BACKGROUND

Parkinson’s disease (PD) affects 2% of the population above 65 years. It is characterized by the death of dopaminergic neurons in the substantia nigra and its most common clinical traits are bradykinesia, rigidity, and resting tremor (Moore et al., 2005; Obeso et al., 2017; Schulz et al., 2016). Mutations in the Leucine-rich repeat kinase 2 (LRRK2) gene have been unequivocally related to familial late-onset PD (Singleton et al., 2017). The first evidence of the genetic link between LRRK2 and PD dates back to 2002, when a new PD locus, PARK8 (OMIM 609007), was identified in a large Japanese family (Funayama et al., 2002). Soon after, three papers appeared describing that PARK8 codes for LRRK2 and that LRRK2 mutations cause autosomal dominant PD (Funayama et al., 2005; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Since then, eight pathological missense mutations (i.e., N1437H, R1441C/G/H/S, Y1699C, G2019S, and I2020T) have been characterized (Hui et al., 2018; Schulz et al., 2016). In the Taiwanese populations a relatively common LRRK2 variant, G2385R, that moderately increases PD risk has been identified (Tan, 2006; Tan et al., 2009).

Recent epidemiological studies attribute LRRK2 mutations to 13% of familial PD cases, with the most common G2019S mutation accounting for 4% of familial and 1% of sporadic PD [reviewed in (Tolosa et al., 2020)].

THE BASICS OF LRRK2

Leucine-rich repeat kinase 2 (LRRK2) is a very large protein consisting of 2,527 amino acids, encompassing multiple functional domains: from N- to C-terminus armadillo ankyrin, the namesake leucine-rich repeats, a ROC GTPase, a COR dimerization domain, a kinase domain, and WD40 repeats (Bosgraaf & Van Haastert, 2003; Gilsbach et al., 2018; Mills et al., 2012).
LRRK2 exists both as monomer and dimer, being the dimer likely the active species (Berger et al., 2010; Sen et al., 2009). A double enzymatic activity characterizes LRRK2: it behaves as a serine–threonine kinase and possesses a GTPase activity, mediated by the ROC–COR domains.

Besides the well-characterized catalytic domains, the N-terminal Armadillo, the LRR, and Ankyrin repeats as well as the C-terminal WD40 domain host multiple protein interaction sites and allow LRRK2 dimerization. Accumulating evidence indeed describes LRRK2 as a molecular hub orchestrating numerous protein networks (Porras et al., 2015). LRRK2 is phosphorylated by various kinases on an N-terminal stretch of serine residues (Nichols, 2017) and de-phosphorylated by PP1 (Lobbestael et al., 2013). 14-3-3s bind phosphorylated Ser910/Ser935 and maintain LRRK2 in a latent hypoactive state (Mamais et al., 2014; Muda et al., 2014). One striking feature of the majority of LRRK2 pathogenic mutations—except for G2019S—is that they all display moderate to severe loss of S910/S935 phosphorylation (Nichols et al., 2010). Thus, cellular phosphorylation is a key event for controlling the LRRK2 function (Athanasopoulos et al., 2018). The mutations within the LRRK2 kinase domain (G2019S and I2020T) increase LRRK2 activity in vitro (Gloeckner et al., 2006; Greggio et al., 2006) and in vivo (Steger et al., 2016), possibly by disrupting the inhibited kinase domain conformation (Schmidt et al., 2019). The mutations falling within the ROC and Cor domain (N1437H, R1441G/C/H, Y1699C) suppress GTPase activity, promote GTP binding (Nguyen & Moore, 2017), and eventually increase LRRK2 kinase activity (Sheng et al., 2012). Finally, the G2385R mutation modifies WD40 domain folding and interaction (Carrion et al., 2017; Piccoli et al., 2014) and enhances LRRK2 GTPase (Ho et al., 2016) and kinase activity by a yet unknown mechanism (Zhang et al., 2019). It is well accepted that the primary pathogenic mechanism arises from kinase hyper-activation (Greggio et al., 2006). LRRK2 phosphorylates its targets at a conserved Thr/Ser motif (Gloeckner et al., 2010; Pungaliya et al., 2010). The most robust substrates for LRRK2 kinase activity identified so far are a panel of Rab proteins, including Rab8A and Rab10 (Steger et al., 2016). All PD-linked LRRK2 mutants show increased kinase activity on Rabs (Jeong et al., 2018; Steger et al., 2016). Other LRRK2 targets have been suggested, such as endophilin A1 (Matta et al., 2012), ribosomal protein s15 (Martin et al., 2014), N-ethylmaleimide sensitive fusion protein (NSF) (Belluzzi et al., 2016), synaptojanin-1 (Islam et al., 2016), P62/SQSTM1 (Kalogeropulou et al., 2018), auxillin (Nguyen & Krainc, 2018), and synapsin I (Marte et al., 2019).

LRRK2 can be detected in different tissues, with the highest value reached in the brain, kidney, and lung (Gliasson et al., 2006). Of note, LRRK2 expression is quite relevant also in immune cells, including B lymphocytes, monocytes, and neutrophils [reviewed in (Cabezudo et al., 2020; Dzamko, 2017; Wallings et al., 2020)]. Recently, the role of LRRK2 in astrocytes has been gaining interest (di Domenico et al., 2019; Sanyal et al., 2020; Zhao et al., 2018). In mouse and rat brain, strong LRRK2 mRNA and protein expression have been observed in the cerebral cortex, striatum, hippocampus, and amygdala nuclei. Moderate mRNA and protein expression were detected in the thalamus and hypothalamus. Notably, LRRK2 protein expression is relatively low in the substantia nigra and in the ventral tegmental area of the midbrain where dopaminergic neurons are particularly abundant (Higashi et al., 2007; Taymans et al., 2006). KO validated monoclonal antibodies detected LRRK2 at high level in the cortex and striatum in rat and mouse brain. Moreover, the highest level is detected in cortical pyramidal neurons of layer V and in striatal medium spiny neurons (West et al., 2014). In particular, expression of Lrrk2 has been detected in Dopamine receptor-1 and –2–positive striatal medium spiny neurons (Giesert et al., 2013).

Single-cell RNA seq has allowed an unprecedented mapping of gene expression at the cellular level. LRRK2 mRNA has been positively identified in cortical neurons (Zeisel et al., 2015) but not in P7 murine dopaminergic neurons (Hook et al., 2018). However, West and colleagues found that in adult mice (but not in rats), LRRK2 protein is expressed in the substantia nigra pars compacta (West et al., 2014), suggesting that its expression in the dopaminergic system may start at a later developmental stage.

LRRK2 is not observed at the subcellular level in the nucleus but distributed throughout the cytoplasm, where it binds the cytoskeletal system and localizes to organelle membranes (Biskup et al., 2006; Gandhi et al., 2008; Hatano et al., 2007; Meixner et al., 2011). In particular, LRRK2 co-precipitates and interacts with synaptic vesicles (SV) (Biskup et al., 2006; Piccoli et al., 2014).

3 | LRRK2 FUNCTION AT THE PRESYNAPTIC SITE

A vast effort has been dedicated to revealing LRRK2 physiological and pathological function. Several Lrrk2 KO rodent models exist and have been instrumental in gaining insights into the putative role of LRRK2 [reviewed in (Volta & Melrose, 2017; Xiong et al., 2017)]. It is established that Lrrk2 KO animals are viable, fertile, with average lifespan and do not show any overt motor phenotypes. Instead, they share peripheral traits, such as morphological abnormalities in the lungs and kidneys (Herzig et al., 2011). Interestingly, large-scale analyses demonstrated that reduced LRRK2 protein levels are not associated with human-specific phenotype (Blauwendraat et al., 2018; Whiffen et al., 2020). Along with this 16-year long investigation, different and sometimes unexpected functions have been attributed to LRRK2: microtubules and microfilaments dynamics, protein synthesis, cellular signaling, mitochondrial homeostasis, ubiquitin–proteasome system and lysosomal protein clearance, and eventually synaptic transmission [reviewed in (Berwick et al., 2019; Taylor & Alessi, 2020)].

The discovery that Rab proteins are the principal substrate for LRRK2 (Steger et al., 2016) strongly links LRRK2 to the vesicular system. Rabs orchestrate membrane trafficking, including...
the processes of vesicle formation, vesicle movement along actin and tubulin tracks, membrane fusion, and regulate SV trafficking (Wandinger-Ness & Zerial, 2014). In particular, Rab5 is a marker of the early endosome and plays an important role in clathrin-mediated endocytosis (He et al., 2017). Rab5-driven SV fusion with early endosome has been proposed to be crucial for the highly recycling ready releasable pool (RRP) (Hoopmann et al., 2010). Finally, Rab5 manipulations result in defective SV recycling (Fischer von Mollard et al., 1994; Shimizu et al., 2003; Wucherpfennig et al., 2003).

In vitro evidence suggests that LRRK2 binds or phosphorylates key proteins involved in SV cycle other than Rab5, such as endophilinA, NSF, dynamin, synaptotagmin1, auxilin, snapin, synapsin I, AP2 complex, and CaV2.1 channel (Arranz et al., 2015; Bedford et al., 2016; Belluzzi et al., 2016; Heaton et al., 2020; Islam et al., 2016; Matta et al., 2012; Shin et al., 2008; Stafa et al., 2014; Yun et al., 2013). Clearly, LRRK2 is potentially well-positioned to govern different steps along with both SV endo- and exocytosis (see Box 1).
TABLE 1  The table lists the papers discussed in this review. It provides first author name and year of publication (paper), kind of genetic or pharmacological manipulation of LRRK2 (LRRK2 status), neuronal population or tissue investigated (neuron/tissue), model organism (organism), primary experimental approach (technique), and sum-up of the results (outcome).

| Paper | LRRK2 status | Neuron/tissue | Organism | Technique | Outcome |
|-------|--------------|---------------|----------|-----------|---------|
| Arranz et al., 2015 | KO | striatal culture | rat Long Evans | SypHy; TEM | impairment of endocytosis |
| Arranz et al., 2015 | KO | hippocampal cultures | mouse C57BL/6J | electrophysiology | impairment of endocytosis |
| Beccano-Kelly, Kuhlmann, et al., 2014 | KI G2019S | cortical culture | mouse C57BL/6J | electrophysiology | increased mEPSC frequency |
| Beccano-Kelly, Kuhlmann, et al., 2014 | KO | cortical culture | mouse C57BL/6J | electrophysiology | no alteration |
| Beccano-Kelly, Kuhlmann, et al., 2014 | OE human wild-type (BAC, endogenous promoter) | cortical culture | mouse C57BL/6J | electrophysiology | no alteration |
| Beccano-Kelly, Volta, et al., 2014 | KO | Cortico-striatal slices (SPN) | mouse C57BL/6J | electrophysiology | no alteration |
| Beccano-Kelly, Volta, et al., 2014 | OE human wild-type (BAC, endogenous promoter) | Cortico-striatal slices (MSN) | mouse C57BL/6J | electrophysiology | no alteration in sEPSC |
| Beccano-Kelly, Volta, et al., 2014 | KO | Cortico-striatal slices (glutamatergic neurons) | mouse C57BL/6J | electrophysiology | no alteration |
| Beccano-Kelly, Volta, et al., 2014 | OE human wild-type (BAC, endogenous promoter) | Cortico-striatal slices (glutamatergic neurons) | mouse C57BL/6J | electrophysiology | no alteration in PPR |
| Beccano-Kelly, Volta, et al., 2014 | OE human wild-type (BAC, endogenous promoter) | dopaminergic system | mouse C57BL/6J | microdialysis | decreased basal DA release; increased presynaptic D2R activity |
| Belluzzi et al., 2016 | OE human G2019S (BAC) | cortical culture | mouse C57BL/6J | sypHy | increased SV exo/endocytosis |
| Belluzzi et al., 2016 | pharmacological inhibition (GSK) | cortical culture | mouse C57BL/6J | sypHy | decreased SV cycle |
| Chou et al., 2014 | OE human G2019S (CMV) | striatal slices | mouse FVB/N | fast scan cyclic voltammetry; electrophysiology | decreased evoked DA release; no high-frequency LTD in MSN |
| Chou et al., 2014 | OE human G2019S (CMV) | hippocampal slices | mouse FVB/N | electrophysiology | no alteration in STP |
| Chou et al., 2014 | OE human G2019S (CMV) | cerebellar slices | mouse FVB/N | electrophysiology | no alteration in STP |
| Cirnaru et al., 2014 | pharmacological inhibition (IN-1) | cortical culture | mouse C57BL/6J | Anti-synaptotagmin uptake assay; electrophysiology | decreased SV endocytosis; reduced mEPSC |
| Creed et al., 2019 | KO | Dopaminergic, glutamatergic, cholinergic system | rat Long Evans | In vivo microdialysis | no alteration |
| Giesert et al., 2017 | Knock-down (constitutive) | cortical culture | mouse C57BL/6J | Anti-synaptotagmin uptake assay | no alteration |
| Giesert et al., 2017 | KI R1441C | cortical culture | mouse C57BL/6J | Anti-synaptotagmin uptake assay | no alteration |
| Hinkle et al., 2012 | KO | dopaminergic system | mouse C57BL/6J | In vivo microdialysis | no alteration |
| Li et al., 2010 | OE murine wild-type (BAC, endogenous promoter) | striatal slices | mouse C57BL/6J | fast scan cyclic voltammetry | increased evoked DA release |
| Li et al., 2010 | OE murine G2019S (BAC) | striatal slices | mouse C57BL/6J | fast scan cyclic voltammetry | decreased evoked DA release |

(Continues)
| Paper                        | LRRK2 status         | Neuron/tissue            | Organism       | Technique                           | Outcome                                                                 |
|------------------------------|----------------------|--------------------------|----------------|-------------------------------------|-------------------------------------------------------------------------|
| Liu et al., 2015             | OE human wild-type   | dopaminergic system      | mouse C57BL/6J | Microdialysis; fast scan cyclic     | increased evoked DA release                                              |
|                             | (only in DA neurons) |                          |                | voltammetry                         |                                                                         |
| Liu et al., 2015             | OE human G2019S      | dopaminergic system      | mouse C57BL/6J | Microdialysis; fast scan cyclic     | decreased evoked DA release                                              |
|                             | (only in DA neurons) |                          |                | voltammetry                         |                                                                         |
| Maas et al., 2017            | KO                   | hippocampal cultures    | mouse C57BL/6J | SypHy; FM-dye; electrophysiology    | no alteration                                                           |
| Maas et al., 2017            | KO                   | Cortico-striatal cultures| mouse C57BL/6J | sypHy                              | increased SV endocytosis                                                |
| Marte et al., 2019           | OE human G2019S (BAC)| cortical culture         | mouse C57BL/6J | SypHy                              | increased SV exo/endocytosis                                            |
| Marte et al., 2019           | pharmacological      | glutamatergic system     | mouse C57BL/6J | SypHy; microdialysis; electrophysiology | decreased SV exocytosis                                                |
| Matikainen-Ankney et al., 2016 | KI G2019S            | corticostriatal slices  | mouse C57BL/6NTac | electrophysiology | increased sEPSC frequency due to glutamatergic afferents                |
| Matikainen-Ankney et al., 2016 | KI kinase dead      | corticostriatal slices  | mouse C57BL/6NTac | electrophysiology | no alteration in sEPSC                                                 |
| Matta et al., 2012           | KO                   | NMJs                     | drosophila     | FM-dye; electrophysiology;TEM      | decreased SV endocytosis                                                |
| Melrose et al., 2010         | OE human wild-type   | dopaminergic system      | Mouse FVB/N    | microdialysis                       | decreased basal DA                                                      |
| Mercatelli et al., 2019      | KO                   | striatal synaptosome     | mouse C57BL/6J | microdialysis                       | increased evoked DA release                                              |
| Mercatelli et al., 2019      | KI G2019S            | striatal synaptosome     | mouse C57BL/6J | microdialysis                       | no alteration in DA release                                             |
| Mercatelli et al., 2019      | KI kinase dead       | striatal synaptosome     | mouse C57BL/6J | microdialysis                       | increased evoked DA release                                              |
| Mercatelli et al., 2019      | pharmacological      | striatal synaptosome     | mouse C57BL/6J | microdialysis                       | decreased evoked DA release; decreased evoked Glut release;             |
| Mercatelli et al., 2019      | pharmacological      | cortical synaptosome     | mouse C57BL/6J | microdialysis                       |                                                                         |
| Nguyen & Krainc, 2018        | KI R1441C            | iDA                      | human          | TEM                                 | reduced SV number                                                       |
| Pan et al., 2017             | OE murine wild-type  | midbrain culture         | mouse C57BL/6J | sypHy                              | no alteration in SV exo-end                                             |
| Pan et al., 2017             | OE murine G2019S (BAC)| midbrain culture         | mouse C57BL/6J | sypHy                              | reduced SV endocytosis                                                  |
| Pan et al., 2017             | OE murine wild-type  | cortical culture         | mouse C57BL/6J | sypHy                              | no alteration in SV exo-end                                             |
| Pan et al., 2017             | OE murine G2019S (BAC)| cortical culture         | mouse C57BL/6J | sypHy                              | increased SV exocytosis                                                |
| Parisiadou et al., 2014      | KO                   | Cortico-striatal slices  | mouse C57BL/6J | electrophysiology;TEM               | reduced mEPSC frequency; no PPF                                          |
| Piccoli et al., 2011         | Knock-down (acute)   | cortical culture         | mouse C57BL/6J | Anti-synaptotagmin uptake assay;    | Increased SV fusion; paired-pulse depression                            |
| Paper                          | LRRK2 status                  | Neuron/tissue       | Organism        | Technique                  | Outcome                                      |
|-------------------------------|-------------------------------|---------------------|-----------------|----------------------------|----------------------------------------------|
| Plowey et al., 2014           | OE human wild-type (CMV       | cortical culture    | rat Sprague–Dawley | electrophysiology          | no alteration in mEPSC                       |
|                               | promoter)                     |                     |                 |                            |                                              |
| Plowey et al., 2014           | OE R1441C                    | cortical culture    | rat Sprague–Dawley | electrophysiology          | increased mEPSC frequency                    |
| Plowey et al., 2014           | OE human G2019S (CMV)        | cortical culture    | rat Sprague–Dawley | electrophysiology          | increased mEPSC frequency                    |
| Qin et al., 2017              | KO                            | striatal slices     | Mouse FVB/N     | fast scan cyclic voltammetry; electrophysiology | no alteration in DA release                  |
| Qin et al., 2017              | pharmacological inhibition (GSK; GNE; IN−1) | striatal slices     | Mouse FVB/N     | fast scan cyclic voltammetry; electrophysiology | no alteration in DA release                  |
| Sanchez et al., 2014          | OE R1441G (BAC)              | striatal slices     | Mouse FVB/N     | fast scan cyclic voltammetry | no alteration in DA release                  |
| Shin et al., 2008             | OE human G2019S (CMV)        | hippocampal cultures | rat Sprague–Dawley | SypHy; FM-dye | decreased SV endocytosis                     |
| Shin et al., 2008             | Knock-down (acute)            | hippocampal cultures | rat              | SypHy; FM-dye | impairment of endocytosis                    |
| Sloan et al., 2016            | OE human wild-type (BAC, endogenous promoter) | striatum            | rat Sprague–Dawley | fast scan cyclic voltammetry | decreased evoked DA release                  |
| Sloan et al., 2016            | OE human G2019S (BAC, endogenous promoter) | striatum            | rat Sprague–Dawley | fast scan cyclic voltammetry | decreased evoked DA release                  |
| Sloan et al., 2016            | OE human R1441C (BAC, endogenous promoter) | striatum            | rat Sprague–Dawley | fast scan cyclic voltammetry | decreased evoked DA release                  |
| Tong et al., 2009             | KI R1441C                    | chromaffin cell     | mouse B6/129    | electrophysiology          | decreased evoked DA release; increased evoked Glut release; |
| Tozzi et al., 2018            | KI G2019S                    | corticostriatal slices | mouse C57BL/6J  | electrophysiology          | decreased D2 mediated sEPSC                  |
| Tozzi et al., 2018            | KI Kinase dead               | corticostriatal slices | mouse C57BL/6J  | electrophysiology          | no alteration in EPSC                       |
| Tozzi et al., 2018            | KO                           | corticostriatal slices | mouse C57BL/6J  | electrophysiology          | no alteration in amplitude                   |
| Volta et al., 2015            | OE human G2019S (BAC)        | striatum            | mouse C57BL/6J  | microdialysis              | no alteration in basal or evoked DA release; reduced response to D2 mediated inhibition |
| Volta et al., 2017            | KI G2019S                    | striatal slices     | mouse C57BL/6J  | fast scan cyclic voltammetry; electrophysiology | increased sEPSC frequency; no alteration in DA release |
| Xiong et al., 2018            | OE human G2019S (only in DA neurons) | DA neuron           | mouse C57BL/6J  | TEM                        | impairment of endocytosis                    |
| Yue et al., 2015              | KI G2019S                    | striatum            | mouse C57BL/6J  | In vivo microdialysis      | decreased evoked DA release                  |
| Yun et al., 2013              | OE human G2019S (CMV)        | hippocampal cultures | rat Sprague–Dawley | sypHy | Decreased RRP size |

Abbreviations: BAC, bacterial artificial chromosome; LTD, long-term depression; mEPSC, miniature excitatory post-synaptic current; PPR, paired-pulse ratio; sEPSC, spontaneous excitatory post-synaptic current; sypHy, synaptopHluorin assay; TEM, transmission electron microscopy.
down the rate of synaptic vesicle endocytosis and eventually caused an increase in FM-dye loading. However, different outcomes have been reported for glutamatergic terminals in other LOF models.

In detail, SV dynamics is normal in hippocampal neurons prepared from KO mice (Maas et al., 2017) or in cortical cultures obtained from mice expressing constitutively a LRRK2 shRNA (Giesert et al., 2017). Similarly, another pre-synaptic controlled parameter, namely the frequency of post-synaptic currents, is not affected in LRRK2 KO murine cortical culture (Beccano-Kelly, Kuhlmann, et al., 2014) or cortico- striatal slice (Beccano-Kelly, Volta, et al., 2014). It may well be that the impact of LRRK2 LOF gets compensated along with embryonic development while it becomes evident upon acute LRRK2 manipulation in post-natal models. Lastly, the possibility of off-target effects of shRNA strategies has to be taken into account.

Studies focusing on other neuronal populations appear to provide a more robust outcome. LRRK2 down-regulation correlates with abnormal endocytic vesicles, such as clathrin-coated endocytic intermediates in rat striatum (Arranz et al., 2015). Syph4 assay revealed that LRRK2 KO delayed the endocytosis of recycling pool SV in striatal cultures prepared from rats (Arranz et al., 2015). Excitatory post-synaptic current (EPSC) frequency resulted in impaired striatal cultures prepared from LRRK2 KO mice (Parisiadou et al., 2014). Similarly, imaging studies showed that SV endocytosis is deficient in striatal culture prepared from LRRK2 KO mice (Maas et al., 2017) or rats (Arranz et al., 2015). GABAergic cells encompass about 90% of striatal neurons. These findings open the possibility that LRRK2 sustains SV recycling in a synapse-specific manner.

However, neurotransmitter release does not seem to be particularly sensitive to LRRK2 LOF.

LRRK2 KO does not affect basal and evoked dopamine (DA) release as judged by in vivo microdialysis (Creed et al., 2019; Hinkle et al., 2012) or fast-scan cyclic voltammetry (FSCV) (Qin et al., 2017). Similarly, basal and evoked glutamate release resulted being normal in isolated synaptosome prepared from LRRK2 KO mice (Mercatelli et al., 2019).

It has to be mentioned that the very same experimental setting revealed an increased DA release in LRRK2 KO mice (Mercatelli et al., 2019). Isolated synaptosome constitutes indeed a powerful tool to dissect potential synapse-specific effect. However, DA transmission in basal ganglia is finely tuned by a complex inter-neuronal cross-talk: approaches such as in vivo microdialysis or FSCV may catch the final outcome of the entire circuitry.

We can conclude that LRRK2 down-regulation does not have a drastic impact on the amount of neurotransmitter released.

Still, some reports claim that LRRK2 LOF may affect SV exocytosis at the glutamatergic terminal. In particular, a functional link between LRRK2 and RRP SV has been proposed. Paired-pulse protocol and bath-sucrose pulse are convenient methods to engage SV belonging to the RRP. siRNA-mediated LRRK2 ablation mimics sucrose application in terms of recruitment of SV and affects paired-pulse plasticity in murine cortical cultures (Piccoli et al., 2011). Accordingly, sucrose fails to further increase post-synaptic current frequency in LRRK2 KO murine hippocampal cultures (Arranz et al., 2015). Finally, LRRK2 silencing increased the fusion of docked SV in a heterologous cellular system (Carrion et al., 2017). Thus, reduced LRRK2 protein level may stimulate exocytosis limited to the RRP. Such a subtle event may escape the detection limit of many experimental approaches.

5 | THE IMPACT OF LRRK2 OVER-EXPRESSION AT THE PRE-SYNAPTIC SITE

Among the first models generated to investigate LRRK2 function, bacterial artificial chromosome (BAC) over-expressing animals have been the object of many studies. However, BAC models have a considerable caveat. BAC strategy implies the non-physiological expression of human/murine mutant protein on the top of endogenously expressed rodent LRRK2.

BAC-mediated over-expression (OE) of human (Beccano-Kelly, Volta, et al., 2014) or murine (Pan et al., 2017) wild-type LRRK2 did not alter synaptic activity in striatal medium spiny (MSN) or cortical neurons. A similar outcome was reported in rat cortical neurons upon LRRK2 acute OE (Plowey et al., 2014).

Instead, the dopaminergic terminal seems to suffer LRRK2 OE. BAC-mediated OE of human wild-type LRRK2 resulted in decreased basal DA release in mice (Beccano-Kelly, Volta, et al., 2014; Melrose et al., 2010) and decrease evoked DA release in rats (Sloan et al., 2016). Conversely, the introduction of murine LRRK2 locus via BAC (Li et al., 2010) or the selective expression of human LRRK2 in DA neurons (Liu et al., 2015) brought to an elevated evoked release of dopamine in mice.

It has been noted that the expression of LRRK2 protein from mouse BAC constructs closely mimics endogenous LRRK2 distribution in the mouse brain. Instead, human BAC constructs drive LRRK2 expression in additional tissues, such as the hippocampus (Melrose et al., 2010). The different tissue expression may account for the divergent outcome on DA release reported in murine versus human BAC rodent models.

6 | THE HYPO- AND HYPER-ACTIVATION OF LRRK2 AT THE SYNAPTIC SITE

We contributed to describing LRRK2 as a critical scaffolding protein at the pre-synaptic site, modulating SV dynamics via protein interaction. The ongoing development of (ever more) specific LRRK2 kinase inhibitors has helped to dissect the impact of its enzymatic activity at the pre-synaptic site. Acute treatment with IN-1 (Cirnaru et al., 2014), PF-06447475 (Marte et al., 2019) severely impairs pre-synaptic activity in murine cortical cultures or hippocampal slices in terms of EPSC frequency. Accordingly, GSK2578215A (Mercatelli et al., 2019), IN-1, and PF-06447475 (Marte et al., 2019), reduce evoked glutamate release from isolated cortical synaptosomes. This outcome has been reported also in a G2019S transgenic mouse line: LRRK2 pharmacological inhibition
FIGURE 1  (a) Physiological role at the pre-synaptic bouton. LRRK2 is well-positioned to influence the entire SV cycle. (1) LRRK2 interacts with actin and synapsin I, which tether SV belonging to the reserve pool. (2) LRRK2 facilitates inward Ca\(^{2+}\) current via CaV2.1. (3) Local Ca\(^{2+}\) increase drives the assembly of the SNARE complex that eventually triggers SV fusion. LRRK2 binds SNAP25, syntaxin, and the associated proteins synaptotagmin and snapin. (4) Upon SV fusion, NSF, a well-characterized LRRK2 interactor, allows SNARE complex disassembly. (5) In the kiss-and-run model, SV do not entirely fuse with pre-synaptic membrane, detach from the membrane, and are acidified by the V-ATPase proton pump before re-entering the cycle. (6) In case SV completely fuse to the active zone membrane, clathrin-mediated endocytosis (CME) intervenes to recycle SV lipids and proteins. (7) Upon clathrin deposition, endophilinA promotes membrane invagination. The GTPase dynamin is required for the fission of endocytic membrane vesicle. (8) Auxillin and synaptojanin facilitate clathrin uncoating. LRRK2 interacts with endophilin, dynamin, auxillin, and synaptojanin. (9) SV are now ready to re-enter the cycle. (10) Rab5 drives SV fusion with early endosome, in a pathway suggested being crucial to maintaining RRP. LRRK2 binds and phosphorylates Rab5. (b) Impact of LRRK2 mutation. (1) LRRK2 phosphorylates synapsin I, thus promoting SV mobilization from the recycling pool. (2) LRRK2 G2019S stimulates inward Ca\(^{2+}\) current in a kinase-dependent manner. (3) Increased local Ca\(^{2+}\) facilitates SV fusion. (4) Upon LRRK2 phosphorylation, NSF catalyzes more efficiently SNARE disassembly. (5) SV recycle to be ready for the next round of fusion. At the glutamatergic terminal, LRRK2 mutations increase exocytosis. (6) LRRK2 kinase activity impacts on CME. Upon clathrin deposition, LRRK2 phosphorylation perturbs vesicle budding acting on endophilinA and (8) uncoating acting on auxillin and synaptojanin. (9) SV uncoating is mandatory for re-entering the cycle. (10) Rab5 favors the fusion with the endosome of SV retrieved via clathrin-dependent or -independent mechanisms. LRRK2 phosphorylation prevents Rab binding to the membrane. In the DA bouton, LRRK2 hyper-activation hampers SV clathrin-mediated endocytosis. (c) LRRK2 within basal ganglia. Striatal GABAergic MSN neurons receive glutamatergic stimulation from layer V cortical pyramidal neurons and modulatory dopaminergic input from substantia nigra (SN) pars compacta DA neurons. MSN neurons express either D1 (stimulatory, direct pathway) or D2 (inhibitory, indirect pathway) DA receptor. D2 receptors are also present at glutamatergic pre-synaptic terminals and dampen glutamate release. MSN within the indirect pathway produce and release endocannabinoids via PLC. Endocannabinoids inhibit pre-synaptically glutamatergic afferents via the CB1 receptor. An increased striatal glutamatergic release characterizes G2019S models. Such glutamatergic tone may inhibit DA release directly via mGluR1 localized on DA terminals or indirectly via AMPAR-mediated production of H\(_2\)O\(_2\) in MSN. However, G2019S mice show a strong endocannabinoids-driven glutamatergic LTD. These findings open the possibility that G2019S mutation strikes the direct and indirect pathway differently
hampered exocytosis only in cortical neurons (Pan et al., 2017). Acute LRRK2 inhibition increased (Mercatelli et al., 2019) or left unaltered (Qin et al., 2017) DA release. Therefore, the suppression of neurotransmitter release appears to be quite selective for the glutamatergic synapses. The cell-specific expression of LRRK2 may explain this difference.

Similarly, the genetic ablation of LRRK2 kinase activity resulted in equal (Tozzi et al., 2018) or even increased (Mercatelli et al., 2019) evoked DA release. However, it has to be mentioned that in the models presented above, MSN electrophysiological properties were almost normal, suggesting no major defects in the excitatory cortical afferent upon LRRK2 genetic inactivation (Matikainen-Ankney et al., 2016; Tozzi et al., 2018). The different outcomes upon acute versus constitutive LRRK2 kinase inhibition may be because of potential compensatory phenomena or off-target effects upon prolonged incubation with kinase inhibitors.

Given the pathological relevance of LRRK2 mutations, a colossal effort has been spent to investigate their functional consequence at the synaptic site. Again, different outcomes have been reported in each specific neuron. BAC-driven expression of human LRRK2 G2019S boosts glutamatergic neurons, as witnessed by increased SV dynamics in murine cortical culture (Belluzzi et al., 2016; Marte et al., 2019), higher frequency of post-synaptic currents in rat cortical cultures (Plowey et al., 2014) and increased glutamate release in the rat striatum (Sloan et al., 2016). Conversely, human LRRK2 G2019S OE reduces SV number (Xiong et al., 2018) and basal (Melrose et al., 2010) or evoked (Chou et al., 2014; Liu et al., 2015; Sloan et al., 2016) DA release in the dopaminergic system. Similarly, murine LRRK2 G2019S over-expression via BAC increased SV exocytosis in cortical cultures while it reduced SV endocytosis in DA neurons (Pan et al., 2017) as well as DA release (Li et al., 2010). These reports strongly suggest that BAC-driven LRRK2 G2019S expression increases glutamatergic neuron activity while it depresses DA release.

As mentioned above, the OE of human wild-type LRRK2 per se was sufficient to impair basal DA release in mice (Beccano-Kelly, Volta, et al., 2014; Melrose et al., 2010) and decreased evoked DA release in rats (Sloan et al., 2016). LRRK2 is barely detectable in the substantia nigra in rodents (Giesert et al., 2013; West et al., 2014). Upon over-expression, it might instead reach the critical level necessary to interfere with DA release. These observations implicate the physiological interpretation of results coming from BAC models.

A knock-in (KI) model represents the ultimate resource to gain insights into the synaptic impact of LRRK2 G2019S mutation. Complementary approaches confirmed that G2019S mutation stimulates glutamatergic neurons, as witnessed by increased EPSC frequency (Beccano-Kelly, Kuhlmann, et al., 2014) or enhanced exocytosis (Pan et al., 2017) in cortical cultures and increased glutamatergic transmission in striatal slices (Matikainen-Ankney et al., 2016; Volta et al., 2017). Instead, no major alteration has been reported in DA release (Mercatelli et al., 2019; Volta et al., 2017). Therefore, the DA phenotype described in BAC LRRK2 G2019S models may depend more on the excessive LRRK2 protein level than the G2019S mutation itself. Still, Pan et al. described a specific impairment of endocytosis in G2019S KI midbrain neuronal cultures (Pan et al., 2017). Interestingly, rodent KI models of R1441C mutation demonstrate a decreased evoked DA release and an increased evoked glutamate release (Tong et al., 2009) as well as increased frequency of cortical EPSC (Plowey et al., 2014). Finally, Yue et al. found an impairment of DA release in 12- but not in 6-month-old G2019S KI mice, despite a normal TH-positive neuron count (Yue et al., 2015), suggesting the relevance of aging.

7 A MODEL OF LRRK2 AT THE SYNAPTIC SITE

Altogether the reports discussed so far depict an integrated and synapse-specific role for LRRK2 at the pre-synaptic bouton.

Identifying LRRK2 substrates may help to decipher the connection between its kinase activity and the bouton-specific SV-dynamics. LRRK2-mediated phosphorylation of synapsin I abolishes its binding with actin (Marte et al., 2019). Furthermore, the heterologous expression of G2019S LRRK2 increases Ca^{2+} inward current via pre-synaptic CaV2.1 channel (Bedford et al., 2016). Eventually, biochemical evidence report that LRRK2 phosphorylates NSF on T645, increasing its ATPase activity and, eventually, its capability to dissociate the SNARE complex (Belluzzi et al., 2016), that is, the first step of SV recycling. Altogether, these in vitro data may link LRRK2 kinase activity with the increase of exocytosis.

Recent evidence attributes a crucial role to LRRK2 in clathrin-dependent mechanisms (Heaton et al., 2020). Auxilin, endophilin, and synaptojanin 1 have been proposed as LRRK2 targets and might provide a mechanistic link between LRRK2 and endocytosis. LRRK2 phosphorylation of auxilin at Ser627 abolishes auxilin association with clathrin and eventually disrupts SVE (Nguyen & Krainc, 2018). Recruitment of endophilin to clathrin coated pit is crucial for SV un-coating. Phosphorylation of the Endophilin A BAR domain by LRRK2 hampers its ability to dissociate from membranes and impairs SV endocytosis (Matta et al., 2012). In vitro, LRRK2 phosphorylates synaptojanin 1 in two sites, T1131, and S1142 within the C-terminal proline-rich domain, crucial for the interaction with endophilin A (Islam et al., 2016). Therefore, LRRK2-dependent phosphorylation of synaptojanin-1 may interfere with the binding to endophilin, and thus negatively influences endophilin-dependent endocytosis. Finally, upon phosphorylation, Rab proteins lose their ability to bind upstream and downstream proteins, get trapped on intracellular membranes, and hamper vesicle endocytosis [reviewed in (Pfeffer, 2018)].

Thus, an evident paradox exists: while in vitro data show that LRRK2 phosphorylation stimulates SNARE disassembly via NSF, other experimental evidence reports that it impairs the subsequent clathrin-mediated endocytosis. The mechanistic description of endocytosis may help to solve this issue.

After the necessary disassembly of SNARE complex catalyzed by NSF, SV recycling can occur through clathrin-dependent
and independent mechanisms [reviewed in (Mayor et al., 2014; Milosevic, 2018)]. Stimuli that are just sufficient to deplete the RRP typically imply a fast clathrin-independent retrieval, while stronger stimulation recruits the larger recycling pool and requires the slow clathrin-dependent mechanism. If this holds, LRRK2 may have a different impact depending on the activity taking place at the given terminal and, consequently, on the specific SV pool mobilized: it boosts basal release while undermining any high-frequency events that require clathrin-mediated endocytosis.

Still, it is difficult to understand why LRRK2 kinase activity plays opposing roles at glutamatergic versus dopaminergic terminal. Mutations in auxilin (Edvardson et al., 2012; Köröglü et al., 2013) and synaptojanin 1 (Quadri et al., 2013) have been recognized as causative in a rare form of familial PD. These findings enlighten the physiological relevance of clathrin-mediated endocytosis in the DA system. Indeed, DA neurons are spontaneously active with firing patterns that range from regular pacemaker rhythm to high-frequency burst firing (Bunney et al., 1991). Therefore, it is tempting to speculate that DA neurons rely on clathrin-mediated endocytosis to sustain their activity. Furthermore, glutamatergic and dopaminergic pre-synaptic machinery differs at the molecular level (Liu et al., 2018). For example, synapsin I, II, and III are differentially expressed in each terminal, with each of them playing a specific role in glutamate or GABA and DA release, respectively (Gitler et al., 2004; Kile et al., 2010).

Therefore, one intriguing hypothesis is that LRRK2 supports exocytosis at the glutamatergic terminal. If confirmed, the LRRK2-driven phosphorylation of NSF, synapsin I, and CaV2.1 may sustain this phenomenon. Instead, at DA terminal, LRRK2 might impair clathrin-mediated endocytosis acting on Rabs or on other suggested targets such as endophilinA, auxilin, and synaptojanin 1.

Finally, locating LRRK2 within the circuitry acting in basal ganglia may provide further hints. The striatum is composed essentially by GABAergic neurons decorated by spines (the MSN) that receive modulatory dopaminergic input from the SN pars compacta and a massive excitatory signal from cortical pyramidal neurons. Striatal MSN neurons express either D1 (stimulatory, direct pathway) or D2 (inhibitory, indirect pathway) DA receptor. Anatomical evidence indicates that D2Rs are present at glutamatergic pre-synaptic terminals in the striatum (Wang & Pickel, 2002), where they dampen glutamate release (Hsu et al., 1995). In turn, glutamate inhibits DA release indirectly via AMPAR-mediated production of H2O2 in MSN and directly via mGlur1 localized on DA terminals [reviewed in (Zhang & Sulzer, 2012)]. Finally, MSN within the indirect pathway produce and release endocannabinoids, such as anandamide, in response to simultaneous depolarization and D2 receptor activation. Endocannabinoids activate CB1 receptors on the glutamatergic pre-synaptic terminal and eventually result in a long-lasting decrease in glutamate release [reviewed in (Lovingier & Mathur, 2012)].

G2019S models demonstrate an increased glutamatergic activity together with an impaired DA release. Indeed, glutamate and dopamine release are tightly interconnected: G2019S mutation may dampen DA release acting from the glutamatergic afferents. Evoking a role for LRRK2 in cortico-striatal plasticity may also conciliate the inconsistent results regarding LRRK2 expression at the DA terminal; LRRK2 G2019S could reduce DA release even if not expressed in DA neurons themselves.

Intriguingly, in G2019S KI mice, Tozzi et al. found a strong D2 receptor-dependent reduction of the glutamatergic activity via pre-synaptic CB1Rs (Tozzi et al., 2018). In particular, their work indicates that the G2019S LRRK2 mutation increases the sensitivity of the D2R/endocannabinoid pathway in MSNs and triggers glutamatergic long-term depression. Furthermore, preliminary experimental evidence shows that LRRK2 G2019S modulates D2R membrane expression influencing its intracellular trafficking (Rassu et al., 2017).

These findings open the possibility that G2019S mutation has a different impact on the direct and indirect pathway. In particular, the retrograde D2R/endocannabinoids pathway may buffer the glutamatergic action on DA release in the indirect pathway. A recent work has enlightened the impact of LRRK2 R1441C and, to a lesser extent, G2019S mutations on the structure and function of the striatal spiny projection neurons synapses belonging to the direct pathway (Chen et al., 2020); clearly, a pathway-specific investigation of LRRK2 physiological and pathological action is needed.

8 | CONCLUDING REMARKS

Notwithstanding some experimental incongruence, there is full agreement that LRRK2 plays a key role at the pre-synaptic site and that LRRK2 pathological kinase activity may detrimentally affect basal ganglia functionality. Brain imaging studies showed alterations of the nigrostriatal system in asymptomatic G2019S carriers, such as an increased striatal dopamine turnover (Sossi et al., 2010), a reduced F-DOPA uptake (Gersel Stokholm et al., 2020), and a reorganization of corticostriatal circuits (Heilmich et al., 2015; Vilas et al., 2015, 2016) in the absence of any overt degeneration. These observations suggest that LRRK2 affects cortico-striatal plasticity early in the pathological progression and a recent study in a LRRK2 G2019S KI mouse model supports this hypothesis (Guevara et al., 2020). DAT imaging indicates a milder phenotype in G2019 LRKR2-PD patients (Simuni et al., 2020). Indeed, further studies are needed to elucidate how LRRK2 mutations eventually bring to PD. But the strong impact of the G2019S mutation on the corticostriatal afferents leaves open the possibility that LRRK2 causes PD starting from the glutamatergic site.

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ORCID
Giovanni Piccoli ID https://orcid.org/0000-0001-5262-7903
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