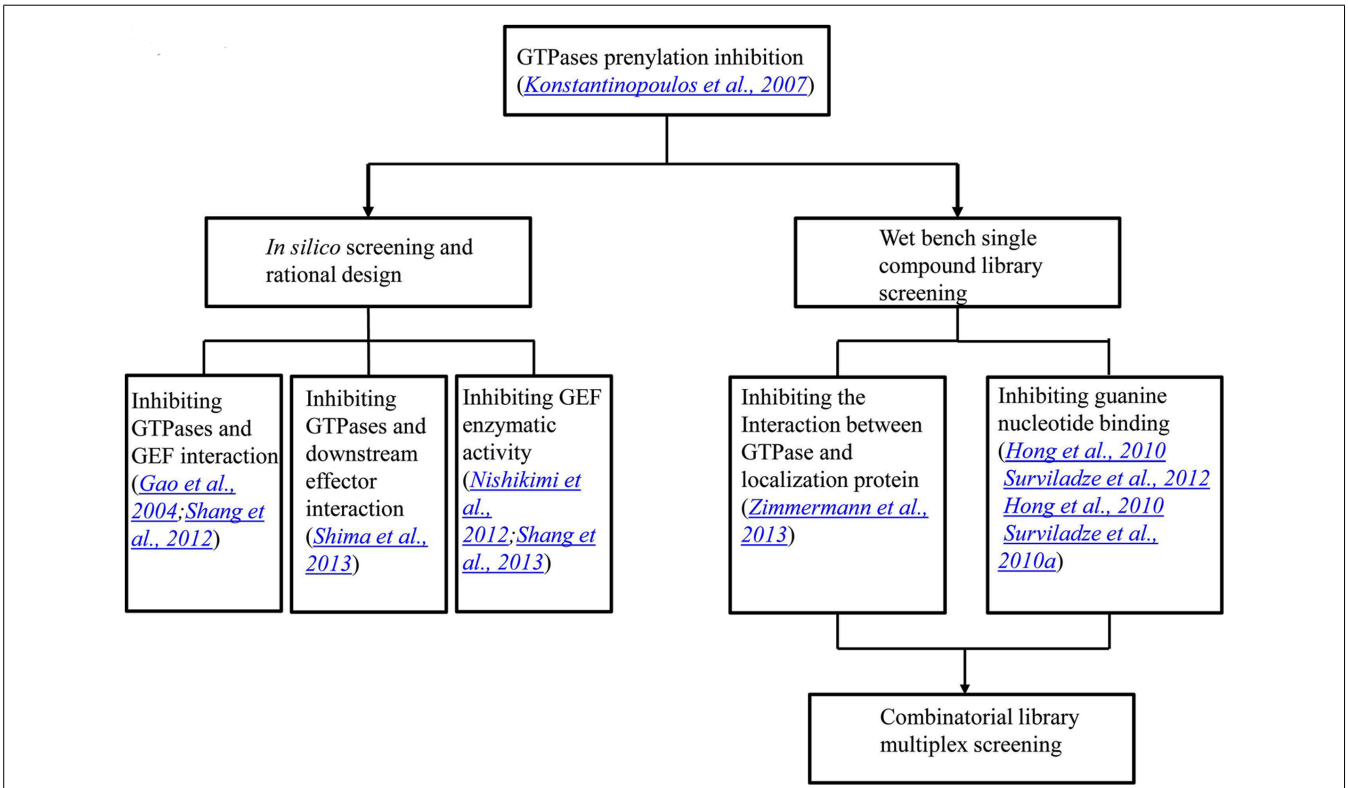

FIGURE 3 | A flow chart showing the progress in the development of GTPase activity modulators. The initial GTPase inhibitors were natural products that inhibit GTPase prenylation and signal transduction. In the last several years, through *in silico* screening and rational design, inhibitors that blocked protein interactions and GEF enzymatic activities were identified. On the other hand, single compound library screening revealed molecules that interfere with either protein interactions or nucleotide binding. The most recent progress lies in the combinatorial library screening in a multiplex format.

We have previously reported the results of a collaborative screening effort with Torrey Pines Institute for Molecular Studies (TPIMS) involving libraries generated by combinatorial synthesis and a duplex of G protein coupled receptors (GPCRs) which resulted in a large number of the most active small molecules for the formyl peptide receptors (FPRs) ever reported (Medina-Franco et al., 2013; Pinilla et al., 2013; Santos et al., 2013). The combinatorial library contains more than 5 million small molecules and 26 million peptides. These are grouped into 37 scaffolds each of which has combinatorial derivatives of multiple functional groups (Pinilla et al., 2013). Recently, we probed this chemical library for activity modulators for GTPases in a multiplex of Rab5, Rab7, Cdc42, Ras wild type and Ras mutant Q61L with Rho and Rac screened as individual targets[1]. The GTPases used either have been associated with Parkinson's disease or have significant roles in other illnesses. Interestingly, activators selective toward subfamilies of GTPases, including Ras and its mutant, were identified. Although the Ras inhibitors would usually be pursued, the activators of Ras can have their own uses, such as to generate cellular and animal models with aberrant Ras signaling which could be more convenient than the genetic approach. Moreover, if structures of the activator bound GTPases could be obtained, they would provide instructive information on designing GTPase activity regulators. Future collaborative studies will focus on the deconvolution of the combinatorial mixtures and the characterization of the individual or subgroups of compounds. The progress in the development of GTPase activity modulators has been recapitulated in a flow chart, as shown in **Figure 3**.

In summary, although the causes of the Parkinson's disease have not been elucidated, the accumulating studies have shown that several cellular physiological processes are likely to be implicated in the initiation and progression of the disease, such as organelle homeostasis, axon growth and maintenance, mitochondria dynamic changes, organelle traffic, oxidative homeostasis, and neuronal cell differentiation. Enhanced by GWAS, genes implicated in the familial forms of Parkinson's disease have been increasingly identified and their links to the disease have been studied. Multiple GTPases are involved in the disease related processes and some genes identified from GWAS encode GTPases. GTPases are therefore rational therapeutic targets for Parkinson's disease. However, GTPase drug discovery has been progressing more slowly and only more recently when compared to its kinase counterpart. This is likely due to the fundamentally different nature of the two classes of proteins, and the different roles they have in cell physiology. Yet, lessons learned from the kinase field, such as rational design and combinatorial approaches, joined by the rapid growth of structural information, promise a leap forward in GTPase drug discovery and development in the near future.

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[1]The available reagents, Rho and Rac proteins, suffered from technical difficulties that created interference in the multiplex assay.

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Parkinson's disease-implicated kinases in the brain; insights into disease pathogenesis

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Substantial evidence implicates abnormal protein kinase function in various aspects of Parkinson's disease (PD) etiology. Elevated phosphorylation of the PD-defining pathological protein, α-synuclein, correlates with its aggregation and toxic accumulation in neurons, whilst genetic missense mutations in the kinases PTEN-induced putative kinase 1 and leucine-rich repeat kinase 2, increase susceptibility to PD. Experimental evidence also links kinases of the phosphoinositide 3-kinase and mitogen-activated protein kinase signaling pathways, amongst others, to PD. Understanding how the levels or activities of these enzymes or their substrates change in brain tissue in relation to pathological states can provide insight into disease pathogenesis. Moreover, understanding when and where kinase dysfunction occurs is important as modulation of some of these signaling pathways can potentially lead to PD therapeutics. This review will summarize what is currently known in regard to the expression of these PD-implicated kinases in pathological human postmortem brain tissue.

Keywords: kinase, LRRK2, JNK, PLK, PINK1, GAK, MAPK, brain

INTRODUCTION

Studies of the postmortem human brain have been invaluable in gaining insights into the etiology of Parkinson's disease (PD), an increasingly common movement disorder resulting from the early selective loss of dopamine producing neurons in the substantia nigra. Dr. Fritz Lewy discovered the eponymous intracellular inclusion bodies synonymous with PD (Rodrigues et al., 2010). Drs. Tretiakoff, Hassler, and others were able to demonstrate degeneration of the substantia nigra in PD (Hassler, 1938). Postmortem brain studies were integral to the experiments of Carlsson and colleagues and their discoveries on the therapeutic potential of exogenous dopamine treatment for PD patients (Carlsson, 1959) and more recently, the work of Braak and others has suggested that PD spreads through the brain in a predictable or staged fashion (Braak et al., 2003; Halliday et al., 2008).

The Braak staging hypothesis is modeled on the toxic spread and accumulation of α-synuclein, a 17–18 kDa presynaptic protein encoded by the *SNCA* gene. Point mutations in, or multiplications of, the *SNCA* gene cause familial PD in an autosomal-dominant fashion (Polymeropoulos et al., 1997), whilst genome-wide association studies conclude that common variations in the *SNCA* gene increase the risk of sporadic PD (Pihlstrom and Toft, 2011). Moreover, α-synuclein is the predominant component of Lewy bodies, where it accumulates in an aggregated form (Spillantini et al., 1997). Hence, α-synuclein is proposed as a key protein in the pathogenesis of PD. Accumulating evidence suggests that α-synuclein acts in a prion-like manner, inducing the aggregation of healthy α-synuclein and propagating the spread of PD from neuron to neuron (Olanow and Brundin, 2013). The aggregated and proposed toxic form of α-synuclein is hyperphosphorylated (Oueslati et al., 2010). In disease free conditions only 4% of total α-synuclein is phosphorylated in brain, but in PD and related synucleinopathies, >90% of α-synuclein deposited in Lewy bodies is phosphorylated (Fujiwara et al., 2002; Anderson et al., 2006). In particular, phosphorylation of pathological α-synuclein on serine 129 (S129) is prevalent in PD postmortem brain (Fujiwara et al., 2002; Anderson et al., 2006; Zhou et al., 2011; Lue et al., 2012; Walker et al., 2013). Although the biological consequences of α-synuclein phosphorylation remain inconclusive, there is much interest in the identification of the kinases mediating this event. A number of candidate kinases, including members of the polo-like kinase (PLK), casein kinase (CK), and G protein coupled receptor kinase (GRK) families have subsequently been identified.

In addition to the hyperphosphorylation of α-synuclein, kinase dysfunction is also genetically linked to PD. In particular, missense mutations in the leucine-rich repeat kinase 2 (LRRK2) are causal for autosomal-dominant familial PD (Paisan-Ruiz et al., 2004; Zimprich et al., 2004), whilst multiple mutations in the PTEN-induced putative kinase 1 (PINK1) protein are causative for familial PD in a recessive fashion (Valente et al., 2004). Moreover, common polymorphisms identified by genome-wide association in loci encoding cyclin G-associated kinase (*GAK*) and serine/threonine kinase 39 [*STK39*, more commonly known as STE20-related proline alanine-rich kinase (SPAK)], have implicated these kinases as susceptibility enzymes for sporadic PD (Pankratz et al., 2009; Nalls et al., 2011; Sharma et al., 2012).

Finally a myriad of laboratory studies have focused on kinase signaling in PD. Kinases remain attractive targets for the treatment of many human diseases. Kinases of the MAPK and PI3K signaling pathways including extracellular signal related protein kinase (ERK), c-Jun N-terminal kinase (JNK), p38, protein kinase

B (PKB), and mammalian target of rapamycin (mTOR) make particularly attractive targets for PD through their ability to coordinate and regulate cell survival, apoptosis, inflammation, and autophagy.

KINASES MEDIATING α-SYNUCLEIN S129 PHOSPHORYLATION IN PD

The exact mechanism resulting in the pathological accumulation of S129 phosphorylated α-synuclein is unclear. A number of kinases phosphorylate α-synuclein at this residue *in vitro* with accumulating evidence for a role *in vivo* (**Figure 1**). Understanding how this major pathological protein becomes hyperphosphorylated and the extent to which post-translational modifications impact upon the aggregation and prion-like spread of α-synuclein could provide key insight into PD etiology.

POLO-LIKE KINASES (PLKs)

Polo-like kinases (PLKs) comprise a serine/threonine kinase family containing an N-terminal kinase catalytic domain and a C-terminal polo-box domain (PBD) that is involved in substrate binding and regulation of kinase activity. Five mammalian PLK family members from three subfamilies have been identified, including the PLK1 subfamily, the PLK4 subfamily, and the PLK2 subfamily (containing PLK2, PLK3, and PLK5; de Carcer et al., 2011b). The study of PLKs has focused primarily on their critical roles in the cell cycle (Winkles and Alberts, 2005); however, recent studies suggest PLKs also have important roles in terminally differentiated cells of the nervous system (Seeburg et al., 2005).

In particular, PLKs 1–3 are capable of phosphorylating α-synuclein (de Carcer et al., 2011a,b). Comparative studies suggest that PLK2 and PLK3 directly phosphorylate α-synuclein at Ser129 *in vitro* with high stoichiometry, whilst PLK4 is unable to phosphorylate α-synuclein at this residue (Anderson et al., 2006; Inglis et al., 2009). The low kinase activity of PLK4 against α-synuclein, and other substrates, is partially explained by its unique structure, with only a single polo-box in the PBD, resulting in a much-reduced electropositive environment in its substrate-binding site (Mbefo et al., 2010). Human PLK5 lacks a functional kinase domain due to a premature stop codon in exon 6 and is therefore unable to phosphorylate α-synuclein.

Increasing PLK2 or PLK3 significantly up-regulates α-synuclein Ser129 phosphorylation (Mbefo et al., 2010; Waxman and Giasson, 2011), whilst their inhibition or reduction remarkably decreases α-synuclein phosphorylation in both cell and animal models (Inglis et al., 2009; Waxman and Giasson, 2011). This has led to efforts to generate small molecule PLK inhibitors for potential therapeutic use (Bowers et al., 2013; Fitzgerald et al., 2013; Bergeron et al., 2014). The utility of such compounds, however, has been questioned by a recent study showing that Ser129 phosphorylation by PLK2 is required for autophagic degradation of α-synuclein (Oueslati et al., 2013). In this study overexpression of PLK2, as opposed to inhibition, prevented the toxic accumulation of α-synuclein in rodent models, suggesting more work is required to delineate the exact role of PLKs in α-synuclein pathology.

In addition, studies investigating the association of PLKs with α-synuclein pathology / phosphorylation in human brain are lacking. The central nervous system has relatively high levels of PLK2, 3, and 5, low levels of PLK1 and seems to lack PLK4 (Winkles and Alberts, 2005; de Carcer et al., 2011a,b). PLK2 and PLK3 are expressed in most regions of the brain, but surprisingly there is almost no expression of either PLK2 or PLK3 in the cerebellum (Winkles and Alberts, 2005; de Carcer et al., 2011b). Whether PLK2 or PLK3, the main family members that can phosphorylate α-synuclein are increased, or indeed more active, in PD brain remains to be determined. The recent identification of autophosphorylation sites on PLK2 (Rozeboom and Pak, 2012) and other potential selective substrates (Salvi et al., 2012) could allow readouts of PLK2 activity to be examined in PD brain. It would be of interest to determine if the phosphorylation of such substrates correlated to levels of α-synuclein phosphorylation in pathology rich brain regions in PD cases.

CASEIN KINASES (CKs)

Casein kinases (CKs) comprise a ubiquitously expressed serine/threonine kinase family (Peters et al., 1999) containing two members, CK1 and CK2, which differ substantially in terms of structure, localization and function (Perez et al., 2011). CK1 consists of a small N-terminal lobe, a large C-terminal lobe and a catalytic cleft where ATP and substrates bind (Cheong and Virshup, 2011). To date, at least seven CK1 isoforms (α, β, γ1–3, δ,

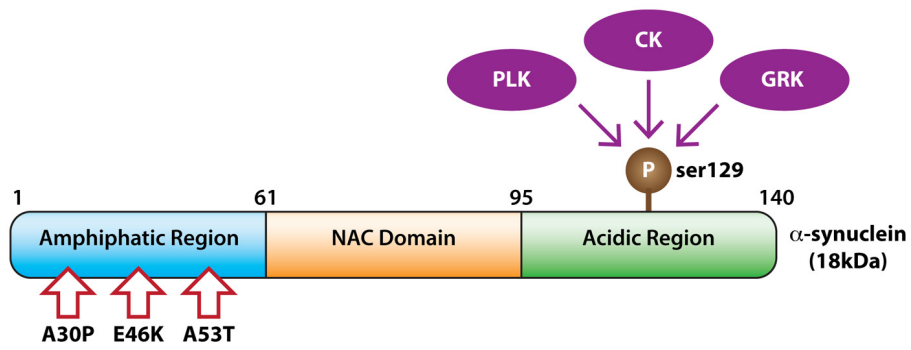

FIGURE 1 | Kinases phosphorylating α-synuclein. The domain structure of α-synuclein showing phosphorylation at serine 129 by members of the polo-like kinase (PLK), casein kinase (CK), and G protein coupled receptor kinase (GRK) families. Pathogenic α-synuclein missense mutations are indicated with arrows.

and ϵ) and their various splice variants, ranging from 22 to 55 kDa, have been localized within the membrane, nucleus, and cytoplasm of eukaryote cells, and additionally in the mitotic spindles of mammalian cells (Fish et al., 1995). All CK1 isoforms are highly homologous in their kinase domains (Knippschild et al., 2005), presenting a strong preference for "primed," pre-phosphorylated substrate. However, they can also phosphorylate related unprimed sites under certain conditions (Cheong and Virshup, 2011).

In contrast to CK1, CK2 is a tetrameric enzyme assembled from two catalytic subunits (CK2α and CK2α′) and a regulatory subunit (CK2β dimer). The two catalytic subunits α and α′ share 90% sequence homology in their N-terminal region, but the regulatory subunit β does not have any similarity to the other two subunits. CK2 is found in many organisms and tissues and nearly every subcellular compartment. It can phosphorylate more than 300 substrate proteins (Meggio and Pinna, 2003) involved in diverse cellular processes including cell division, proliferation, apoptosis, and DNA repair.

Both CK1 and CK2 can constitutively phosphorylate α-synuclein at Ser129 *in vitro* (Okochi et al., 2000; Waxman and Giasson, 2008), and inhibition of CK1 or CK2 reduces α-synuclein Ser129 phosphorylation *in vivo* (Okochi et al., 2000; Ishii et al., 2007; Waxman and Giasson, 2008), with CK2 inhibition seemingly more efficient at reducing phosphorylation (Ishii et al., 2007). However, at least one study failed to find an effect of CK1 inhibition on α-synuclein Ser129 phosphorylation in a cellular model (Waxman and Giasson, 2011). This discrepancy could result from, at least partially, the specificity of CK1 inhibitors and more studies are needed to define the relationship between CKs and α-synuclein.

In pathological human brain CK1δ co-localizes predominantly with tau-containing inclusions such as neurofibrillary tangles, and does not co-localize with α-synuclein in Lewy bodies in PD (Schwab et al., 2000). In contrast, CK2β regulatory subunits are present in the halo region of Lewy bodies in PD substantia nigra (Ryu et al., 2008), suggesting that CK2 may be more pathologically relevant to PD. More work is required to determine any correlations between CK isoforms and the pathological accumulation of phosphorylated α-synuclein in PD.

G PROTEIN COUPLED RECEPTOR KINASES (GRKs)

G Protein coupled receptor kinases comprise a serine/threonine kinase family that regulate G protein-coupled receptors (GPCRs) by phosphorylating their intracellular domains after their associated G proteins have been released and activated (Gurevich et al., 2012). Structurally, GRKs contain a central catalytic domain flanked by an N-terminus containing a regulator of G protein signaling homology domain and a variable length C-terminal end. Based on sequence homology and tissue expression, GRKs are further classified into three subfamilies: the rhodopsin kinase or visual GRK subfamily (GRK1 and GRK7), the β-adrenergic receptor kinases subfamily (GRK2 and GRK3), and the GRK4 subfamily (GRK4, GRK5, and GRK6; Gurevich et al., 2012; Kamal et al., 2012).

Exactly which GRK isoforms phosphorylate α-synuclein under pathological conditions is unclear. *In vitro* GRK2 preferentially phosphorylates α and β synuclein isoforms while GRK5 prefers α-synuclein as a substrate (Pronin et al., 2000). However, knockdown of either GRK5 or GRK2 failed to diminish the phosphorylation of α-synuclein in cell models (Sakamoto et al., 2009; Liu et al., 2010). In contrast, knockdown of GRK3 or GRK6 significantly decreased α-synuclein Ser129 phosphorylation levels (Sakamoto et al., 2009), suggesting further work is required to verify the role of GRK isoforms in phosphorylating α-synuclein.

G protein coupled receptor kinase isoforms, 2, 3, 5, and 6, are highly expressed in the human brain. In PD brain, however, GRK protein levels tend to be lower than controls (Bychkov et al., 2008) with conflicting reports regarding the co-localization of GRK5 in Lewy bodies (Arawaka et al., 2006; Takahashi et al., 2006).

OVERVIEW OF KINASES INVOLVED IN α-SYNUCLEIN S129 PHOSPHORYLATION IN PD

Understanding events that promote α-synuclein pathology is increasingly important as evidence suggests a pathogenic prion-like spread of α-synuclein in PD (Olanow and Brundin, 2013; Recasens et al., 2014). There are now multiple human brain tissue studies using the methods developed by Braak and colleagues to observe the progression of pathology in PD brain showing that substantial α-synuclein S129 phosphorylation precedes the aggregation of α-synuclein in Lewy bodies (Zhou et al., 2011; Lue et al., 2012; Walker et al., 2013). Stoichiometrically, PLK2 seemingly contributes most to such α-synuclein S129 phosphorylation; however, studies with PLK2 knockout mice show that other kinases also contribute (Bergeron et al., 2014). Information from other PD models, however, remains controversial on the role α-synuclein S129 phosphorylation plays in disease pathogenesis, with some studies suggesting that S129 phosphorylation promotes α-synuclein oligomerization and/or toxicity (Chen and Feany, 2005; Febbraro et al., 2013) whilst others suggest that phosphorylation reduces toxicity or has no effect (McFarland et al., 2009; Oueslati et al., 2010; Sato et al., 2013; Escobar et al., 2014). This makes determining other relevant kinases difficult and information on any differences between species and models (acute versus chronic) will need further consideration. More data from informative staged human brain studies as well as from primate models with acute and chronic phases is likely to assist with clarifying the role of α-synuclein S129 phosphorylation over the course of PD. It is also important to note that other post-translation modifications of α-synuclein, such as ubiquitylation or nitrosylation, may equally contribute to the pathological process (Oueslati et al., 2010), with similar staged human brain and primate model data on the relative contributions of different protein modifications yet to be published.

KINASES GENETICALLY IMPLICATED IN PD

Monogenetic causes of PD presently account for less than 10% of all cases (Gasser, 2009). However, the identification of genetic causes has invigorated PD research by providing new avenues of mechanistic investigation and therapeutic treatment. Missense mutations in *LRRK2* and *PINK1* cause PD in an autosomal-dominant or recessive manner, respectively, whilst common variations in the *LRRK2* and loci encoding the *GAK* and *STK39* genes have been implicated as risk factors for PD (Sharma et al., 2012). Understanding how mutations in these kinases alters their

function and the biological processes they regulate has great potential for uncovering initiating events leading to the onset of PD.

LEUCINE-RICH REPEAT KINASE 2 (LRRK2)

Mutations in the *LRRK2* gene were discovered as causal for PD in 2004 (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Subsequently, some 40 missense mutations have been described across the LRRK2 protein with six of these demonstrated as pathogenic (Paisan-Ruiz, 2009). Collectively these *LRRK2* mutations account for the majority of autosomal-dominantly inherited PD (Kett and Dauer, 2012). *LRRK2*-associated PD is largely clinically and pathologically indistinguishable from sporadic PD (Healy et al., 2008), suggesting that understanding LRRK2 function has implications for all forms of PD. Moreover, large-scale genome-wide association studies show that common variations in non-coding regions of the *LRRK2* gene also confer greater risk for sporadic PD (Satake et al., 2009; Simon-Sanchez et al., 2009; Sharma et al., 2012).

Leucine-rich repeat kinase 2 is a 286 kDa multi-domain containing member of the receptor interacting protein kinase (RIPK) family. LRRK2 has N-terminal ankyrin repeats, leucine-rich repeats, a ras of complex (ROC) GTPase domain with adjoining C-terminal of ROC (COR) domain, a serine/threonine protein kinase domain and C-terminal WD40 repeats. Intriguingly, the majority of the pathogenic mutations lie in the catalytic domains of LRRK2. The most common mutation results in the substitution of glycine to serine (G2019S) in the activation loop of the protein kinase domain resulting in a constitutive threefold increase in LRRK2 kinase activity (West et al., 2005; Jaleel et al., 2007). The next most common mutations, substitution of arginine to either histidine (R1141H), cysteine (R1441C), or glycine (R1441G) lie in the GTPase domain. Some evidence suggests that these mutations also increase kinase activity (Sheng et al., 2012), potentially by trapping LRRK2 in a GTP bound active state (Liao et al., 2014). It has previously been shown that GTP binding is required for LRRK2 kinase activity (Taymans et al., 2011) and a complex relationship exists between the two domains (Taymans, 2012). The increase in catalytic kinase activity with LRRK2 mutations has led to the development of LRRK2 kinase inhibitors as potential PD therapeutics (Deng et al., 2012) and much interest has focused on determining the targets of LRRK2 kinase activity (Dzamko and Halliday, 2013). One such robust effect for LRRK2 kinase activity is to mediate the phosphorylation-dependent interaction of LRRK2 with isoforms of the 14-3-3 adaptor protein (Dzamko et al., 2010); however, consensus regarding the PD-relevant physiological functions of LRRK2 has remained largely elusive.

LRRK2 mRNA expression shows a widespread neuronal localization in human brain; however, intriguingly, only weak levels are detected in the substantia nigra (Higashi et al., 2007; Sharma et al., 2011). Moreover, decreased LRRK2 mRNA was found in certain non-nigral regions of PD brain (cerebellum, amygdala, frontal cortex, and cingulate gyrus; Sharma et al., 2011), suggesting a pathogenic role for LRRK2 outside of nigral neurons. This contrasts with the increased levels of LRRK2 protein reported in PD brain regions with pathological accumulation of α-synuclein (Cho et al., 2013; Guerreiro et al., 2013). The exact nature of the relationship between LRRK2 and α-synuclein is somewhat unclear as postmortem localization studies have produced conflicting results. Some studies have demonstrated localization of LRRK2 to α-synuclein pathology (Miklossy et al., 2006; Alegre-Abarrategui et al., 2008; Qing et al., 2009; Sharma et al., 2011) whilst others have not (Giasson et al., 2006; Higashi et al., 2007; Melrose et al., 2007; Waxman et al., 2009). Studies comparing different LRRK2 antibodies have shown that discrepancies in LRRK2 tissue localization likely occurs through use of antibodies unsuitable for immunohistochemistry (Biskup et al., 2007; Melrose et al., 2007; Davies et al., 2013). Indeed, recent data using more rigorous methods shows LRRK2 and α-synuclein co-localize in a small proportion of PD pathologies (Guerreiro et al., 2013). Despite such data, further work is required to define the relationship between LRRK2 expression and protein levels, between LRRK2 and α-synuclein increases and aggregation, and indeed determine if these proteins interact in the same molecular pathway.

As LRRK2 is also expressed by glial cells in normal human brain (Miklossy et al., 2006) and in tissue culture, and its expression in glia is increased by interferon gamma (Gardet et al., 2010) and bacterial lipopolysaccharide (LPS; Moehle et al., 2012), the neuroinflammation prevalent in PD affected regions may promote the expression changes observed for LRRK2 specifically in microglia rather than neurons. It will also be important to correlate any changes in LRRK2 expression to cell type. The recent demonstration that there is a primate specific LRRK2 promoter that differentiates primate expression of the protein in the brain from that observed in rodents (West et al., 2014) underlies the requirement for further observations in staged human tissue specimens in order to determine the role of LRRK2 kinase function in PD pathogenesis.

PTEN-INDUCED PUTATIVE KINASE 1 (PINK1)

Homozygous missense mutations in the *PINK1* gene were identified as a cause of familial PD in 2004 (Valente et al., 2004). Around 50 missense mutations have subsequently been identified across the PINK1 protein in a number of populations (Kawajiri et al., 2011). Mutations in *PINK1* are the second most common cause of recessive PD (following mutations in the ubiquitin ligase *Parkin*) and are thought to contribute to 1–8% of familial PD (Kawajiri et al., 2011). Unlike *LRRK2*, *PINK1* mutations reduce kinase activity and cause an atypical form of PD characterized by an early age of onset and slower clinical progression (Abou-Sleiman et al., 2006; Woodroof et al., 2011).

The PINK1 protein comprises a serine/threonine protein kinase domain, a N-terminal mitochondrial targeting motif and a transmembrane domain located between the two. The mitochondrial targeting motif is required for recruitment of PINK1 to mitochondrial membranes. Following recruitment in healthy mitochondria, PINK1 is enzymatically cleaved to produce a shorter fragment, which is degraded by the proteasome (Narendra et al., 2010). In this way, PINK1 is maintained at very low levels. Pharmacological uncoupling of the mitochondrial membrane leading to a loss of membrane potential; however, results in inhibition of PINK1 cleavage and its accumulation on depolarized mitochondrial membranes (Matsuda et al., 2010; Narendra et al., 2010). The kinase activity of PINK1 is also increased under these conditions with PINK1 undergoing autophosphorylation (Kondapalli et al.,

2012). Intriguingly, Parkin is then also recruited to depolarized mitochondria where it is phosphorylated and activated by PINK1 (Matsuda et al., 2010; Kondapalli et al., 2012). Parkin then mediates the degradation of dysfunctional mitochondria by mitophagy (mitochondrial autophagy). Therefore, the two proteins responsible for the majority of familial early onset PD appear to function in the same pathway important for the regulation of mitochondrial quality control. Whether this is the pathway ultimately responsible for loss of neurons in PD is still unclear (Grenier et al., 2013) and it should be noted that PINK1 has also been implicated in other biological processes such as neurite maintenance (Dagda et al., 2014) and inflammation (Lee and Chung, 2012; Kim et al., 2013) among others.

Detailed *in-situ* hybridization studies using rodent brain demonstrate that *PINK1* mRNA is expressed throughout the brain with the strongest signal in neurons of the olfactory bulb, neocortex, prefrontal cortex, piriform cortex, hippocampus, amygdala, brainstem, and cerebellar Purkinje cells (Taymans et al., 2006). Similarly, in human brain, *PINK1* mRNA is widely expressed in neurons with highest signals recorded for the temporal cortex, amygdala, substantia nigra, cerebellar Purkinje cells and the dentate nucleus (Blackinton et al., 2007). *PINK1* mRNA is undetectable in glial cells (Taymans et al., 2006; Blackinton et al., 2007) and is not different in the substantia nigra of sporadic PD patients compared to controls (Blackinton et al., 2007). The mRNA distribution of *PINK1* has been largely confirmed at the protein level with the exception that PINK1 immunoreactivity was also observed in glia, albeit with weak staining compared to neuronal staining (Gandhi et al., 2006). PINK1 is predominantly localized to mitochondria and does not change in amount or localization in the brain of patients with idiopathic PD, although PINK1 immunoreactivity is detected in ∼10% of brainstem Lewy bodies (Gandhi et al., 2006). Interestingly, a case report describing the neuropathology of an early onset *PINK1* homozygous mutation patient showed a pattern of Lewy body pathology with atypical Braak Lewy body staging (Samaranch et al., 2010). This was due to the absence of Lewy bodies, and indeed cell loss in the locus coeruleus, potentially helping to explain the longer disease duration of *PINK1*-associated PD (Samaranch et al., 2010). Collectively these studies suggest that alterations in PINK1 function, rather than protein levels, likely contribute to PD. This is consistent with observations that the majority of described *PINK1* mutations result in a loss of kinase activity (Woodroof et al., 2011). Whether PINK1 autophosphorylation or PINK1-induced Parkin phosphorylation are altered in sporadic PD, and how this correlates to mitochondrial health and/or neuronal loss, would be interesting to explore.

CYCLIN G-ASSOCIATED KINASE (GAK)

The Ser/Thr protein kinase GAK was originally identified via its interaction with cyclin G and cyclin-dependent kinase 5 (CDK5; Kanaoka et al., 1997). The kinase domain is located at the N-terminus and a leucine-zipper region is located at the C-terminus. The majority of the protein comprises a TAG domain that has 80% identity to the auxilin protein (Kanaoka et al., 1997). Both auxilin and GAK play key roles in the uncoating of clatherin-coated vesicles and the regulation of clatherin-mediated endocytosis (Eisenberg and Greene, 2007). The latter is completely blocked in GAK deficient mouse embryonic fibroblasts (Lee et al., 2008). GAK also plays a key role in brain development. Conditional deletion of GAK in mouse brain resulted in marked cell loss and morphological changes in new-born pups, potentially due to a lack of proliferation of neural progenitor cells in the subventricular zone of the hippocampus. Conditional GAK knockout mice die soon after birth whilst conventional GAK knockouts are embryonic lethal (Lee et al., 2008). Moreover transgenic mice expressing kinase inactive GAK die within 30 min of birth due to respiratory dysfunction (Tabara et al., 2011). Respiratory problems are also associated with use of gefitinib (Tabara et al., 2011), an anticancer epidermal growth factor receptor (EGFR) inhibitor that also inhibits GAK.

Single nucleotide polymorphisms in the *GAK* locus were first associated with PD susceptibility following genome-wide association analysis of a large number of familial PD patients (Pankratz et al., 2009). The association has since been robustly replicated in different populations (Rhodes et al., 2011; Sharma et al., 2012). One *GAK* SNP, rs1564282 is associated with higher expression of α-synuclein in PD brain, and when *GAK* mRNA was knocked down with siRNA, there was accumulation of α-synuclein in cell culture models (Dumitriu et al., 2011). This provides some biochemical evidence for a role for GAK in PD, although the toxic effects of GAK knockdown/inhibition suggest that GAK is unlikely to be dramatically decreased in PD brain. Moreover, an alternative microarray based study has shown that *GAK* mRNA expression is increased in the substantia nigra of PD patients (Grunblatt et al., 2004). Intriguingly, GAK has also been proposed to interact with LRRK2 and potentially help co-ordinate the clearance of trans-Golgi derived vesicles (Beilina et al., 2014); however, GAK protein expression in PD brain and any association with PD pathology has been poorly explored.

SERINE/THREONINE KINASE 39 (STK39)

Serine/threonine kinase is more commonly referred to in the literature as SPAK. The majority of work on SPAK has focused on the enzymes role as a regulator of the Na^+/Cl^- and $Na^+/K^+/2Cl^-$ ion co-transporters, NCC and NKCC, respectively. In response to osmotic stress SPAK is activated by phosphorylation at T233 in its activation loop by isoforms of WNK (with-no lysine) kinases and in turn phosphorylates NCC/NKCC to promote transporter activity (Richardson and Alessi, 2008). These ion co-transporters are major drug targets of current anti-hypertensive medications and evidence suggests that inhibition of SPAK may also lower blood pressure (Richardson and Alessi, 2008; Glover and O'shaughnessy, 2011). Indeed, variations in the *STK39* gene have been implicated in hypertension in the Amish population through genome-wide association, with the resulting non-coding mutations increasing the allelic expression of SPAK (Wang et al., 2009a). This association, however, has failed to reach significance in other populations (Cunnington et al., 2009; Persu and Vikkula, 2011).

Genome-wide association studies have also implicated variations in the *STK39* locus with PD. First identified through large-scale meta-analysis (Liu et al., 2011; Nalls et al., 2011), the association of *STK39* SNPs with PD has been subsequently replicated in Asian and Caucasian populations (Lill et al., 2012; Sharma

et al., 2012). The three reported *STK39* SNPs associating with PD differ from the reported SNP for hypertension. Whether these polymorphisms affect SPAK expression is unknown.

Studies using rats show that SPAK is highly expressed in the nervous system, especially brain where it is detected in neurons, Purkinje cells and choroid epithelial cells (Ushiro et al., 1998). Glial cells do not show immunoreactivity for SPAK (Ushiro et al., 1998). In developing brain, SPAK plays a role in the regulation of Cl^- concentration and in-turn release of the neurotransmitter GABA (Delpire and Austin, 2010). SPAK has also been suggested to act as a stress-response kinase with its overexpression or activation leading to increased phosphorylation of p38 MAPK (Yan et al., 2007). Whether levels of SPAK protein, phosphorylation of SPAK T233 or phosphorylation of the SPAK ion transporter substrates are altered in PD brain has not been investigated.

OVERVIEW OF KINASES GENETICALLY IMPLICATED IN PD

There is much interest in LRRK2 as both a key to understanding PD pathogenesis and a potential therapeutic target, as *PINK1* mutations cause an atypical form of PD and the mechanism/s of increased PD risk due to *SPAK* and *GAK* polymorphisms is presently unclear. The mRNA expression of *LRRK2* is decreased in PD brain; however, LRRK2 protein is increased, at least in Lewy body-rich regions at end-stage disease. Further work is required to determine if LRRK2 protein is altered earlier in PD pathogenesis and in particular, as LRRK2 expression can be induced with inflammatory agonists in microglia (Moehle et al., 2012), the cell types expressing LRRK2 may be important. Localization studies of LRRK2, and indeed PINK1 have proven difficult, as a number of available antibodies are not optimal for this procedure. Moreover, the kinase activity of LRRK2 has not been explored in PD brain. This is potentially important as kinase inhibiting therapeutics are being targeted toward LRRK2, even though it is unclear if the toxic effects of LRRK2 mutations are kinase-dependent. At least one risk variant reportedly decreases LRRK2 kinase activity (Rudenko et al., 2012b) leading to suggestions that other functions of LRRK2 such as GTPase activity may be important (Rudenko et al., 2012a). The identification of *bona fide* substrates for PINK1 and LRRK2 will be important for inferring any changes in enzymatic activity in the PD brain.

KINASES EXPERIMENTALLY IMPLICATED IN PD

KINASES OF THE MITOGEN ACTIVATED PROTEIN KINASE (MAPK) PATHWAY

The MAPK superfamily of serine/threonine protein kinases consists of three major branches, the JNKs, the p38 kinases and the ERKs (**Figure 2**; for review see Kyriakis and Avruch, 2012). The three JNK isoforms (JNK1, JNK2, and JNK3) and four p38 isoforms (p38α, p38β, p38γ, and p38δ) are referred to as stress activated protein kinases (SAPKs). In particular, JNK is activated by a number of environmental stresses implicated in PD including, toxins, inflammatory agonists and misfolded protein-induced ER stress. The activation of p38 is more restricted to inflammatory agonists whilst the two ERK isoforms (ERK1 and ERK2) are activated principally in response to mitogens, although a high level of cross-talk exists between the different MAPK branches. Upon activation, JNK, ERK, and p38 phosphorylate a large number of substrates in a proline-directed manner. In some instances substrates can be specific, such as JNK to phosphorylate the AP-1 transcription factor component c-Jun, or ERK to phosphorylate the p90 ribosomal S6 kinase (RSK), or shared, such as ERK and p38 to phosphorylate the mitogen and stress activated kinase (MSK). Biologically, the MAPKs modulate a number of important functions including development, immunity, apoptosis, cell growth and division, autophagy and cell survival.

JNK and p38

c-Jun N-terminal kinase is robustly activated in common toxin models of PD such as LPS, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA; Choi et al., 1999; Saporito et al., 2000; Xing et al., 2007). Genetic deletion of JNK2 and JNK3 protect against MPTP-induced neurodegeneration in mice (Hunot et al., 2004) and kinase inhibitors of JNK have neuroprotective effects in the MPTP (Saporito et al., 1999; Wang et al., 2004, 2009b; Chambers et al., 2011) and 6-OHDA (Chambers et al., 2013) models of PD. Moreover a host of anti-oxidant and anti-inflammatory compounds offering varying degrees of neuroprotection in these models are thought to have a mechanism of action, at least in part, involving inhibition of JNK activation (Xing et al., 2007; Castro-Caldas et al., 2012; Lee et al., 2013; Zhai et al., 2013). Genetic deletion of the p38 substrate MK2, also protects against MPTP-induced neurodegeneration in rodents, by reducing the neuroinflammation associated with MPTP lesions (Thomas et al., 2008). Both JNK and p38 are also implicated in the death of neuronal cells following treatment with another environmental toxin used to model PD, rotenone (Newhouse et al., 2004; Gao et al., 2013), with inhibition of p38 potentially protective (Choi et al., 2014). Despite the evidence from cellular and animal models, however, a clinical trial of the JNK inhibitor CEP-1347 failed to show benefit in human PD patients (Investigators, 2007), possibly because of an absence of substantial changes in these kinases in patients with chronic PD (Ferrer et al., 2001).

Increased nuclear staining of the JNK substrate, c-Jun, has been observed in the substantia nigra of PD patients (Hunot et al., 2004). Translocation of c-Jun to the nucleus requires JNK phosphorylation and is a surrogate marker of JNK activity. The association between JNK and p38 and α-synuclein pathology has also been explored in the substantia nigra and brainstem regions of control and PD brain. In this study, granular phosphorylated p38 immunoreactivity was observed in association with diffuse α-synuclein pathology, more consistent with Lewy neurites than Lewy bodies in the substantia nigra (Ferrer et al., 2001). In contrast, phosphorylated JNK rarely stained Lewy body containing neurons (Ferrer et al., 2001). There was also no association between phosphorylated JNK immunostaining and apoptosis in PD substantia nigra neurons (Ferrer et al., 2001). These studies suggest a potential early role for p38 in the formation of Lewy bodies whereas JNK appears not to be involved. The protective effects of JNK inhibitors may instead be mediated through glial cells. Further studies could explore how JNK activity in glia relates to PD pathogenesis.

ERK

Extracellular signal related protein kinase is activated following treatment of cells with 6-OHDA and MPTP and inhibitors of ERK provide protection in these PD cell models (Kulich and Chu, 2001; Gomez-Santos et al., 2002). ERK crosstalk also modulates protective effects of neurotrophins and anti-oxidant treatments (Chu et al., 2004; Hetman and Gozdz, 2004).

In neurons in the substantia nigra of PD patients, phosphorylated ERK immunoreactivity shows granular aggregations, distinct from the diffuse cytoplasmic localization of phosphorylated ERK in cortical neurons from control and PD patients (Zhu et al., 2002). The aggregated pattern of phosphorylated ERK staining is also observed in pigmented neurons of the locus coeruleus in PD patients, but is absent in glial cells. The levels of phosphorylated ERK increase in substantia nigra neurons in PD patients, where the granular inclusions partly associate with mitochondria and weakly with endosomes (Zhu et al., 2003). Increased phosphorylation of ERK correlates with increased staining for the ERK substrate RSK1 (Zhu et al., 2002) and total levels of ERK do not differ between control and PD samples (Zhu et al., 2002), collectively demonstrating an increase in ERK activity in PD brain. ERK also associates with Lewy bodies, particularly the halo region (Ferrer et al., 2001; Zhu et al., 2002, 2003). Moreover, granular ERK inclusions are often seen in PD neurons devoid of α-synuclein pathology and sometimes not seen at all in neurons with severe α-synuclein pathology suggesting a potential early role for ERK in PD pathogenesis (Zhu et al., 2002).

KINASES OF THE PHOSPHOINOSITIDE 3-KINASE (PI3K) PATHWAY

The PI3K pathway controls cell survival and proliferation and thus has been studied extensively in the context of cancer. PI3K is classically activated by tyrosine kinase receptors following their binding of insulin or insulin like growth factors (e.g., IGF1; **Figure 3**). Activated PI3K phosphorylates membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP2) to produce phosphatidylinositol 2,4,5-triphosphate (PIP3), which results in recruitment of PKB (also known as AKT; **Figure 3**). PKB is in turn phosphorylated in its activation loop (at Thr308) by phosphoinositide-dependent

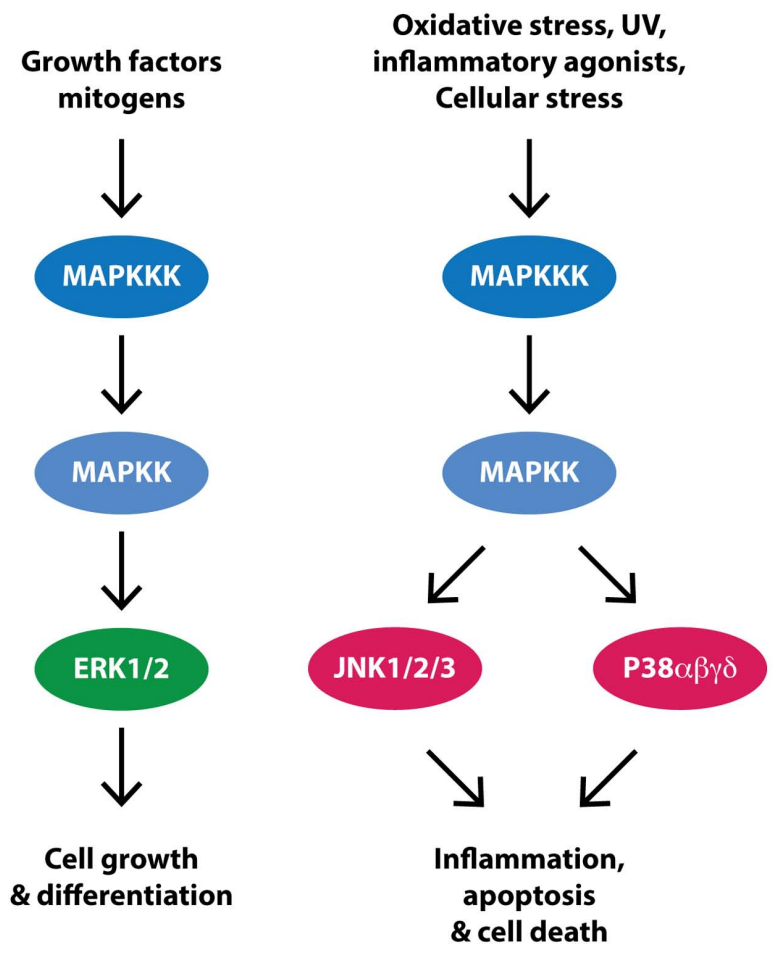

FIGURE 2 | Simplifed MAPK signaling. Receptor activation by growth factors or mitogens triggers a signal cascade in which mitogen activated protein kinase kinase kinases (MAPKKK) are activated and in turn activate mitogen activated kinase kinases (MAPKK) and then the mitogen activated protein kinases (MAPK), ERK, JNK, and P38. Evidence suggests that this pathway is upregulated in PD substantia nigra dopaminergic neurons, potentially contributing to cell death.

kinase 1 (PDK1) and at its C-terminal hydrophobic motif (Ser473) by mTOR complex 2 (mTORC2). Activated PKB phosphorylates a number of substrates including mTOR, to promote protein synthesis and inhibit autophagy, glycogen synthase kinase 3β (GSK3β), to induce glycogen and regulate glucose metabolism and fork head box-O class (FOXO), a transcription factor regulating genes essential for cell growth, proliferation, and survival.

PKB

Administration of 6-OHDA results in reduced PKB Thr308 and Ser473 phosphorylation and marked loss of PKB activity in cell culture and rodent PD models and a number of compounds that stimulate PKB activity have demonstrated neuroprotection in this model, as well as other PD toxin models, including MPTP and rotenone (for review see Greene et al., 2011). Overexpression of PKB in rodent brain also protects dopaminergic neurons from 6-OHDA-induced cell death (Ries et al., 2006) and activation of PKB likely contributes to the neuroprotective effects of trophic factors such as glial cell line-derived neurotrophic factor (GDNF; Ugarte et al., 2003) and potentially to the effects of the monoamine oxidase B inhibitor and PD drug rasagiline (Mandel et al., 2007; Sagi et al., 2007).

In PD midbrain the phosphorylation of PKB Ser473 is reduced in both cytosolic and membrane fractions (Timmons et al., 2009). PKB and its phosphorylation at Ser473 are also robustly detected in dopaminergic neurons of the substantia nigra and are consequently reduced in PD with loss of these neurons. However, PKB immunoreactivity is still detected in surviving PD dopaminergic neurons (Timmons et al., 2009). A second study has confirmed reduced phosphorylation of PKB at both Thr308 and Ser473 in PD substantia nigra dopaminergic neurons (Malagelada et al., 2008). Results of this study suggest that reduced phosphorylation of PKB is restricted to dopaminergic neurons as non-neuromelanin containing neurons of the midbrain expressed similar levels of PKB and phosphorylated PKB in both control and PD states. Interestingly, a robust increase in PKB and phosphorylated Ser473 PKB was detected in cells with glial morphology in the substantia nigra region in PD (Timmons et al., 2009). Whilst reduced PKB pathway activity in neurons may contribute to their loss in PD, the contribution of increased PKB activity in glia to the progression of PD has not been explored.

*GSK3*β

The two isoforms of GSK3, GSK3α, and GSK3β, are ubiquitously expressed in the brain where they predominantly act to regulate glucose metabolism. Inhibition of the GSK3β isoform can protect against MPTP, 6-OHDA, and LPS-induced neurotoxicity (Kozikowski et al., 2006; Wang et al., 2007; Morales-Garcia et al., 2013) whilst its activation has been implicated in rotenone toxicity (Hongo et al., 2012). GSK3β has also been implicated in microglial-mediated inflammation (Yuskaitis and Jope, 2009) and the neuroprotective effects of GSK3β inhibitors may be mediated, at least in part, through anti-inflammatory actions (Yuskaitis and Jope, 2009; Morales-Garcia et al., 2013).

In brain, increased total and phosphorylated GSK3β is detected as punctate structures in the cytosol of pigmented neurons in PD

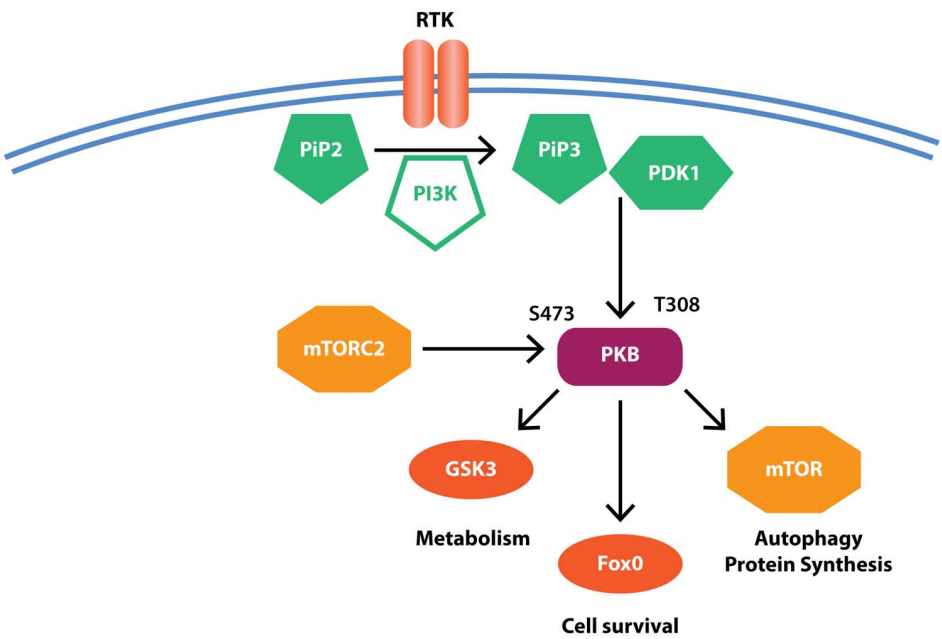

FIGURE 3 | Simplified PI3K signaling. Receptor-ligand binding results in the activation of phosphoinositide 3-kinase (PI3K) that in turn mediates the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 2,4,5-triphosphate (PIP3). PIP3 recruits protein kinase B (PKB) where it is activated by phosphorylation at Thr308 by phosphoinositide-dependent kinase 1 (PDK1) and Ser473 by mammalian target of rapamycin (mTOR) complex 2 (mTORC2). PKB then further phosphorylates downstream substrates to regulate cell survival and metabolic pathways. Evidence suggests reduced PKB but increased mTOR and GSK3β activity in PD, potentially contributing to protein accumulation and reduced cell survival.

substantia nigra (Nagao and Hayashi, 2009). GSK3β and phosphorylated GSK3β partly co-localize to the halo region of Lewy bodies and also to Lewy neurites (Nagao and Hayashi, 2009). GSK3β protein is also significantly increased in the striatum of PD brains where its phosphorylation correlates with both tau and α-synuclein pathology (Wills et al., 2010). This suggests a potential role for GSK3β in promoting the early stages of tau interaction with α-synuclein, leading to α-synuclein pathology in PD. This could be important as genome-wide association studies implicate polymorphisms in the *MAPT* and *SNCA* genes as the most robustly reproducible risk factors for sporadic PD (Satake et al., 2009; Simon-Sanchez et al., 2009).

mTOR

The mTOR kinase exists in two complexes termed mTORC1 and mTORC2, with mTORC2 regulating PKB activity and mTORC1 regulating protein synthesis and autophagy (Laplante and Sabatini, 2012). The phosphorylation of mTORC1 by PKB promotes protein synthesis and inhibits autophagy. Reduced phosphorylation of PKB in PD brain may therefore be expected to promote

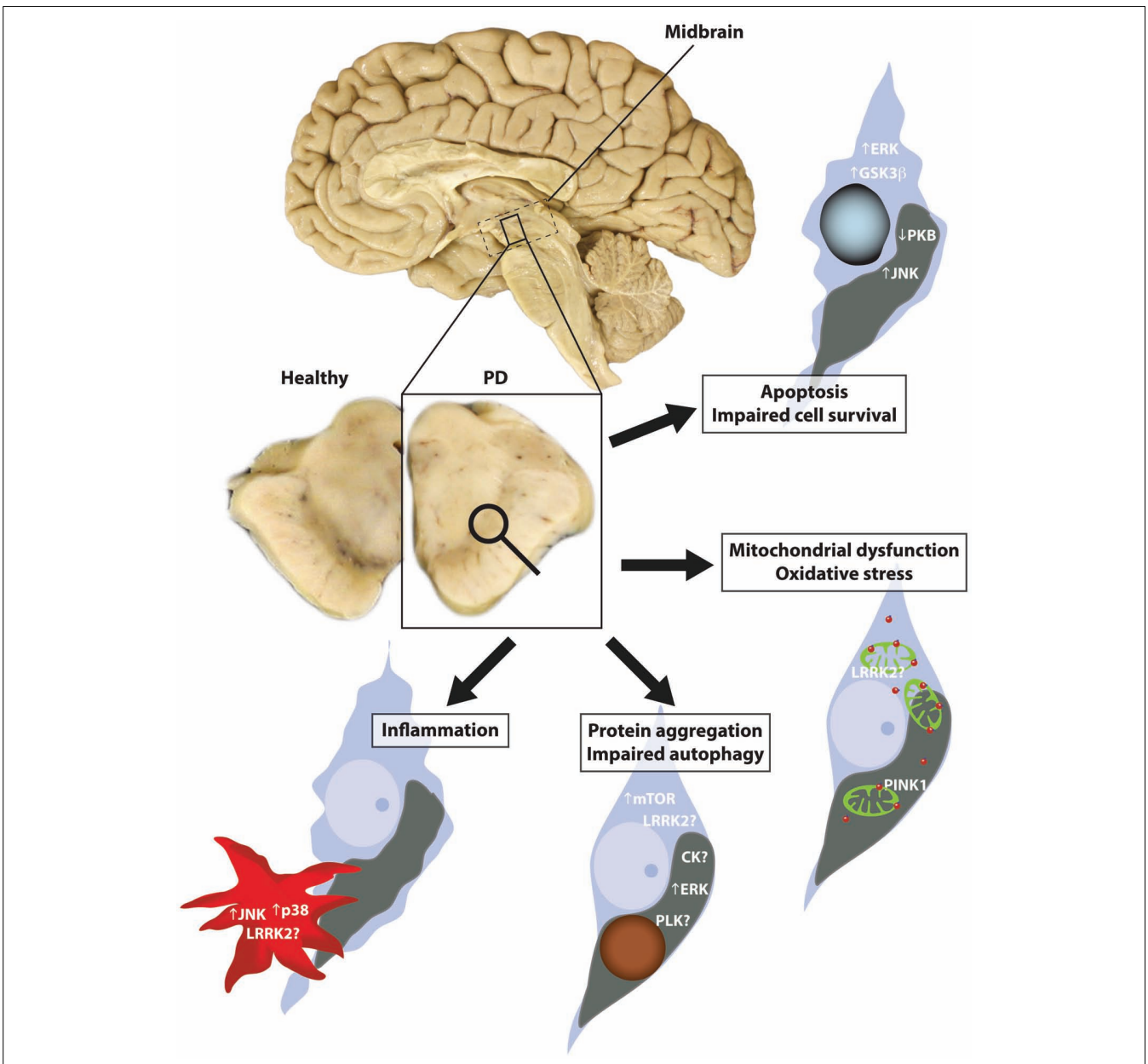

FIGURE 4 | Kinases implicated in Parkinson's disease (PD). PD is characterized by the loss of pigmented dopaminergic neurons in the substantia nigra region of the midbrain. A number of biological processes have been implicated in this neuronal loss including mitochondrial dysfunction, oxidative stress, autophagy, and inflammation. In the substantia nigra, a number of kinases impacted by these processes combine to promote the accumulation of phosphorylated α-synuclein and induce conditions that reduce cell viability.

autophagy; however, this process is clearly dysregulated in PD as autophagy markers are also significantly decreased in PD substantia nigra (Chu et al., 2009; Alvarez-Erviti et al., 2010). Indeed, evidence suggests that dysfunctional autophagy pathways play a key role in the pathogenesis of PD (Lynch-Day et al., 2012). Moreover, the mTOR inhibitor (and therefore autophagy inducing) rapamycin prevents MPTP-induced neurodegeneration (Dehay et al., 2010; Liu et al., 2013). Rapamycin also has protective properties in rotenone and α-synuclein PD models (Pan et al., 2009; Spencer et al., 2009; Crews et al., 2010; Xiong et al., 2011) suggesting inhibition of mTORC1 has potential as a treatment for PD.

Protein levels of neuronal mTOR were significantly increased in the temporal cortex of cases with dementia with Lewy bodies, particularly in neurons displaying accumulation of α-synuclein (Crews et al., 2010). In comparison, brain tissue from cases with Alzheimer's disease had normal levels of mTOR in the temporal cortex (Crews et al., 2010). Up-regulation of mTOR is consistent with a phenotype of increased protein synthesis and reduced autophagy, promoting the accumulation of potentially toxic proteins. In the context of PD, increased mTOR would likely aid the propagation of α-synuclein, however, whether changes in mTOR are associated with the spread of α-synuclein pathology in PD brain is unknown.

OVERVIEW OF KINASES EXPERIMENTALLY IMPLICATED IN PD

It is evident from human tissue studies, particularly those focused on the nigral dopaminergic system, that inflammatory pathways are activated in PD and autophagy pathways are impaired. PD brain tissue samples from different brain regions at different stages of pathology could inform on the order of these events and provide more insight into whether certain kinases are causal for PD pathologies. The discovery that certain toxins (MPTP, rotenone, LPS, 6-OHDA) induce a selective loss of dopaminergic neurons in rodent models has facilitated a wealth of information regarding the order of the biological processes leading to such neuronal death as well as signaling proteins mediating these events. While these toxin-based models do not replicate all the features of sporadic PD, such as an age-dependent phenotype and the presence of α-synuclein pathology, they have implicated a range of kinases as important in the process. It will be important in many instances to use pathologically staged human brain tissue to validate the expression of kinases and their isoforms and any disease-associated changes identified experimentally in mice.

ADDITIONAL KINASES AND OVERALL CONCLUSIONS

In addition to the kinases discussed above, a number of other kinases are emerging as having potential roles in PD pathogenesis and/or potential therapeutic targets. These include CDK5, a kinase whose activity is increased by MPTP treatment and inhibition attenuates MPTP-induced neuronal loss (Smith et al., 2003; Qu et al., 2007). The eIF2alpha kinase (also known as PERK), whose inhibition was recently shown to attenuate neurodegeneration in prion-infected mice (Moreno et al., 2013), and AMP-activated protein kinase (AMPK), a major metabolic regulatory enzyme whose activation has been associated with neuroprotection in a number of PD models (Wu et al., 2011; Bayliss and Andrews, 2013; Dulovic et al., 2014; Li et al., 2014). AMPK is also activated by thiazolidinones (Fryer et al., 2002), compounds that have neuroprotective properties in a number of settings (Carta, 2013). Of these kinases, CDK5 and PERK have been studied in PD brain, with CDK5 localizing to Lewy bodies (Brion and Couck, 1995; Nakamura et al., 1997) and PERK increased in PD substantia nigra neurons (Hoozemans et al., 2007). PERK levels also correlated with α-synuclein deposition (Hoozemans et al., 2007), making PERK in particular a very interesting candidate for further study.

Thus, a number of protein kinases have been implicated in the pathogenesis of PD covering a diverse array of biological functions including oxidative stress, inflammation, and autophagy (**Figure 4**). However, delineating the exact order by which these biological functions go wrong in PD brain is still a major challenge, despite the staging methods now in more common use. It is likely that some kinases are more important for initiating the disease whilst others are more important for disease propagation. In this regard, the majority of brain tissue work to date has focused on the substantia nigra region in PD, a region mostly at end-stage pathology in patients dying with PD. Thus brain tissue studies using this region are not informative on early pathogenic events, and assessment of this region provides limited information on the cause or consequence of many findings. With a greater understanding of how PD spreads throughout the brain in a staged fashion, brain regions can be selected to determine biochemical responses across the disease spectrum, particularly assessing regions with evidence of only early perturbations indicative of PD. Such an approach should provide some insight into which processes may precede PD pathology and which processes may propagate PD pathology. This is important for determining when potential therapies, such as kinase inhibitors, are likely to exert maximum efficacy. It is also important to recognize potential caveats of postmortem studies such as postmortem delay, comorbidities, and drug regimes, even though many caveats can be controlled with appropriate sample selection. In the absence of animal models that replicate all the cardinal features of PD, human pathological postmortem brain tissue remains an important resource to understand the biochemical details of PD and to verify cell and animal model hypothesis testing.

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