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Parkinson's Disease and Parkin: Insights from *Park2* Knockout Mice

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1 Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder distinguished by resting tremor, bradykinesia, rigidity, postural instability and gait disturbances. Non-motor symptoms including dysfunction of the autonomic nervous system, neuropsychiatric changes, sensory and sleep disturbances are also common. PD may be diagnosed at any age but is most common in aged populations - affecting approximately 1% of individuals over 60 years of age and rising to approximately 4% in age groups above 85 years of age (de Lau and Breteler, 2006; Van Den Eeden et al., 2003). It has become apparent that differential subgroups may be categorised by age of onset, dominant symptoms and progression. Two key subsets include late- and early-onset PD. Late-onset PD is typically identified in individuals over the age of 70 and is characterised by postural imbalance and gait impairment with accompanying rigidity and akinesia. Early-onset PD is typified by a dominant tremor and slow progression in motor decline, and is primarily identified in individuals less than 50 years of age (Lewis et al., 2005; Selikhova et al., 2009).

The lead pathological identifier of PD is moderate to severe dopaminergic neuronal loss within the substantia nigra pars compacta with accompanying Lewy pathology in surviving neurons (Daniel and Lees, 1993; Dickson et al., 2009; Gelb et al., 1999). It is thought that the combination of Lewy pathology and dopaminergic cell loss in PD leads to striatal dopamine depletion, and this accounts for the motor symptoms (Obeso et al., 2008). Earlier diagnosis of PD is important as motor symptoms do not become apparent until approximately 60% of dopaminergic neurons are lost (Fearnley and Lees, 1991; Pakkenberg et al., 1991).

Current treatments are available to manage the symptoms of PD. Medications such as the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) and inhibitors of dopamine metabolism are used to supplement the reduced dopamine level. Deep brain stimulation is an alternative treatment. The patient undergoes surgery to implant an electrical stimulation device into the affected region of the basal ganglia. The electrical impulses generated by the device interfere with the abnormal signals that are causing the tremor, thereby alleviating some of the symptoms of the disease (reviewed in (Hurelbrink and Lewis, 2010)). To identify treatments that address or arrest the progressive nature of PD, an understanding of the molecular mechanisms responsible for the loss of nigrostriatal dopaminergic neurons and associated Lewy pathology must be delineated.

The aetiology of the majority of PD cases remain unknown, however, gene mutations in familial forms of PD account for up to 10% of cases. Over 15 PD loci have been reported and greater than 10 genes have been identified (reviewed in (Shulman et al., 2010)). Mutations in *alpha-synuclein* and *LRRK2* are the predominant cause of autosomal dominant PD, while mutations in *parkin* and *PINK1* cause autosomal recessive PD. Several genome wide association studies using large idiopathic PD cohorts have demonstrated an unequivocal role for common genetic variation in familial PD genes in the aetiology of idiopathic PD, and identified new genetic players including *microtubule associated protein tau* in sporadic disease aetiology (Satake et al., 2009; Simon-Sanchez et al., 2009).

Mutations in *PARK2* (*parkin)* account for 50% of all familial early-onset PD cases, at least 20% of young-onset sporadic PD and also contribute to late onset sporadic disease (Foroud et al., 2003; Lucking et al., 2000; Mata et al., 2004). *Parkin* encodes a 465 amino acid multi-domain protein with homology to a class of enzymes termed E3 ubiquitin-protein ligases that function in the ubiquitin proteasome system (UPS) (Shimura et al., 2000). The UPS is the predominant cellular pathway for the turnover of misfolded and short-lived intracellular proteins (Ciechanover et al., 2000). Parkin mediates the formation of a lysine-48 polyubiquitin chain linked to the target protein, which functions as a signal for degradation by the proteasome. Parkin is also capable of alternative modes of ubiquitination including monoubiquitination and lysine-63 polyubiquitination, which appear to function in signalling and autophagy, respectively (Chew et al., 2011; Olzmann and Chin, 2008).

The pathological hallmark of many neurodegenerative diseases is the accumulation of proteins in aggregates/inclusions. There is very little information about the neuropathology observed in the brains of individuals with *parkin*-proven PD as only a seven cases have gone to autopsy. These cases displayed variable degree of cell loss in the substantia nigra pars compacta, and in some cases the locus coeruleus. Several cases displayed evidence of gliosis and astrocytosis. With the exception of two cases, Lewy pathology was not identified. Evidence of other types of protein aggregation including neurofibrillary tangles, alpha-synuclein-positive dendritic inclusions and tau accumulation were evident to varying degrees (reviewed in (Cookson et al., 2008)). As Lewy pathology is often cited as a significant pathological difference between idiopathic and *parkin*-proven PD some have hypothesised that parkin may play an integral role in Lewy formation (von Coelln et al., 2006). However, due to the small number of autopsies and the limitations of studying end stage disease tissues, animal and cell models provide a more detailed mechanistic understanding of *parkin*-mediated PD and the contribution of parkin to idiopathic PD.

A number of animal and cellular models of parkin dysfunction have been described. Parkin knockout flies demonstrated a reduced lifespan, muscle degeneration mitochondrial abnormalities, sensitivity to oxidative stress and male infertility (Greene et al., 2003; Pesah et al., 2004). While the first reports suggested *parkin* deficient drosophila did not have significant loss of dopaminergic neurons, a subsequent study utilising more sensitive analytical techniques indicated a reduction in the number of a subtype of dopaminergic neurons (Whitworth et al., 2005). *Park2* knockout mice did not appear to display extensive behavioural or dopaminergic abnormalities. As such the utility of such models to evaluate new pharmaceutical agents is unclear. A number of independent *parkin* deficient mouse models have now been generated. This review will outline the insights provided and look ahead to how *Park2* knockout mice may help to better understand *parkin*-mediated PD and by extension idiopathic PD.

2. *Parkin* mouse models

To date, eleven models of parkin dysfunction have been reported, seven of which are independently generated *Park2* knockout models. Studies on these mice will provide the basis of the discussion within. Models noted that are outside the scope of this paper include the quaking viable mouse which is a knockout of *parkin* and *PArkin Co-Regulated Gene* with dysregulation of *quaking* gene (Lockhart et al., 2004; Lorenzetti et al., 2004;), a recently identified spontaneous CH3-*Park2*^{E398Q} mutant (Ramsey and Giasson, 2010), a model of transgenic overexpression of a parkin truncated mutant (Lu et al., 2009) and a model of overexpression of wildtype parkin (Yoshida et al., 2010).

The majority of *parkin*-proven PD cases are the result of large genomic alterations (deletion, duplication or inversion) that affect one or more exons. *PARK2* spans an exaggerated genomic interval of approximately 1.4 Mb due to super-expanded introns and is located in a region of genomic instability (Denison et al., 2003; Palumbo et al., 2010). Several mouse models were generated to replicate known deletions that affect a single exon. Exons targeted in the *Park2* knockout models were exon 2, exon 3 or exon 7, which are predicted to result in a peptide/protein of 4aa, 57aa or 243aa truncated, respectively (Table 1). This suggests that parkin function would be abrogated in all of these models in the event translation occurs. Consistent with loss of function, immunoreactivity corresponding to full length parkin could not be detected in any of the knockout mice lines (Table 1 for references).

2.1 Behavioural characteristics

PD is a progressive adult onset neurodegenerative disorder, therefore, it would be anticipated that disease associated mutations within animal models would replicate this feature. For this reason it is important to consider mouse to human age equivalents. It is generally considered that between the ages of 3-6 months mice have finished development, the rapid maturational growth of most biological processes and structures, but are not affected by senescence. These mice are considered to represent 'mature adults' equivalent to a 20-30 year old human. Changes in senescence can be detected in some but not all mice between the ages of 10-15 months, which is considered 'middle age' and corresponds to 38-47 years in humans. Senescence markers can be detected in almost all 'old' mice between 18-24 months of age and these represent approximately 56-69 years in humans. Although for each inbred strain it varies, the average life span (50% survivorship) of a laboratory mouse is 28 months and the upper limit near 36 months, considered the equivalent of approximately 78 and 94 human years, respectively (Harrison, 2011).

Park2 knockout mice appear to develop normally, have normal general motor function and do not show any obvious clinical phenotype or behavioural abnormality. In one study, Kaplan-Meier survival analysis using the *Park2*^{tm1Roo} knockout model suggested an increase in mortality, which is consistent with data from PD patients' pre-L-DOPA therapy (Rodriguez-Navarro et al., 2007). However, in another study using the *Park2*^{tm1Roo} knockout model on a different background a difference in survival was not found (Guerrero et al., 2008). A reduction in body weight was identified in three lines but was not replicated in three other lines (Table 2) (Itier et al., 2003; Oyama et al., 2010; Palacino et al., 2004; Perez and Palmiter, 2005; Von Coelln et al., 2004; Zhu et al., 2007). In addition, a reduced body temperature was detected in *Park2*^{tm1Roo} knockout mice but was not replicated in the *Park2*^{tm1Rpa} knockout line. The findings for adhesive-removal tests and acoustic-startle were also conflicting (Goldberg et al., 2003; Itier et al., 2003; Perez and Palmiter, 2005; Von Coelln et al., 2004).

B6;129S2-*Park2*^{tm1Roo} (Itier et al., 2003)[1] Partial replacement of exon 3 with PGK-Neo^R cassette resulting in a truncated protein. If exon 3 is skipped, the predicted result is a frame shift after parkin amino acid 57 with the addition of 49 novel amino acids.
B6;129S4-*Park2*^{tm1Shn} (Goldberg et al., 2003) Partial replacement of exon 3 with in frame insertion of EGFP followed by PGK-Neo^R cassette resulting in a truncated protein consisting of the first 95 amino acids of parkin followed by EGFP (expression of EGFP protein was not detected). If exon 3 skipping occurs, the predicted result is a frameshift after parkin amino acid 57 with the addition of 49 novel amino acids.
B6;129S7/S4-*Park2*^{tm1Tmd} (Von Coelln et al., 2004)[2] Cre-mediated deletion of exon 7 resulting in a frameshift after parkin amino acid 243 with the addition of 8 novel amino acids.
B6;129S4-*Park2*^{tm1Rpa} (Perez and Palmiter, 2005)[1] Complete replacement of exon 2 with Polr2a-Neo^R cassette. If exon 2 is skipped, the predicted result would be a change in the reading frame producing a 4-aa peptide.
B6;CBA-*Park2*^{tm1Hn} (Sato et al., 2006)[3] Partial replacement of exon 2 with in frame insertion of tauGFP fusion protein followed by MC1-Neo^R cassette to produce tauGFP driven by the parkin promoter (expression of tauGFP protein was not detected). If exon 2 skipping occurs, the predicted result would be a change in the reading frame producing a 4-aa peptide.
B6;129P2-*Park2*^{tm1Oga}(Kitao et al., 2007) Complete replacement of exon 3 with PGK-Neo^R cassette. If exon 3 is skipped, the predicted result is a frame shift after parkin amino acid 57 with the addition of 49 novel amino acids.
B6;129S1/X1-*Park2*^{tm1Ccs} (Stichel et al., 2007) Complete replacement of exon 3 with a Neo^R cassette. If exon 3 is skipped, the predicted result is a frame shift after parkin amino acid 57 with the addition of 49 novel amino acids.

Table 1. *Park2* knockout mouse models
Nomenclature guidelines for naming genetically modified mouse strains were followed. Information regarding the genetic background of the mouse strain was extracted from the embryonic stem cell line strain and strain used in initial chimeric breeding. Only the first reported mouse strain is indicated, mutant alleles may have subsequently been bred to other strains or isogenicity for subsequent publications. [1]Also reported the allele on 129 isogenic background but unless indicated experiments were performed on a mixed background. [2]Strain information was obtained from Perez and Palmiter (2005) Supplementary Table 3. [3]A laboratory code for Nobutaka Hattori, the corresponding author of the publication by Sato et al., (2006) could not be identified with the Institute for Laboratory Animal Research (ILAR) therefore his initials (Hn) were used. This is only for the purposes of differentiating these strains in this chapter and does not represent the true allele designation.

It was anticipated that *parkin*-deficient mouse models would display motor defects, which would validate their suitability for PD research. Initial experiments indicated that only *Park2*^{tm1Roo} knockout line showed a basal reduction in locomotion (Table 3) (Itier et al., 2003). However, when investigated in the same line at 24 months, only a non-significant tendency for reduced locomotion was reported (Rodriguez-Navarro et al., 2007). The *Park2* knockout lines did not show significant deficits on the rotarod, which is used to measure balance,

	Body weight		*Body temperature*		*Adhesive-removal*		*Acoustic-startle*	
Park2^{tm1Roo}	↓	1-16	↓	4				
Park2^{tm1Shn}	↓	1-12			↓[3]	2-7		
Park2^{tm1Tmd}	–	NA					↓	9
Park2^{tm1Rpa}	–[1]	3-24	–	3, 22	–	19	–[2]	12-15
Park2^{tm1Hn}								
Park2^{tm1Oga}	↓	NA						
Park2^{tm1Ccs}	–	6, 18						

Table 2. General behavioural attributes of *Park2* knockout mice
Attributes investigated in two or more *Park2* knockout models. *Park2* knockout showed no difference (-) or a significant difference compared to wildtype (decreased = ↓, increased = ↑). The age(s) of mice (in months) is indicated, if unreported it is shown as not available (NA). Gray cells indicate that attribute has not been reported. [1]Decrease in weight was identified at 6 months of age in the B6:129S4 background but was not reproduced in the 129S4 background. [2]An increased sensitivity was detected in the B6:129S4 background but was not reproduced in the 129S4 background. [3]A significant decrease was not identified at 18 months. The table should be used as a guide only as methodology and analysis varies for each model.

	Locomotion		*Amphetamine response*		*Rotarod*		*Balance-beam*		*Pole Test*	
Park2^{tm1Roo}	↓	6	↓	6						
Park2^{tm1Shn}	–	6-18			–	6-18	↓	2-18		
Park2^{tm1Tmd}	–	18			–	3-24				
Park2^{tm1Rpa}	–[1]	3-22	–	3	–[3]	3-18	–	19	↓	6, 18
Park2^{tm1Hn}	–	12	–	12	–	12				
Park2^{tm1Oga}					–	3-12			–	3-12
Park2^{tm1Ccs}	–	6-21	↓[2]	3	–	6-24				

Table 3. Motor function of *Park2* knockout mice.
Attributes investigated in two or more *Park2* knockout models. *Park2* knockout showed no difference (-) or a significant difference compared to wildtype (decreased = ↓, increased = ↑). The age(s) of mice (in months) is indicated. Gray cells indicate that attribute has not been reported. [1]*Park2*^{tm1Rpa} knockout mice exhibited greater locomotor activity specifically during the second dark cycle at 12 months only. [2]Reported as a lack of amphetamine to induce thigmotaxic behaviour. [3]*Park2*^{tm1Rpa} knockout mice were more likely to grip the rotarod at 6 months. The table should be used as a guide only as methodology and analysis varies for each model.

coordination, physical condition, and motor-planning (Goldberg et al., 2003; Oyama et al., 2010; Perez and Palmiter, 2005; Sato et al., 2006; Von Coelln et al., 2004; Zhu et al., 2007). Two of the *Park2* knockout models appeared to have a reduced response to amphetamine, which increases the amount of dopamine in the synaptic cleft and enhances the response of post-synaptic neurons, whereas another two did not (Itier et al., 2003; Perez and Palmiter, 2005; Sato et al., 2006; Zhu et al., 2007). Furthermore, alternative results were reported for motor co-ordination determined by the balance beam test and the pole test (Goldberg et al., 2003; Oyama et al., 2010; Perez and Palmiter, 2005).

Cognitive function has been investigated in three of the *Park2* knockout lines (Table 4). Two of the models displayed a reduction in exploratory behaviour which was suggestive of an increased anxiety (Itier et al., 2003; Zhu et al., 2007). However, in other tests of cognition, including light/dark exploration and T-maze alteration the finding varied depending on the *Park2* knockout model analysed (Itier et al., 2003; Perez and Palmiter, 2005; Zhu et al., 2007).

For the most part, based on the aforementioned investigations of the behavioural attributes of the *Park2* knockout mouse lines it would appear that *parkin* deficiency does not cause a PD-like phenotype in mice. The discrepancies reported for the *Park2* knockout lines may be attributable to the genetic background of the mouse strain harbouring the mutant allele (discussed in (Perez and Palmiter, 2005)). The relative inability to identify a significant difference from wildtype for a number of the attributes investigated may be because the effect of parkin deficiency is very subtle and the experimental approach lacks the required sensitivity. As PD is a progressive disorder, an alternative explanation may be the age of *Park2* knockout model when the attribute was investigated. For example, 18 month old *Park2*^{tm1Roo} knockout mice showed a significant decrease in the length of their hind limb stride, and at 24 months a number of motor and non-motor irregularities were apparent in the *Park2*^{tm1Roo} knockout line that were suggested to parallel symptoms observed in PD sufferers (Rodriguez-Navarro et al., 2007). Further investigation when *parkin* deficiency is combined with old age in the *Park2* knockout models is required to address this issue.

	Refuse to perform tests		*T-maze alternation*		*Exploratory behaviour*		*Light/dark exploration*		*Morris water maze*	
Park2^{tm1Roo}	↓	15	↓	4	↓	6				
Park2^{tm1Shn}										
Park2^{tm1Tmd}										
Park2^{tm1Rpa}	–	3-22	–	12-20			–	12,18	–	18
Park2^{tm1Hn}										
Park2^{tm1Oga}										
Park2^{tm1Ccs}					↓	3	↓	3	↑	NA

Table 4. Cognitive-related behaviour in *Park2* knockout mice.
Attributes investigated in two or more *Park2* knockout models. *Park2* knockout showed no difference (-) or a significant difference compared to wildtype (decreased = ↓, increased = ↑). The age(s) of mice (in months) is indicated, if age was unreported it is shown as not available (NA). Gray cells indicate that attribute has not been reported. The table should be used as a guide only as methodology and analysis varies for each model.

2.2 Pathology

The pathological hallmark of PD is the loss of dopaminergic neurons within the substantia nigra and Lewy pathology. Other regions may also display loss of neurons and/or Lewy pathology, including the locus ceruleus, the hypothalamus and some regions of the cortex (Halliday and McCann, 2010). The cell body of dopaminergic neurons are predominantly localised to three regions of the brain: the substantia nigra pars compacta, the ventral tegmental area and the hypothalamus. These neurons project into the putamen to form the nigrostriatal dopaminergic system, the ventral striatum or the cortex to form the mesolimbic or the mesocortical dopaminergic pathways, and the pituitary gland to form the tuberoinfundibular pathway, respectively. In contrast, the largest population of noradrenergic neurons is found in the locus ceruleus, and these project to most central nervous system areas (Grimm et al., 2004).

The number and morphology of tyrosine hydroxylase staining neurons in the substantia nigra of *Park2* knockout mice was not found to be remarkably different from wildtype mice (Table 5). However, in the *Park2*^{tm1Roo} knockout line age was an important factor influencing loss of dopaminergic neurons. When examined at 2 or 15 months there was no difference in the

	Morphology of tyrosine hydroxylase staining neurons		*Number of tyrosine hydroxylase staining neurons*			
Park2^{tm1Roo}	−	2, 15	−	2, 15	↓[1]	18, 24
	C, BS, H, SN, ST		SN			
Park2^{tm1Shn}	−	NA	−		12-24	
	LC, SN, ST		SN			
Park2^{tm1Tmd}	−	18	−	12-18	↓[2]	2,12-18
	SN, ST		SN		LC	
Park2^{tm1Rpa}	−	NA	−		NA	
	SN		SN			
Park2^{tm1Hn}	−	3	−		3	
	SN		LC, SN			
Park2^{tm1Oga}			−		26	
			SN			
Park2^{tm1Ccs}	−	6-18	−		6-18	
	LC, SN, ST		LC, SN			

Table 5. Catecholaminergic neuron pathology in *Park2* knockout mice.

The morphology and number of catecholaminergic neurons in *Park2* knockout mice were identified by staining with anti-tyrosine hydroxylase antibody. *Park2* knockout showed no difference (-) or a significant difference compared to wildtype (decreased = ↓, increased = ↑) The age(s) of mice (in months) is indicated, if unreported it is shown as not available (NA). Gray cells indicate that attribute has not been reported. Brain stem (BS), cerebellum (C), locus ceruleus *(LC),* hippocampus (H), substantia nigra (SN), striatum (ST). [1]Significant reduction in the number of tyrosine hydroxylase staining neurons at 18 month was only identified in female *Park2*^{tm1Roo} knockout mice. [2]Phenotype shows reduced penetrance, ~30% of *Park2*^{tm1Tmd} knockout do not show a significant reduction in the number of tyrosine hydroxylase staining neurons in the LC. The table should be used as a guide only as methodology and analysis varies for each model.

number of dopaminergic neurons in the substantia nigra, but when investigated at 24 months a loss of ~35% of neurons was detected (Itier et al., 2003; Rodriguez-Navarro et al., 2007). In addition, a significant loss of tyrosine hydroxylase reactivity was demonstrable at 18 months in females but not males mice (Rodriguez-Navarro et al., 2008). Notably, in two other models examined at or above 24 months no significant loss of dopamineric neurons in the substantia nigra was reported (Goldberg et al., 2003; Kitao et al., 2007). Alternatively, in ~70% of *Park2*^{tm1Tmd} knockout mice there was significant reduction in the number of catecholaminergic neurons in the locus coeruleus. Such pathology was present at 2 months of age, suggesting dysfunctional development of catecholaminergic neurons in the locus ceruleus (Von Coelln et al., 2004). As ~30% *Park2*^{tm1Tmd} knockout mice showed no significant decrease, it is indicative of reduced penetrance and suggests other factors must also influence the trait.

It is generally considered that *parkin* deletion alone in humans is sufficient to cause PD with the onset of symptoms typically earlier than observed for idiopathic PD. The analyses summarised in Tables 2 to 5 suggests mouse models of *parkin* deficiency do not replicate this phenotype. However, some of the *Park2* knockout models display subtle behavioural and pathological phenotypes that parallel PD. Therefore, *parkin* deficiency in the mouse may alter pathways common to human pathogenesis but for as yet unknown reasons do not cause a pronounced PD-like phenotype. Observations reported suggest that the mechanistic pathways in which parkin functions are conserved between the human and mouse, and *Park2* knockout mice may be a useful model to further refine the function of parkin and its role in disease pathogenesis.

2.3 Neurochemistry

It is hypothesised that dysfunction in dopamine metabolism may precede loss of dopaminergic neurons and represent a presymptomatic disease state. This is supported by studies of dopamine metabolism using positron emission tomography in both *parkin*-proven and idiopathic Parkinson's disease, and unaffected individuals with heterozygous mutations in *Parkin* and *PINK1* (Guo et al., 2010; Sioka et al., 2010; Tang et al., 2010). The biosynthetic pathway for dopamine production in catecholaminergic neurons is the sequential conversion of phenylalanine to tyrosine, dihydroxyphenylalanine (L-DOPA) and dopamine. In dopaminergic neurons the synthesis of dopamine is the final product of the process whereas in noradrenergic neurons dopamine may be further modified to norepinephrine (Daubner et al., 2011). Within dopaminergic neurons, newly synthesised cytoplasmic dopamine is translocated by vesicular monoamine transporter 2 (VMAT2) into vesicles for storage until synaptic release. Synaptic dopamine interacts with D1 and D2 receptors on postsynaptic neurons in the striatum, which mediate the direct and indirect dopaminergic pathways, respectively. Within the substantia nigra and the basal ganglia synaptic dopamine is inactivated primarily via reuptake by the dopamine transporter (DAT) on presynaptic neurons. Intracellular dopamine is then sequestered in vesicles via VMAT for re-use or converted by monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetic acid (DOPAC) for degradation. DOPAC then diffuses out of the nerve terminal and into the extracellular space, where it can be transformed into the more stable metabolite homovanillic acid (HVA) via catechol-*O*-methyltransferase (COMT). To a lesser extent, extracellular dopamine can be metabolised without reuptake into presynaptic neurons. In this process, extracellular dopamine is converted to 3-methoxytyramine (3-MT) by COMT then to HVA by MAO (reviewed in (Standaert and Galanter, 2007)).

Investigation of the levels of dopamine and its metabolites DOPAC and HVA in the striatum of the *Park2* knockout lines did not reveal significant alterations (Table 6) (Itier et al., 2003; Oyama et al., 2010; Palacino et al., 2004; Perez and Palmiter, 2005; Von Coelln et al., 2004). The notable exception was the increase in HVA in the *Park2*^{tm1Ccs} model (Zhu et al., 2007). In addition, in a later study, a significant increase in DOPAC and HVA in the striatum at 3 months old *Park2*^{tm1Roo} knockout mice was reported (omitted from table) (Menendez et al., 2006). This may be reflective of an increase dopamine metabolism. Likewise, an increase in the ratio of DOPAC to dopamine or 3-MT was identified in the striatum of the *Park2*^{tm1Roo} knockout line, perhaps indicative of increased intracellular dopamine metabolism via MAO as opposed to extracellular metabolism via COMT (Itier et al., 2003). Although no significant difference of total dopamine levels were identified in the striatum of the *Park2*^{tm1Shn} knockout model, the amount of extracellular dopamine in this region was significantly increased. In addition, dopamine reuptake was unaltered, which suggests that the increased extracellular dopamine was due to increased release of dopamine from presynaptic dopaminergic neurons (Goldberg et al., 2003). In contrast, in the striatum of the *Park2*^{tm1Hn} knockout model a reduction in dopamine synthesis and an increase in D1 and D2 receptor

	Dopamine	DOPAC	HVA	Noradrenaline
Park2^{tm1Roo}	− 11 BS, D, ST | ↑ 11 L	− 11 BS, D, ST | ↑ 11 L	− 11 BS, D, L, ST	− 11 BS, D, L, ST
Park2^{tm1Shn}	− 6-24 ST	− 6-24 ST		
Park2^{tm1Tmd}	− 18 ST	− 18 ST	− 18 ST	− 18 BS, C, CC, D, H, PC | ↓ 18 OB, SC
Park2^{tm1Rpa}	− 22 ST	− 22 MB, ST	− 22 ST	− 18-22 OB, SC, ST
Park2^{tm1Hn}	↑ 12 MB | − 12 ST	− 12 MB, ST	− 12 MB, ST	
Park2^{tm1Oga}	− 3-12 ST	− 3-12 ST	− 3-12 ST	
Park2^{tm1Ccs}	− NA ST	− NA ST	↑ NA ST	

Table 6. Neurochemical analysis of *Park2* Knockout mice
The level of dopamine, its metabolites 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) and its derivate noradrenaline were analysed. *Park2* knockout showed no difference (-) or a significant difference compared to wildtype (decreased = ↓, increased = ↑). The age(s) of mice (in months) is indicated, if unreported it is shown as not available (NA). Gray cells indicate that attribute has not been reported. Brain stem (BS), cerebral cortex (CC), cerebellum (C), diencephalon (D), hippocampus (H), limbic region (L), olfactory bulb (OB), midbrain (MB), prefrontal cortex (PC), spinal cord (SC), substantia nigra (SN), striatum (ST). The table should be used as a guide only as methodology and analysis varies for each model.

binding was detected using receptor specific antagonists. This suggests decline in striatal dopamine release may result in decreased synaptic dopamine (Sato et al., 2006). Similar analysis in the *Park2*^{tm1Shn} knockout line however, did not indicate an alteration in the amount of D1 or D2 receptor binding in the striatum (Goldberg et al., 2003; Kitada et al., 2009a). In the *Park2*^{tm1Hn} line the binding index of DAT and VMAT was unchanged and suggests that the levels of these proteins are not altered (Sato et al., 2006). Likewise, the level of DAT in the *Park2*^{tm1Shn} knockout mice, measured using radiolabeled dopamine uptake, suggested that DAT levels were not altered (Kitada et al., 2009a). However, a reduction of the DAT and VMAT protein levels were identified in the *Park2*^{tm1Roo} line (Itier et al., 2003).

Alterations in dopamine metabolism were also found in other brain regions. An increase in the level of dopamine and DOPAC was identified in the limbic region from *Park2*^{tm1Roo} knockout mice, and an increase in dopamine but not DOPAC was identified in the midbrain, which includes the limbic region, from the *Park2*^{tm1Hn} knockout line (Itier et al., 2003; Sato et al., 2006). In addition, in the *Park2*^{tm1Tmd} knockout model, which showed loss of noradrenergic neurons within the locus coeruleus, a significant reduction in the dopamine derivative norepinephrine was identified in two regions that are innervated by the locus ceruleus, the olfactory bulb and the spinal cord (Von Coelln et al., 2004). Although a number of discrepancies related to dopamine metabolism are evident between the different *Park2* knockout models, collectively these analyses have suggested parkin may play a role in the presynaptic release of dopamine. The loss of parkin function may alter dopamine release, potentially by affecting vesicular packaging. Furthermore, they suggest that alterations in presynaptic dopamine function are evident prior to the development of PD-associated pathology.

2.4 Synaptic transmission

Deficits in dopamine-related synaptic transmission have been identified in *parkin*-mediated PD (Guo et al., 2010). Studies in a number of the *Park2* knockout lines suggest the presynaptic dopamine transmission is perturbed. In the *Park2*^{tm1Shn} knockout line, the evoked dopamine signal of medium spiny-neurons, the major class of striatal neurons whose excitability is influenced by dopamine levels, was reduced and could not be restored with DAT inhibition, suggesting dopamine re-uptake was not affected (Goldberg et al., 2003). Long term depression (LTD), which is activity-dependent reduction in the efficacy of neuronal synapses that lasts for an extended period, was also found to also be dysfunctional in these neurons. LTD could be restored by increasing synaptic dopamine with amphetamine but not by inhibition of dopamine re-uptake by antagonism of DAT. In addition, simultaneous antagonism of both D1 and D2 receptors, but not individual receptor antagonism, was required to restore LTD, and L-DOPA treatment was also effective. Furthermore, using primary dissociated adrenal chromaffin cells, which enable real time analysis of catecholamine release from single vesicles, exocytosis was found to be reduced (Kitada et al., 2009a). These observations suggest neurons from *Park2*^{tm1Shn} knockout have an intrinsic impairment in synaptic transmission that may result from defective presynaptic dopamine release.

The amplitude and half-life of evoked dopamine overflow was reduced in nigrostriatal fibers of the medial forebrain bundle of *Park2*^{tm1Oga} knockout mice. These are reflective of competing mechanisms of synaptic release and re-uptake, indicating that these mice also have alterations in dopamine synaptic transmission. Furthermore, a progressive age related

reduction in facilitation of these neurons was identified, which may be reflective of presymptomatic age-related changes (Oyama et al., 2010). The synaptic strength of hippocampal neurons from *Park2*^{tm1Rpa} line was significantly weaker in mice carrying one *Park2*^{tm1Rpa} mutant allele but not two mutant alleles. Hemizygous mice consistently showed reduced pair-pulse ratio whereas *Park2*^{tm1Rpa} knockout mice were only affected under small inter-stimulus ranges. Changes in pulse-pair ratio are consistent with presynaptic abnormalities, suggesting that in *Park2*^{tm1Rpa} mouse line parkin deficiency also affects presynaptic process.

Long term potentiation (LTP) is a long-lasting enhancement in signal transmission between two neurons that results from stimulating them synchronously, and is considered one of the major cellular mechanisms that underlies learning and memory. In the *Park2*^{tm1Rpa} knockout line, the LTP of 24 month old, but not 2 month old, appeared to be more robust, which may suggest that there is an absence of normal age related decline in the hippocampus of these mice (Hanson et al., 2010). In contrast, LTP was unchanged in the hippocampus of *Park2*^{tm1Roo} knockout line of a similar age (Itier et al., 2003). Furthermore, LTP was also unaltered in the hippocampus of *Park2*^{tm1Shn} knockout mice but could not be induced in striatal medium spiny neurons (Kitada et al., 2009a). Studies carried out to date on *Park2* knockout mice suggest that these mice have presynaptic abnormalities affecting dopamine function, which may be differentially modulated by the homozygous or heterozygous state of the mutant allele, cellular and regional specificity and/or age.

2.5 Neuroinflammation

Neuroinflammation is the chronic presence of activated microglia and reactive astrocytes, and associated mediators of the immune response in the central nervous system (reviewed in (Lee et al., 2009)). Postmortem analyses have demonstrated activated glia in both the striatum and in the substantia nigra of patients with idiopathic PD and in *parkin*-proven PD (reviewed in (Cookson et al., 2008; Lee et al., 2009)).

In vivo, Park2^{tm1Ccs} and *Park2*^{tm1Roo} knockout mice did not appear to have increased astrocytic or microglial markers in the substantia nigra, striatum or midbrain (Rodriguez-Navarro et al., 2007; Schmidt et al., 2011). However, a significant increase in the number of astrocytes in the striatum and the number of microglia in the midbrain occurs with age in the *Park2*^{tm1Roo} mice, independent of parkin deficiency, indicating an age-associated increase in the inflammatory environment may contribute to neuronal degeneration (Rodriguez-Navarro et al., 2007). *In vitro* however, alterations of glial populations are evident. Neuronal enriched cultures derived from *Park2*^{tm1Roo} knockout embryos appear to be enriched for microglial and glial progenitor cells (Casarejos et al., 2005), and glial cultures appear to have a reduced proportion of astrocytes compared to wildtype derived cultures (Solano et al., 2008). Furthermore, glial conditioned medium from *Park2*^{tm1Roo} and *Park2*^{tm1Ccs} knockout mice were found to have reduced neuroprotective capabilities that suggest a reduction in a trophic factor production or excretion by astrocytes (Schmidt et al., 2011; Solano et al., 2008).

Park2^{tm1Shn} knockout mice have been used to investigate if parkin deficiency can affect vulnerability to inflammation-mediated dopaminergic degeneration, using sustained low dose intraperitoneal injection of lipopolysaccaride (LPS). LPS is a bacterial endotoxin that is used as a glial activator for the induction of inflammatory dopaminergic neurodegeneration (Dutta et al., 2008). *Park2*^{tm1Shn} knockout mice showed increased vulnerability to LPS treatment and developed fine motor deficits and a loss of dopaminergic neurons (Frank-

Cannon et al., 2008). This suggests that *parkin* deficiency selectively increases the vulnerability of dopaminergic neurons to the effects of LPS induced inflammatory dopaminergic neurodegeneration. Collectively, these studies suggest that there may be inherent dysregulation of glial cells in *parkin* deficiency. Alterations in the function of these mediators of neuroinflammation within *parkin*-proven PD, together with endogenous age related changes in the neuroimmune system may play a role in the pathogenesis of PD.

2.6 Oxidative stress

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability to rapidly detoxify the reactive intermediates or repair the resulting damage to cellular constituents. Markers of oxidative stress are increased in PD patients (reviewed in (Tobon-Velasco et al., 2010)), and dopamine metabolism facilitates production of ROS (Lotharius and Brundin, 2002). Glutathione (GSH) is a free radical scavenger that acts on redox reactive molecules. Both astrocytes and neurons have the capacity to synthesis GSH, but astrocytes are known to play an important role in supply of GSH and other substrates to neurons. One of the earliest biochemical changes observed in PD patients is a decrease in GSH levels. It is thought the decrease in GSH levels may be due to increased oxidative stress and recent research suggests that GSH depletion itself may have a role in the pathogenesis of PD (reviewed in (Martin and Teismann, 2009)).

Park2^{tm1Roo} knockout mice aged 2 months were found to have significantly increased GSH in the midbrain, striatum and limbic system (Itier et al., 2003; Rodriguez-Navarro et al., 2007). However, when GSH levels were investigated in *Park2*^{tm1Roo} knockout mice aged 24 months, the level of GSH in the midbrain was not significantly different from either the 24 month old wildtype or 2 month old knockout mice. While GSH levels in the striatum of 24 month *Park2*^{tm1Roo} knockout mice were significantly reduced compared to 2 month old knockout mice they were not significantly different to 24 month old wildtype mice. In the limbic system of 24 month old *Park2*^{tm1Roo} knockout mice the level of GSH was significantly increased compared to 24 month old wildtype mice. Therefore, regional and age-related alterations of GSH are present in the *Park2*^{tm1Roo} knockout mouse line. Consistent with these observations, *in vitro* age-related studies of alterations in GSH levels have been identified in glial cells derived from *Park2*^{tm1Roo} knockout mice. When knockout glia are cultured for an extended period (6-9 months) the intracellular levels of GSH were significantly reduced compared to wildtype control glia, whereas knockout glia cultured for a shorter time (1-3 months) were shown to have a significant increase in the level of intracellular GSH compared to wildtype (Solano et al., 2008). However, conditioned media generated from glial cultures derived from *Park2*^{tm1Roo} knockout mice that had been cultured for a shorter period had lower levels of GSH and higher levels of hydrogen peroxide, and appeared to be less neuroprotective to wildtype neurons than conditioned media from wildtype glial cultures (Solano et al., 2008). These observations suggest that although intracellular GSH levels are increased in 2 month old *Park2*^{tm1Roo} knockout, there may be defective exocytosis of GSH out of the glia and into the extracellular space.

Mitochondria are a major source of ROS within cells and mitochondrial dysfunction has been robustly implicated with PD. The capacity for electron transport in mitochondria was found to be impaired in the *Park2*^{tm1Shn} and *Park2*^{tm1Ccs} knockout mice (Palacino et al., 2004; Stichel et al., 2007). Dopaminergic neurons in the *Park2*^{tm1Rpa} and *Park2*^{tm1Tmd} knockout lines do not appear to be more sensitive to the mitochondrial toxin MPTP (Perez et al., 2005;

Thomas et al., 2007). However, glial cultures derived from *Park2*^{tm1Roo} knockout mice appeared more sensitive to MPTP (Solano et al., 2008). Furthermore, neuronal cultures derived from *Park2*^{tm1Roo} knockout mice were more sensitive to the mitochondrial toxin rotenone. Co-culture of glia from *Park2*^{tm1Roo} knockout mice with neurons derived from wildtype mice increased the sensitivity of wildtype neurons to rotenone (Casarejos et al., 2006). Furthermore, wildtype neurons exhibited significantly shorter processes and smaller neuronal areas when they were co-cultured with *Park2*^{tm1Ccs} knockout derived astrocytes as opposed to wildtype astrocytes (Schmidt et al., 2011). Collectively, this suggests that lack of parkin leads to a functional disturbance of neuron-glia interactions, and that environmentally induced mitochondrial dysfunction when combined with parkin deficiency may be detrimental to dopaminergic neurons and stimulatory of neuroinflammatory processes.

The ultrastructure of mitochondria from *Park2*^{tm1Ccs} knockout mouse has been investigated in both neuronal and glial populations *in vivo*. Compared to 3 month old mice, neurons in the substantia nigra of 12 month old *Park2*^{tm1Ccs} knockout mice displayed an increased proportion of abnormal mitochondria. This age related change was not identified in wildtype mice (Stichel et al., 2007). Mitochondria within glial populations of *Park2*^{tm1Ccs} knockout mice were found to exhibit an elevated number of structural deficits that included abnormal disintegration, a reduction of mitochondrial cristae and mitochondrial enlargement. The proportion of abnormal mitochondria identified in *Park2*^{tm1Ccs} knockout glia depended on both age and glial cell type. Nonetheless, the amount of mitochondrial damage was significant at just 16 days of age in all glial cell types, and the mitochondrial burden was much greater in glial than neuronal cells (Schmidt et al., 2011). Oxidative stress is thought to be a major contributor to the development of PD. Several pathways associated with oxidative stress, notably GSH and the mitochondria, may be dysfunctional within *Park2* knockout mice. Furthermore, it appears that a number of factors including age and cell type may mediate the extent of dysfunction associated with lack of parkin.

2.7 Ubiquitin Proteasome System (UPS)

Parkin was originally identified as an E3 ubiquitin ligase that functions in the UPS, and a number of putative interacting proteins and substrates have subsequently been identified (reviewed in (Dawson and Dawson, 2010)). Knockout mice allow the *in vivo* analysis of interacting proteins, substrates and pathway deficits that have been identified *in vitro.* A simple working hypothesis is that if a protein is a substrate of parkin mediated ubiquitination and subsequent degradation via the UPS, that protein would be expected to accumulate in a *Park2* knockout mouse model. A number of groups have investigated some of the putative parkin substrates in *Park2* knockout mice, but in general very few studies have replicated the *in vitro* results and shown increased levels of putative substrates *in vivo*.

Two comprehensive analyses of parkin substrates have been reported using the *Park2*^{tm1Tmd} knockout mice. The steady state level of aminoacyl-tRNA synthetase cofactor (AIMP2) was increased in the ventral midbrain/hindbrain of *Park2*^{tm1Tmd} knockout mice but no alteration in the amount of seven other putative parkin substrates, including β-tubulin and α-synuclein, was identified. An accumulation of AIMP2 in brain tissue from individuals with *parkin*-proven PD was also shown, and AIMP2 was shown to interact with parkin *in vitro*, suggesting that AIMP2 is an authentic substrate of parkin (Ko et al., 2005). AIMP2 is a known interacting protein of far upstream element (FUSE)-binding protein 1 (FBP1),

promoting its ubiquitination and degradation. Subsequent studies demonstrated that the level of FBP1 was increased in the brain stem and cortex of *Park2*^{tm1Tmd} knockout mice and also patients with *parkin*-proven PD (Ko et al., 2006). In contrast to the results in the ventral midbrain/hindbrain of *Park2*^{tm1Tmd} knockout mice, the level of AIMP2 in the cortex, cerebellum, brain stem, and striatum, and the level of FBP1 in the ventral midbrain, cerebellum or striatum was not altered (Ko et al., 2006; Ko et al., 2005). This may imply functional redundancy for turnover of these proteins in some regions of the brain. However, an alternative explanation may be that the rate of new protein synthesis is greater than the rate of protein degradation in these regions and so the effect of parkin absence is masked. A similar phenomenon was observed with estrogen related receptor (ERR) isoforms and α-tubulin. The consequence of loss of parkin on accumulation of ERRs and α-tubulin was only demonstrated after new protein synthesis was inhibited by treatment with puromycin (Ren et al., 2010).

In general, *in vitro* studies suggesting a role for parkin in the turnover of putative substrates have not been replicated in the different *in vivo* models. However the extent to which investigation has been reported is limited. Such apparent discrepancy between *in vitro* and *in vivo* models may due in part to experimental methodology. For example, the *in vitro* data suggesting a role for parkin in ubiquitin-proteasome mediated degradation may be an artefact of exogenous expression of parkin and putative substrates, or due to specificity of the cell type utilised. In addition, there is compelling evidence to suggest that parkin is able to function as a multifaceted ubiquitin ligase, capable of modulating alternative forms of ubiquitination not associated with ubiquitin-proteasome mediated degradation of proteins.

2.8 Autophagy

For a substrate to be recognised by the proteasome it needs to labelled with at least four ubiquitin molecules attached via lysine-48 linkage. Most recently, parkin-mediated lysine-63 polyubiquitylation was shown to be an important mediator of the aggregation and turnover of damaged mitochondria via autophagy (Geisler et al., 2010; Narendra et al., 2008; Narendra et al., 2010; Suen et al., 2010). Autophagy is the major pathway involved in the degradation of long-lived proteins and organelles and has been shown to play an integral role in protection against neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). It is comprised of three distinct pathways, macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy (hereafter referred to as autophagy) involves the sequestration of organelles and proteins in a double-membrane vesicle, called an autophagosome or autophagic vacuole, which subsequently fuses with a lysosome and the contents are degraded by lysosomal hydrolases (reviewed in (Klionsky and Emr, 2000)). Emerging data suggests that the UPS and autophagy are functionally coupled and inhibition of the UPS can result in aggresome formation and elevated autophagy (Pandey et al., 2007). Several markers of the autophagic pathway have been shown to be elevated in the substantia nigra of PD cases and it has been proposed that Lewy bodies may be the *in vivo* representation of aggresome. It is not yet clear if the elevated numbers of autophagosomes identified in PD brains represent increased autophagy induction or impaired completion of autophagic degradation (reviewed in (Chu, 2011)). However, the ability of the autophagic vacuole to engulf organelles and large protein aggregates suggests upregulation of autophagy may represent a therapeutic target for PD (reviewed in ((Arduino et al., 2010; Banerjee et al., 2010)). Recent studies have defined a pathway linking parkin and PINK1 to

mitophagy, a form of autophagy selective for mitochondria. Depolarisation of mitochondria results in the accumulation of parkin at the mitochondrion in a PINK1-dependent manner with subsequent degradation via mitophagy (Kawajiri et al., 2010; Narendra et al., 2008; Narendra et al., 2010; Vives-Bauza et al., 2010).

The effect of parkin deficiency on autophagy in *Park2* knockout mice has not been extensively reported. However, one study has investigated autophagy in neuronal cultures derived from *Park2*^{tm1Roo} knockout mice. Midbrain neuronal cultures derived from *Park2*^{tm1Roo} knockout mice were treated with epoxomicin to partially inhibit the proteasome. *Park2*^{tm1Roo} knockout cultures appeared to be less susceptible to the toxic effects of proteasomal inhibition. The autophagic substrate p62 was increased in wildtype neuronal cultures but not in *Park2*^{tm1Roo} knockout neuronal cultures, suggestive of increased autophagic activity. A second indicator of autophagic activity is the relative ratio of LC3I and LC3II. LC3 II is generated by site specific proteolysis and lipidation of LC3I and serves as a specific marker of autophagic activation. In basal conditions, the LC3II/I ratio was unchanged in neuronal cultures derived from wildtype mice, but it was significantly increased in cultures from derived *Park2*^{tm1Roo} knockout mice, and this was further potentiated by epoxomicin treatment. These observations suggest that neuronal cultures derived from *Park2*^{tm1Roo} knockout mice have increased autophagy and that partial proteasomal inhibition further potentiates autophagy in this model (Casarejos et al., 2009). The majority of cell culture models have suggested that Parkin regulates mitochondrial degradation through autophagy, and an early step in this process is the requirement for PINK1 to recruit Parkin to the mitochondria (Vives-Bauza et al., 2010). Therefore, it seems counter intuitive that neuronal cultures derived from *Park2*^{tm1Roo} knockout mice would display increased autophagy following proteasomal inhibition. However, a study of neurons taken from rat substantia nigra suggested that mitophagy was parkin independent in these cells. It was suggested that the difference in neurons may be due to the high dependence of neurons on mitochondrial respiration (Van Laar et al., 2011). Much of what is currently understood about the potential role of parkin in autophagy is derived from studies in cell culture models. However, this is an expanding field in PD research and future studies utilising *Park2* knockout mice will provide considerable insight into the significance of this role *in vivo*.

2.9 Trafficking/signalling

Parkin-mediated ubiquitination has also been suggested to play a role in signal transduction and trafficking. Monoubiquitination is associated with the regulation of endocytosis of membrane proteins and signalling (Mukhopadhyay and Riezman, 2007). The interaction between parkin and PICK1 is an example of this function. PICK1 regulates the trafficking and stability of a number of synaptic proteins, including neurotransmitter receptors, transporters and ion channels (Madsen et al., 2005). *In vitro* analysis suggested that parkin monoubiquitinated rather than polyubiquitinated PICK1. In neurons derived from *Park2*^{tm1Roo} knockout mice steady-state PICK1 levels were not altered, suggesting turnover of the protein is independent of parkin. PICK1 has been shown to interact with the proton-gated ion channel ASIC2a, and potentiation of ASIC2a currents is suggested to be PICK1 dependent. Hippocampal neurons from *Park2*^{tm1Roo} knockout mice show deficits of ASIC2a current potentiation, which suggest that monoubiquitination of PICK1 by parkin is required for ASIC2a channel function (Joch et al., 2007).

The interaction of parkin and the endocytic protein Endophilin A1 provides additional evidence that the ubiquitination activity of parkin is not limited to proteasome turnover. Endophilin A1 is a protein that is abundant in neural synapses and is involved in formation of presynaptic endocytic vesicles. Limited ubiquitination of Endophilin A1 by parkin was shown *in vitro* and Endophilin A1 did not appear to accumulate in synapses from *Park2*^{tm1Roo} knockout mice. *In vivo* the interaction of the two proteins appears to promote co-localisation from the cytosol to the plasma membrane and synaptic vesicle enriched fractions. In synaptosome preparations from wildtype mice relocalisation leads to increased ubiquitination of synaptic proteins, however, this effect is abrogated in *Park2tm1Roo* knockout mice (Trempe et al., 2009). Further understanding of the functional deficits associated with this loss of relocalisation and ubiquitination activity in *Park2* knockout mice may shed light on the defects in synaptic transmission observed in both *Park2* knockout mice and *parkin*-mediated PD.

2.10 Genetic modification of *Park2* knockout mice

Mutations in the genes *parkin*, *PINK1* and *DJ-1* have been associated with familial recessive early-onset PD. A number of lines of evidence suggest that these genes may function in a common pathway(s) important for mitochondrial function (Geisler et al., 2010; Narendra et al., 2010; Thomas et al., 2011). The effect of knockout of all three genes in a single mouse model revealed that *Park2*^{tm1Shn}*/Pink1/Dj-1* knockout mice at 24 months of age do not show significant dopaminergic degeneration (Kitada et al., 2009b). This may suggest a functional redundancy between these genes. An alternative interpretation may be that these genes are not essential for neuronal survival, but rather may play a protective role against cellular insults.

A major component of Lewy bodies in PD is alpha-synuclein, which is also genetically linked with autosomal dominant PD and sporadic PD (reviewed in (Obeso et al., 2010). The effect of parkin deficiency combined with dysregulation of α-synuclein has been reported for three *Park2* knockout mouse lines. The *Park2*^{tm1Tmd} knockout mouse was crossed with a transgenic mouse overexpressing familial mutant A53T α-synuclein under the control of the mouse prion promoter [Tg(PRP-SNCA^{A53T})]. In this model, parkin deficiency did not appear to exacerbate attributes previously recognised in Tg(PRP-SNCA^{A53T}) mice. Likewise, Tg(PRP-SNCA^{A53T}) did not potentiate the locus coeruleus degeneration of the *Park2*^{tm1Tmd} knockout line (von Coelln et al., 2006). The *Park2*^{tm1Roo} knockout mouse was crossed with a transgenic mouse overexpressing familial mutant A30P α-synuclein under the control of the thymus cell antigen 1 promoter [Tg(THY1-SNCA^{A30P})]. In contrast to the previous model, parkin deficiency appeared to delay the progressive neurodegenerative motor phenotype of Tg(THY1-SNCA^{A30P}) mice, and decreased neuritic pathology that is associated with these symptoms. Furthermore, co-staining of ubiquitin with phosphorylated α-synuclein positive structures was reduced in this model (Fournier et al., 2009). The *Park2*^{tm1Ccs} knockout mouse was crossed with two different mutant α-synuclein lines. Both lines expressed human α-synuclein carrying two familial mutations, A30P and A53T, under the control of either the chicken beta-actin (BA) promoter [Tg(BA-SNCA$^{A30/PA53T}$)] or the mouse tyrosine hydroxylase (TH) promoter [Tg(TH-SNCA$^{A30/PA53T}$)]. The most prominent feature identified in neurons from these mutant mice was an age-related increase in the proportion of abnormal mitochondria and a reduction in mitochondrial complex I activity in the substantia nigra (Stichel et al., 2007). Collectively, these studies suggest that the effect of

parkin deficiency on α-synuclein mediated phenotypes may be independent (von Coelln et al., 2006), delayed (Fournier et al., 2009) or enhanced (Stichel et al., 2007). Whether this is related to differential attributes of the *Park2* knockout mice or the differential regional effects of α-synuclein overexpression remains to be investigated.

The normal age related increase of tau pathology is potentiated in the *Park2*^{tm1Roo} knockout mouse (Rodriguez-Navarro et al., 2007). Tauopathies are neurodegenerative diseases principally identified by dementia and Parkinsonism. Idiopathic forms are thought to be due to post-translational alteration of tau whereas familiar tauopathies are the result of mutations in the gene encoding tau, *microtubule associated protein tau* (*MAPT*) (Avila et al., 2004; Hutton et al., 1998). *MAPT* has also recently been genetically implicated in sporadic PD aetiology (Satake et al., 2009; Simon-Sanchez et al., 2009) and aggregation of tau has been shown to be a feature of some cases of *parkin*-proven PD (reviewed in (Cookson et al., 2008).

The consequence of combining the over-expression of a human 4-repeat tau isoform with known familial alterations G2727V, P301L, R406W under the control of human thymus cell antigen 1 promoter [Tg(THY1-Tau^{VLW})] with the *Park2*^{tm1Roo} knockout mouse was investigated. Like *Park2*^{tm1Roo} knockout mice, Tg(THY1-Tau^{VLW}) mice have slight behavioural and molecular changes that do not manifest in a clinical phenotype. However, when combined a number of age-progressive behavioural attributes including reduced hind limb length, uncontrolled movements, loss of balance and postural abnormalities as well as increased self-injury of the face that is a reflection of compulsive long-term grooming, were identified. This was coupled with a significant loss of motor neurons, and dopaminergic neurons in the substantia nigra. In addition, tau pathology was identified, as was abnormal expression glia in the substantia nigra and the hippocampus (Menendez et al., 2006; Rodriguez-Navarro et al., 2008a). Furthermore, phosphorylated tau plaques and tangles, as well as endogenous β-amyloid plaques were found in the hippocampus. Notably, dietary supplementation with the disaccharide trehalose, which is thought to enhance autophagy, was found to ameliorate the severity of the symptoms and pathology in this model. (Rodriguez-Navarro et al., 2010). By combining parkin deficiency with abnormal tau expression, a number of attributes that were modestly perturbed in the single monogenetic mutants were significantly dysregulated. This suggests that parkin and tau may be functionally coupled *in vivo*, and their perturbation is capable of eliciting a progressive neurodegenerative phenotype with characteristics of both PD and Alzheimer's disease.

Alzheimer disease is a degenerative dementia characterised by loss of neurons in the cerebral cortex. Pathological features include extracellular amyloid plaques, which are composed predominately of β-amyloid, and intracellular neurofibrillary tangles that mainly consist of hyperphosphorylated tau. The identification of β-amyloid plaques in the *Park2*^{tm1Roo}/Tg(THY1-Tau^{VLW}) mouse led to the investigation of the effect of parkin deficiency on β-amyloid expression. The APP_{swe} is a transgenic mouse line that overexpresses the β-amyloid precursor protein containing two mutations, K670N and M671L, under the control of the prion protein promoter. Parkin deficiency appears to reduce the severity of behavioural deficits of APP_{swe} mice including reduced weight gain, exploratory activity and working memory. Furthermore, parkin deficiency appears to reduce β-amyloid plaque pathology within the cerebral cortex and hippocampus, and in the hippocampus the amount of astrogliosis and phosphorylated tau was also reduced. This

was coupled with alterations in a number of autophagic markers consistent with induction of autophagy (Perucho et al., 2010).

Therefore, in contrast to the *Park2*^{tm1Roo}/Tg(THY1-Tau^{VLW}) mouse, where combining the two mutant alleles appeared to enhanced the disease process, parkin deficiency combined with APP_{swe} over-expression can ameliorate a number of pathological characteristics of these mice. Consequently, it appears parkin has the potential to be both neuroprotective and a neurotoxic to the neurodegenerative process, depending on context. Furthermore, the effect of active autophagy when either APP_{swe} or Tg(THY1-Tau^{VLW}) were combined with *Park2*^{tm1Roo} knockout appears to be protective. This suggests further investigation into factors that enhance autophagy may identify potential treatments for individuals with PD and other neurodegenerative disorders.

3. Discussion

PD is differentiated from other neurodegenerative disorders by defined motor disturbances resulting from the pathological loss of dopaminergic neurons. Seven *Park2* knockout models have been generated and characterised. In the current context, loss of function of parkin in these mouse models does not appear to reproduce a PD-like phenotype that is reflective of *parkin*-proven PD. However, two models showed a significant loss of catecholaminergic neurons, one in the substantia nigra and the other in the locus coeruleus. Notably, the extent of neuronal loss within the substantia nigra was not equivalent to that associated with the onset of symptomatic PD in humans. However, both neurochemical and electrophysiological studies have identified disturbances in dopamine pathways in *Park2* knockout mice that are consistent with altered presynaptic release of dopamine. Glial and redox dysfunction was also identified, indicating that the capacity to protect neurons against cellular insult may be diminished in *Park2* knockout mice. Furthermore, mitochondrial abnormalities highlight the importance of parkin function in maintaining mitochondrial integrity. Accumulation of specific proteins within *Park2* knockout mice has confirmed *in vivo* that parkin functions as an E3 ubiquitin ligase in the UPS. The additional role of parkin in autophagy and trafficking/signalling pathways are indicative of a broad role in normal cellular function. With regard to the involvement of parkin with neurodegeneration the most important findings were identified by genetic modification of *Park2* knockout mice. *Park2* knockout mice, when combined with other mutant transgenic models, appeared to have the capacity to both exacerbate and ameliorate disease associated characteristics in a context dependent manner. Therefore, dysfunction of parkin has the potential to be both neuroprotective and neurotoxic.

The inability of *Park2* knockout mice to adequately recapitulate the clinical and pathological manifestation of PD is not an isolated observation. Knockout of other PD associated genes such as *Pink1*, *a-synuclein* and *Lrrk2* also fail to produce robust PD-like phenotypes, and the results of transgenic overexpression of disease associated genes and mutants vary considerably depending on the promoter used to drive expression (reviewed in (Dawson et al., 2010)). In addition, a knockin model of a common pathogenic variant of LRRK2 does not show evidence of dopaminergic degeneration. However, like *Park2* knockout mice these *Lrrk2* knockin mice appear to have dysregulation of the dopamine system (Tong et al., 2009). In addition, a number of other features identified in *Park2* knockout mice, including

alterations of the neuroimmune system, mitochondrial dysfunction and oxidative stress are shared between the models. These observations suggest a commonality to the perturbations that occur prior to manifestation of pathogenic phenotypic features.

An explanation as to why *Park2* knockout mice fail, for the most part, to recapitulate the loss of dopaminergic neurons that is a feature of human disease may be the short lifespan of the species. Aging research carried out in mice indicate that a number of molecular markers of aging follow a similar course to that observed in humans, although over a shorter duration. (reviewed ((Yuan et al., 2011)). This suggests that at the cellular and systemic level aging follows a parallel path in mice and humans. The symptoms of *parkin*-mediated PD typically present before 45 years of age. However, there is considerable disparity in age of onset, presentation, progression and response to drug treatment, even for patients within the same sibship, with the same mutations (reviewed in (Mata et al., 2004)). Therefore, loss of function of parkin alone may not be responsible for dopaminergic neuronal loss in individuals with *parkin*-mediated PD. Three lines of evidence from studies carried out in *Park2* knockout mice support this notion. 1) A significant loss (35%), of dopaminergic neurons occurs in the substantia nigra of the *Park2*^{tm1Roo} knockout in an age-related progressive manner over the duration of the life-span of the mouse, although not to the extent that is observed (~60%) at symptom onset in humans. This indicates that parkin deficiency can cause dopaminergic neuronal loss in a mouse model. 2) The loss of catecholaminergic neurons in the locus coeruleus in the *Park2*^{tm1Tmd} knockout line shows reduced penetrance. This suggests that additional factors mediate this trait. 3) The increased vulnerability of *Park2*^{tm1Shn} knockout mice to loss of dopaminergic neurons in the substantia nigra induced by repeated low-dose systemic LPS treatment. This indicates that in this model, although parkin deficiency alone was not sufficient to cause dopaminergic neuronal loss, dopaminergic integrity was compromised in a neuroinflammatory environment.

A number of the phenotypic discrepancies between the *Park2* knockout mice may be attributable to differences between the genetic background of the mouse strains used (discussed in (Perez and Palmiter, 2005)). The majority of studies used mice of variable mixed backgrounds. Isogenic strains are preferred when investigating the effect of gene knockout as genetic similarity tends to result in phenotypic uniformity, increasing the power to identify significant effects. It would be an advantage when comparing traits between different knockout mice for them to be on the same isogenic background, particularly if the genetic effect is modest, as appears to be the case of *parkin*. Therefore, until these carefully controlled studies are undertaken, it remains difficult to determine which of the identified phenotypic features are likely to be attributable to parkin.

Isogenic strains may also be a hindrance if pathogenicity has undefined multigenic influences, as potentially could be the case for parkin. During the process of generating an inbred strain, mice undergo a process called inbreeding depression. In this process the reproductive fitness reduces as homozygosity increases because repressive alleles are unmasked. Therefore, founders of an inbred strain and their offspring must be considered selective survivors of the inbreeding process, and as such have a level of fitness that does not reflect the species as a whole (McClearn and McCarter, 2011). In a multifaceted disease such as PD, other genetic factors may contribute to disease progression. Therefore, the isogenic inbred strains could limit the affect of *Park2* knockout. However, the negative effects associated with inbreeding depression can be overcome by generating F1 hybrids of

two different inbred strains. In the context of the *Park2* knockout models, it may be noteworthy that the only strain that demonstrates significant loss of dopaminergic neurons in the substantia nigra, the *Park2*^{tm1Roo} knockout mouse, was reported in a mixed 129S2/C57BL6 (50/50) genetic background.

Another factor that could be significant in the development of a disease phenotype is the environmental differences between laboratory mice and humans. It is clear that environmental factors influence PD development, and in some cases may cause PD. Laboratory mice are housed in controlled environments, often pathogen free, with none or limited exposure to environmental toxins or stresses. Therefore these mice may not be exposed to the appropriate triggers that are necessary for dopaminergic protective mechanisms to fail. Loss of parkin may result in a pre-degenerative state where neurons are under stress but not sufficiently compromised as to cause significant loss. Other cues, such as pro-inflammatory factors, could provide the additional stimulus required for degeneration to occur. This model may better explain an inherited autosomal recessive trait that takes multiple decades to manifest symptoms.

The *Park2* knockout mice are useful to understand the mechanistic consequence of loss of parkin function in a mammalian species. An improved understanding of the effect of *parkin* deletion on neuron and glial populations will provide considerable insight into the pathogenesis of PD, and the contribution that dopamine dysregulation plays in the pathogenic process. Cellular models have provided a great deal of information about the role of parkin in the turnover of protein and cellular constituents, in particular mitochondria, via the UPS and autophagy. The *Park2* knockout models provide a sophisticated platform to refine and advance these studies, for example using neuronal and glial cultures derived from *Park2* knockout mice.

There are a number of avenues that can be explored to potentially develop a *Park2* knockout model with a pathological phenotype of clinical relevance. Crossing the *Park2* knockout mice with α-synuclein, tau and β-amyloid models has provided enormous insight into the role of parkin in the neurodegenerative process *in vivo*. Further research following on from these studies, including breeding to the conditional α-synuclein knockin model currently in development (NIH project No. 1R21NS057795-01A1), has the potential to develop these mice into a more clinically relevant model. Likewise, the role of neuroinflammation in the neurodegenerative process could be further explored using *Park2* knockout mice. This could be achieved by breeding to genetic models with perturbations in the inflammatory response or treatment with agents such as LPS. In addition, a second rodent model, knockout of parkin in the rat, has recently been developed (SAGE Labs). The phenotypic outcome will be of great interest to compare and contrast with the results already obtained in mouse models.

4. Conclusion

A quote by Michael FW Festing, an expert in the field of laboratory animal genetics, eloquently encapsulates the use of mouse models in disease research. *"Models are subject to improvement through further research. A lot of animal research is aimed at understanding the animal as a potential model for particular human conditions, without being too precise as to what those conditions might be. Models are not just found. They need to be developed, and this requires an understanding of the biology of the species and the effects of various interventions such as*

inactivating specific genes or manipulating the environment. As our understanding increases, so the chance of choosing the most appropriate models for a specific disease increases" (Festing, 2011). To date, seven *Park2* knockout mice have been generated but *Park2* knockout models still need to be developed in order to understand the clinical manifestation of *parkin*-mediated PD and the contribution of parkin to idiopathic PD.

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Parkinson's disease (PD) results primarily from the death of dopaminergic neurons in the substantia nigra. Current PD medications treat symptoms; none halt or retard dopaminergic neuron degeneration. The main obstacle to developing neuroprotective therapies is a limited understanding of the key molecular mechanisms that provoke neurodegeneration. The discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are pivotal to PD pathogenesis. Previously implicated culprits in PD neurodegeneration, mitochondrial dysfunction, and oxidative stress may also act in part by causing the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons. Neurotoxin-based models have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. PD models based on the manipulation of PD genes should prove valuable in elucidating important aspects of the disease, such as selective vulnerability of substantia nigra dopaminergic neurons to the degenerative process.

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