# EXHIBIT B
# FILED UNDER SEAL

## Qualifications

I received my PhD from the Department of Biological Chemistry in the David Geffen School of Medicine at the University of California, Los Angeles. I received additional research training in the Department of Neurogenetics at Massachusetts General Hospital in Boston. Prior to arriving at the Buck Institute for Research- on Aging, I held a faculty position in the School of Gerontology at the University of Southern California.

I have published more than 170 scientific papers and hold three current patents. I have been recognized for my research with a Parkinson's Pioneer Award from the National Parkinson's Foundation, a Glenn Award for Research in Biological Mechanisms of Aging, and a senior scholarship from the Ellison Medical Foundation. I was elected a fellow of the Society for Free Radicals in Biology and Medicine in 2013. I currently serve on the scientific advisory board for the University of Pittsburgh Medical Center's Biology of Aging Program, on the editorial board of e-Neuro (Journal of Neuroscience's e-journal), as a member of the Brookdale Institute on Aging, and as a council member for the Society of Neurotoxicity. I have extensive experience working with both biotech companies and medical foundations, including Roche, the Michael J. Fox Foundation, the National Parkinson's Disease Foundation, and the American Parkinson's Disease Foundation.

My curriculum vitae is attached to this report.

## Parkinson's Disease Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder with two major histopathologic hallmarks: Lewy body formation and the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Lewy bodies are dense clusters of α-synuclein and other proteins within neurons that impair and eventually kill neurons. The absence of these particular neurons results in loss of dopamine, the neurotransmitter required for movement. Sixty to eighty percent of neurons are lost in these areas before the well-known motor symptoms of PD, such as resting tremors and akinesia, appear.

The probability of a PD diagnosis is greater with increasing age. The average age of a PD diagnosis is 62. Both genetics and environment can play a role, however. The pesticide paraquat (PQ) produces the PD disease hallmarks of α-synuclein aggregation and neuron loss in the SNpc in mammals. PQ can get into human bodies via inhalation, oral ingestion, or dermal absorption,

and is able to cross the blood-brain barrier (BBB). Once in the brain, PQ damages the same parts of the brain that are affected in people with PD, leads to an increase in α-synuclein aggregation, causes oxidative stress within neurons and glia, and causes neuroinflammation. Due to these effects, PQ has been used in worldwide research to model PD in laboratory animal models (Andersen and Di Monte, 2014).[1]

**Paraquat Neurotoxicity Involves Oxidative Stress**

In the brain the toxicity mechanism of PQ is derived from the molecule's ability to transfer electrons with enzymes called oxidoreductases and to participate in redox cycling with molecular oxygen (Dinis-Oliveira et al., 2006). Oxidative stress is a well-known feature of PQ toxicity in neurons, and occurs when molecules with unpaired electrons (called radicals) form. These radicals are highly reactive and cause cell damage. The redox cycling property of PQ was described 60 years ago in relation to its mechanism as an herbicide (Homer 1960). PQ is a di-cation with a 2+ charge which can be reduced (gain an electron) to 1+ by enzymes in the cell, forming a $\bullet PQ^+$ radical (the "•" symbol represents an unpaired electron). In a process termed redox cycling (redox = reduction and oxidation), $\bullet PQ^+$ donates the extra electron to an oxygen molecule ($O_2$), which then becomes a superoxide radical, $\bullet O_2^-$. PQ becomes a di-cation once again, and is able to repeat the process. The superoxide radical instigates subsequent reactions leading to the formation of reactive oxygen species (ROS) in the cell, such as hydrogen peroxide, $H_2O_2$, and the hydroxyl radical, •OH. The compound NADPH is an electron donor which is necessary in cellular metabolism. Normally the transition from NADPH to $NADP^+$ and back again is required for metabolic reactions occurring in the mitochondria, but when PQ gets involved, it grabs an electron from NADPH, resulting in the formation of $\bullet PQ^+$ and $NADH^+$, and contributing to the redox cycle.

This redox cycling mechanism was shown in experiments focused on rodent lungs (Bus et al., 1976). *In vitro* experiments also demonstrated the production of ROS. Purified mitochondria from rat brains produced $H_2O_2$ in the presence of $\bullet PQ^+$ (Castello et al., 2007). A human dopaminergic cell line was utilized to demonstrate that PQ interferes in the pentose phosphate pathway of metabolism in mitochondria, leading to the production of NADPH which then becomes an electron donor for PQ redox cycling (Lei et al., 2014).

[1] I incorporate by reference as though set out in full in this report the publication Andersen JK and Di Monte DA. (2014) Paraquat and Parkinson's disease (PD). Encyclopedia of the Neurological Sciences Vol 3, 792-794.

Redox has also been reported to occur in the brain. McCormack showed that oxidative damage occurs selectively in the SNpc of mice dosed with PQ, using a lipid peroxidation marker to measure oxidative stress (McCormack et al., 2005, 2006). Measurements of glutathione (an antioxidant) and lipid peroxidation in the brains of rats showed that both were elevated after PQ treatment, indicating oxidative stress (Somayajulu-Nitu et al., 2009). The activity of ROS-scavenging enzymes increased significantly in the brains of mice treated with 10 mg/kg PQ twice per week for 4 weeks, showing that ROS species were produced (Mitra et al., 2011). An additional area where oxidative stress occurs is the dorsal motor nucleus of the vagus nerve (DMV). Researchers injected adeno-associated viral vectors carrying human α-synuclein into the vagus nerve of mice and then exposed the mice to PQ (Musgrove et al., 2019). The results demonstrated that the DMV was vulnerable to oxidative stress, as shown by the accumulation of ROS, accumulation of α-synuclein modified by oxidation, increased aggregation of α-synuclein, cell-to cell transfer of α-synuclein, and neurodegeneration (Musgrove et al., 2019).

Loss of DA-containing neurons in the SN is a predictable feature of PD (Damier et al., 1999). Oxidative stress has been identified as a major factor in the development of PD, and is known to cause damage to dopaminergic neurons of the SN (Henchcliffe and Beal, 2008; Dias et al., 2013; Subramaniam and Chesselet, 2013; Jiang et al., 2016). The SN is especially vulnerable to oxidative damage because the neurons in that location are involved in DA synthesis and metabolism, which is a process that generates ROS (discussed in Trist et al., 2019). The pro-oxidant environment in the SN explains in part why researchers see more damage to DA neurons when animals are treated with PQ (McCormack et al., 2005; Kang et al., 2009).

Another characteristic of the SN that makes it vulnerable to oxidative damage is the presence of high amounts of iron (Hare and Double 2016). The enzyme superoxide dismutase (SOD) converts superoxide to $H_2O_2$, which will react with ferrous iron to produce harmful hydroxide radicals (see Trist et al., 2019). Empirical support for the activity of PQ in causing neurotoxicity via superoxide generation was provided by Peng et al. in 2005[2] when they used mimetics of SOD (EUK-134 and EUK-189) to dose-dependently prevent oxidative damage induced by PQ. A transgenic mouse strain with elevated ferritin levels – ferritin is a protein that sequesters iron – was protected from PQ-induced oxidative damage (McCormack et al., 2005). Mice fed iron as neonates, and a control group not fed iron, were dosed with PQ after weaning showing a similar loss of neurons at 2 and

[2] Work done in my lab.

6 months that was not enhanced by the neonatal iron feeding (Peng et al., 2007[3]). However, the iron/PQ groups at 12 and 24 months had an increased loss of neurons compared to the control/PQ groups, demonstrating that there is an age-related effect when iron is present. The same research team used a SOD mimetic, EUK-189, to prevent the iron/PQ toxicity (Peng et al., 2007). The increase in oxidative stress due to aging in humans results from a decrease in the antioxidants present in the brain and an increase in the enzymes that produce oxidant molecules.

Considering iron and aging together, the PQ mouse model of PD replicates the interactions between risk factors and predisposition in human disease. These include environmental exposures to PQ during development and/or adulthood (Barlow et al., 2007; Brown et al, 2006); traumatic brain injury (Lee et al., 2013); genetic susceptibilities (Fernagut et al., 2007; Peng et al., 2010; Goldman et al., 2012); co-exposure with maneb (Bastias-Candia 2013; Desplats 2012; Pezzoli and Cerada 2013); and aging (Li et al., 2005).

## Oxidatively-Induced Pathways Involved in PQ Neurotoxicity

The consequences of oxidative stress have further implications for the neurotoxicity of PQ. Experiments conducted in my lab demonstrated that the oxidative stress caused by PQ activated an intracellular molecular cascade ultimately resulting in apoptosis, programmed cell death which would not otherwise occur in a healthy brain (Peng & Andersen, 2003; Peng et al., 2004[4]). A few years later, an *in vitro* study showed that PQ oxidized thioredoxin in the cytosol, which led to apoptosis in a similar manner (Ramachandiran et al., 2007).

## How PQ Enters the Brain

The blood-brain barrier (BBB) serves functionally as a security system for the brain, preventing potentially harmful substances from accessing neurons and glial cells, while permitting entry of necessary compounds such as glucose or amino acids (Abbott et al., 2010). Interactions between the cells of the neurovascular unit control the passage of materials between the blood and the neural tissue. The neurovascular unit is composed of the capillary, surrounded by endothelial cells, pericytes, a basement membrane, and astrocyte end-feet, along with nearby microglia (Abbott et al., 2010). Tight junctions formed by proteins are found between endothelial cells. Substances can be transported across the BBB via passive diffusion, transporter proteins, solute

[3] Work done in my lab.
[4] Studies conducted in my lab.

carriers, and receptor-mediated or adsorptive-mediated transcytosis. The properties of the substance to be let in or kept out determine which route is accessible. Mammals have highly developed central nervous systems, and so natural selection has preserved well-developed BBBs which are very similar across mammalian species (Abbott et al., 2010).

Numerous studies have determined that PQ crosses the BBB. PQ has been found in the brains of rodents 24 hours after a single 20 mg/kg intravenous injection (Litchfield et al., 1973). PQ was also found in the brains of rats fed 120 ppm PQ for 8 weeks (Litchfield et al., 1973). Both age- and dose-dependent amounts of PQ in rat brains were demonstrated in 1991 by Corasaniti and colleagues. When a single dose of PQ was administered to rats, most of the PQ was found in areas of the brain unprotected by the BBB after 4 or 24 hours, yet a small amount penetrated the BBB (Naylor et al., 1995). In 1996, it was reported that PQ passed through the BBB of neonatal mice and aged mice in greater quantities than in adult mice, suggesting a porous BBB for younger and older animals (Widdowson et al., 1996a). After repeated oral dosing, PQ was found to accumulate in mouse brain (Widdowson et al., 1996b). Microdialysis with HPLC/UV detection in rats revealed that PQ was able to cross the BBB through Lat1, a neutral amino acid transporter, in a dose-dependent manner (Shimizu et al., 2001). Prevention of PQ-induced TH+ neuron loss when L-valine and L-DOPA were administered before PQ demonstrated that PQ uses the same transporter (the System L carrier) to cross the BBB (McCormack and Di Monte, 2003). Levels of PQ in the mouse brain peaked rapidly after a single dose, but PQ was still found in the brain up to 12 h later (Barlow et al., 2003). The presence of other toxicants, such as dithiocarbamate pesticides, can increase PQ amounts in the brain, perhaps by inhibiting efflux transporters (Barlow et al., 2003). Another study demonstrated that once PQ is in the mouse brain, it has a 1-3 month half-life, depending on the strain (Prasad et al., 2009). In 2013, Breckenridge et al. reported a PQ half-life of about 24 days, based on empirical fitting of single-dose data followed by PBPK modeling, while acknowledging that PQ was "slowly eliminated" from the brain. Radiolabeled PQ administered to Rhesus macaques was detected in the brain in very small quantity which seemed to rapidly clear (Bartlett et al., 2009). However, only one small dose was given and data were gathered in a 90-minute PET scan with no follow-up scans, so there may have been a lack of sensitivity in this experiment, leading to an inability to show CNS distribution of PQ. Further, the activation of microglia upon a first dose of PQ can constitute a priming event which causes the brain to be more sensitive to injury upon subsequent exposures (Purisai et al., 2007). In summary, PQ is indeed able get through the BBB. Since PQ it is not metabolized and it accumulates in the brain after repeated doses, what follows is neuronal damage due to oxidative stress.

Once PQ enters the rodent brain, it is found in regions relevant to PD and remains detectable over a long period of time. Rose et al. (1976) showed that PQ is taken up into rat brain slices, with the uptake increasing up to 2 h when the measurements were stopped. This group also measured brain uptake *in vivo* and found that PQ was detectable for up to 30 hours, at which time the experiment ended (Rose et al., 1976). When a tracheal cannula was used to administer 150 nmol/kg in saline to rats, PQ remained in the brain for at least 47 hours, which was the length of the experiment (Chui et al. 1988). Prasad et al. (2007) demonstrated a 28-day half-life of PQ in mouse brain, in addition to prolonged oxidative stress, after just one dose. In 2009, Prasad et al. dosed 5 strains of mice with 10 mg/kg intraperitoneally (ip) 2 or 3 times per week, for a total of 24 doses, reporting a PQ half-life in the brain at 1-3 months depending on mouse strain.

## PQ Can Cause Neurodegeneration Through A Gut-to-Brain Mechanism

A connection suggested by Braak et al. (2003) between the enteric nerves and the DMV was confirmed in a 2017 report, and was also shown to be sensitive to PQ-induced damage (Anselmi et al. 2017). The gavage administration of PQ along with lectins resulted in misfolded α-synuclein in the enteric nervous system, DMV, and SN with a loss of TH+ neurons; the neurodegeneration was prevented by vagotomy (Anselmi et al., 2018). Injection of α-synuclein into the wall of the stomach pylori and the duodenum of mice resulted in transmission aggregation of α-synuclein in the vagus nerve, and this pathology eventually reached the brain, resulting in PD-like neurodegeneration; the spread of α-synuclein pathology and neurodegeneration could be prevented by vagotomy in the mice (Kim et al., 2019). These findings support the proposal by Braak et al. (2003) that PQ-induced α-synuclein pathology could be transmitted along a gut-to-brain pathway to target the SNpc.

## Experimental Low-Dose PQ Exposure

Both *in vitro* and *in vivo* studies demonstrate that repeated low dose PQ exposure causes neurotoxicity. Apoptosis of rat cerebellar granule cells resulted from a low 5 μM dose of PQ, and the apoptosis was prevented by co-treating the cells with the antioxidant Vitamin E (Gonzalez-Polo et al., 2004). In rat primary neuron-glia cultures treated with 0.5 μM and 1 μM PQ, there was a dose-dependent loss of DA function, loss of TH+ neurons, and increase in superoxide production due to the presence of microglia (Wu et al. 2005). These same researchers found that neurotoxicity in primary neuron-glia mouse cultures was mediated by NADPH oxidase in a dose-dependent manner, also facilitated by microglia (Wu et al. 2005). Increased oxidative stress and

apoptosis in a human neural progenitor cell line (Chang et al., 2013), and oxidative stress via interference with the anti-oxidant pathway in a human neural progenitor cell line (Dou et al., 2015) have resulted from treatments with low doses of PQ.

Investigations employing low-dose PQ treatments have provided substantial information regarding PQ neurotoxicity. DA-producing neurons contain tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis. Such neurons are prevalent in the midbrain. Immunostaining for TH reveals the presence of dopaminergic neurons, and the number of tyrosine hydroxylase positive (TH+) neurons is an indicator of the health of the midbrain. The lack of TH+ in a DA producing neuron demonstrates a lack of cell function, i.e. it cannot produce dopamine.

With a dose of 5 mg/kg, (once weekly for 3 weeks), C57BL/6 mice had a loss of 36% of TH+ neurons and an 87% loss in density of striatal dopaminergic terminals (Brooks et al., 1999). Comparing C57BL/6 and DBA2/J mouse strains, Yin et al. (2011) found that 1 mg/kg (once weekly for 3 weeks) produced a statistically significant loss of TH+ neurons in C57BL/6 mice, while the TH+ neuron loss was not significant for the DBA2/J strain. The group also reported a statistically significant loss for both strains when given a dose of 5 mg/kg (once weekly for 3 weeks; Yin et al., 2011).

Lectins are proteins that bind carbohydrates and are present in raw vegetables and grains. Gastric dysmotility, an early PD symptom, occurred in rats treated by gavage with low doses of PQ combined with lectins (Anselmi et al., 2018). When lectins were combined with 1 mg/kg PQ doses in rats and administered by gavage (once daily for seven days), the rats showed features of parkinsonism: α-synucleinopathy, loss of DA neurons, and gastric motility impairment (Anselmi et al., 2018). Severing the vagus nerve prevented these effects, lending support to the gut-brain pathway of PD (Anselmi et al, 2018).

An *in vivo* microdialysis method was used to apply 40 μM PQ in the brains of young or aged rats, and researchers were able to show that the perfusion led to a rapid influx of extracellular $Zn^{2+}$ which induced TPRM2 cation channel activation and subsequent hastening of dopaminergic neuron degeneration (Tamano et al., 2019). Most recently, Cristovao et al. (2020) reported a loss of dopaminergic neurons in the SN, a decrease in DA levels, and a decrease in motor performance for rats exposed to low-dose PQ via osmotic minipumps. The dosing regimen was 2.5 mg/kg/day for four weeks.

Notably, there is a dose-dependent relationship between lifetime cumulative exposure and increased risk. In addition to the studies mentioned above, animal studies demonstrate this long-term risk exposure. PQ exposure resulted in age- and dose-dependent dopaminergic neuron loss in male C57BL/6 mice (McCormack et al. 2002). Studies reported in 2005 demonstrated significant dopaminergic neuron loss in male Wistar rats repeatedly exposed to PQ for 4, 8, or 24 weeks (Ossowska et al 2005). Kuter et al. (2010) used male Wistar rats to show that repeated dosing with PQ increased ROS in the brain, while a single dose did not have that affect. This repeated dosing mimics paraquat application in the field.

**Non-Human Primate Studies**

Non-human primates (NHP) have also been studied to determine PQ effects. Since NHP and humans share 98% of their DNA, NHP are generally considered the gold standard for modeling human disease. Squirrel monkeys were administered 2.5 mg/kg of PQ subcutaneously – this low dose was necessary to prevent lung toxicity – and there was a decrease in nicotinic acetylcholine receptor expression with a concomitant decline in dopaminergic neurotransmission, (O'Leary et al., 2008). Dr. Donato Di Monte, a co-author of the O'Leary publication, presented additional unpublished data regarding the NHP study to Syngenta. PQ administered at a dose of 5 mg/kg/week (for 3 weeks) subcutaneously resulted in death for more than 50% of the squirrel monkeys in the study, with lung fibrosis and decreases in striatal DA.[5] Such a dose is not lethal in mice, which indicates that primates are more sensitive to PQ. In subsequent experiments, a dose of 2.5 mg/kg/week (for 6 weeks) was administered.[6] The data were discussed at a Syngenta Human Safety Potentially Referable Findings meeting in April of 2009.[7] The verbal communication notes have a table on the second page which appears to have errors.[8]

[5] SYNG-PQ-02601797
[6] *Id.*
[7] SYNG-PQ-02601795, SYNG-PQ-01547528
[8] The column designated as "Total TH+ Counts" should be labeled "Total DA Neurons"; these numbers are similar to what was reported for total DA neurons in squirrel monkeys in the SN by McCormack, Di Monte, et al. (2004), which was approximately 60,000 DA neurons. The remaining two columns do not add up to the Total column, so a "TH+ only" column should be added to interpret the data. In the 2004 publication, McCormack, Di Monte and colleagues included three kinds of DAergic neurons: TH+ with neuromelanin, TH+ without neuromelanin (TH+ only), and neuromelanin only, so it makes sense to presume that the TH+ only column is missing above.

This apparent phenotypic change in neuronal staining characteristics is quantified in the table below. They are believed to be the mean of 4 or 5 individuals.

| **Treatment Group** | **Total $TH^+$ Counts** | **$TH^+$ & Neuromelanin Stain Counts** | **Neuromelanin Stain Only Counts** |
|---|---|---|---|
| Saline Control | 61,300 | 47,078 | 7,008 |
| PQ 2 weeks | 61,249 | 42,984 | 6,765 |
| PQ 4 weeks | 61,528 | 37,100 | 16,538 |

The first column should be headed, "Total DA Neuron Counts." The numbers for TH+ only cells can be calculated by subtracting the other two columns from the total. The corrected table follows:

| Treatment Group | Total DA Neuron Counts | TH+ Only Counts | TH+ & Neuromelanin Stain Counts | Neuromelanin Stain Only Counts |
|---|---|---|---|---|
| Saline Control | 61,300 | 7214 | 47,078 | 7,008 |
| PQ 2 weeks | 61,249 | 11,500 | 42,984 | 6,765 |
| PQ 4 weeks | 61,528 | 7890 | 37,100 | 16,538 |

DA cells which have lost TH function that is essential for DA synthesis would be positive for neuromelanin stain only. Overall there was a loss of about 10,000 doubly positive neurons which accounted for an increase of almost 10,000 neuromelanin only cells. In other words, about 16% of dopamine neurons (about 10,000/61,000) became non-functional 4 weeks after paraquat exposure.

Syngenta conducted a study of PQ levels in the frontal cortex of the squirrel monkeys from the above study and issued an internal report in 2011.[9] The monkeys had been sacrificed 2, 4, or 8 weeks after the last dose of PQ was given, and the levels of PQ in the brain remained constant up to 8 weeks beyond the last PQ dose. The study demonstrated PQ is persistent in the primate brain.

**Paraquat Specifically Targets the Same Midbrain Neurons Preferentially Affected in PD**

In 1973, Bernheimer et al. reported that SN damage and loss of melanin-containing cells are pathological features common in PD, and Braak et al. (2004) published similar findings. In human brains with Parkinson pathology, DAergic neurons of the midbrain sustain the most damage (Hirsch et al., 1988). Once PQ gets into the brain, it targets dopaminergic neurons in the SN, the

[9] SYNG-PQ-00044965.

neurons that are TH+. An experiment in which radiolabeled PQ was delivered to frogs systemically revealed that PQ does concentrate in neural cells containing neuromelanin (Lindquist et al., 1988). In mice, which have few to no pigmented brain cells, the radiolabeled PQ was evident throughout the brain (Lindquist et al., 1988). C57BL6 mice dosed with 5 or 10 mg/kg PQ (once weekly for three weeks) had significant losses of both TH+ neurons and dopaminergic terminal density (Brooks et al., 1999). Mice receiving one ip injection weekly of 10 mg/kg/ PQ for 3 weeks showed a loss of both TH+ and total neurons (McCormack et al., 2003). Mixed glial-neuron cultures showed a loss of 36% of TH+ neurons when treated with PQ (Wu et al., 2005). A few research groups have not been able to demonstrate a loss of dopaminergic neurons in mice, and they all used young mice aged 8-16 weeks, equivalent to late teens and 20s in human years (Barlow et al., 2004; Breckenridge et al., 2013; Minnema et al., 2014; Smeyne et al., 2016; Syngenta sponsored the Breckenridge, Minnema and Smeyne studies). The difference in these findings may be primarily because of the use of very young mice. The vast majority of the weight of the evidence shows that neuroscientists worldwide have found a loss of dopaminergic neurons in mice due to PQ treatment (McCormack et al., 2002; Manning-Bog et al., 2003; McCormack et al., 2003; Peng et al., 2004; McCormack et al., 2005; Peng et al., 2005; Choi et al., 2006; Fei et al., 2006; McCormack et al., 2006; Fernagut et al., 2007; Kwaja et al., 2007; Peng et al., 2007; Kang et al., 2009; Peng et al. 2010; Mangano et al,. 2011; Rappold et al., 2011; Yin et al., 2011; Zhou et al., 2011; Jiao et al., 2012). Thus, the weight of the evidence demonstrates that PQ induces a loss of dopaminergic neurons, which is a pathologic hallmark of PD.

Recently, I learned that Dr. Louise Marks conducted research into the PQ mouse model between 2003 and 2006 at Syngenta Central Toxicology Laboratory in Alderley Park, United Kingdom. Over four separate experiments, Dr. Marks administered 10 mg/kg/bw PQ via ip injection to the Charles River black mouse once or twice weekly for three weeks, and sacrificed them at 7, 28 or 90 days following the final dose[10]. Dr. Marks used stereology to examine the SNpc and initially found loss of dopaminergic cells that was not significant (SYNG-PQ-00492889). She upgraded her stereology equipment and software and conducted three more experiments, each of which found significant losses in dopaminergic neurons (18.1%-24.0%) (SYNG-PQ-00116782, SYNG-PQ-00490903, and SYNG-PQ-00492785). Dr. Marks concluded the "failure to detect a significant degree of cell loss in the first study is likely to be attributable to the differences in the stereology methodology, software and hardware used" (SYNG-PQ-00116782). Dr. Marks also conducted a study to determine whether the dopaminergic cell loss she had observed in prior studies could be

[10] SYNG-PQ-00492889, SYNG-PQ-00116782, SYNG-PQ-00490903, SYNG-PQ-00492785.

attributed to general toxicity rather than to PQ treatment (SYNG-PQ-00084920). Mice were given caffeine, N-ethylmaleimide, antimycin and paracetamol once a week for three weeks at the maximum tolerated doses and were terminated 7 days after the final dose. Dr. Marks reported that these compounds "failed to induce a statistically significant reduction in dopaminergic cell number" in the SNpc, suggesting that PQ neurotoxicity in the prior studies was not due to non-specific systemic toxicity but rather was due to a "selective mechanism of action specific to [paraquat]" that targeted dopaminergic neurons in the SN. *Id.*

**In Vitro Dopamine Transporter Investigations**

The *in vitro* evidence of PQ's route for entry into DAergic neurons is not entirely clear. *In vitro* experiments conducted by Barlow et al. (2003) demonstrated that PQ did not compete with [$^{3}$H]DA for passage through the DA transporter (DAT). Experiments with the SK-N-MC human neuroblastoma cell line expressing DAT indicated that DAT is not an entry point for PQ into neurons (Richardson et al., 2005). However application of DAT inhibitor GBR-12909 followed by PQ to rat brain slice cultures reduced DAergic neuron loss in the cultures compared to cultures treated with PQ alone (Shimizu et al., 2003b). Using a neuroblastoma cell system with controlled expression of DAT, Richardson et al. (2005) showed that PQ toxicity does not depend on DAT expression and that PQ did not inhibit DA uptake through DAT suggesting that PQ is not a DAT substrate. Working with the SH-SY5Y neural cell line, Yang and colleagues found that DAT inhibition decreases PQ neurotoxicity (2005). *In vitro* evidence for DAT as a portal for PQ entry is mixed, however there is at least one other way for PQ to access neurons. In the dopaminergic neuron cell line, SH-SY5Y, PQ can bind to the outer membrane and be endocytosed through a clathrin-dependent mechanism (Li et al., 2017).

**In Vivo DAT Investigations**

Barlow and colleagues injected mice intraperitoneally with [$^{14}$C]PQ and, since no difference was observed in the amount of [$^{14}$C]PQ in any region of the brain between 0.5 and 12 h after injection, concluded that the DAT was not utilized by [$^{14}$C]PQ to get into DAergic neurons (Barlow et al., 2003). Experiments with 8-12 month-old C57BL/6 mice performed by Richardson et al. (2005) suggested that PQ-induced damage to DAergic neurons is not facilitated by entry through the DAT. Delivery of PQ with GBR-12909 via microdialysis in freely-moving rats resulted in reduced uptake of the pesticide into the striatum and dopaminergic terminals (Shimizu et al., 2003a). One ip administration of PQ to rats decreased the level of [$^{3}$H]GBR-12935 (a DAT marker) in the

caudate putamen at 2 h and 24 h after dosing, but levels returned to the same as controls 7 d later, indicating at least a temporary interaction of PQ with DAT (Ossowska et al., 2005). In the same set of experiments, [$^3$H]GBR-12935 in the striatum of rats was decreased compared to controls at 24 weeks of dosing, indicating a slow progressive DAergic neurodegeneration (Ossowska et al., 2005). The conclusion reached by these researchers was that PQ does interact with DAT in the striatum. A thorough series of studies conducted by Rappold and colleagues (2011) provided evidence that $PQ^{2+}$ is reduced to $PQ^{+}$ outside of neurons by microglia. $PQ^{+}$ is a substrate for DAT and when $PQ^{+}$ enters DA neurons through DAT, it is then able to cause damage via redox cycling (Rappold et al., 2011). In addition, this team established that $PQ^{+}$ is a substrate for the organic cation transporter 3 (Oct3) suggesting the compound has at least two pathways into DA neurons (Rappold et al., 2011). DAT-knockdown (DAT-KD) mice showed resistance to PQ-maneb toxicity and the study's authors concluded that lower levels of DAT prevented reduced PQ from being taken up by DAergic neurons (Richter et al., 2017). Additionally, the higher concentration of DAT in the neurons of the SNpc (Rao 2007) suggests that $PQ^{+}$ can do more harm to that area of the brain.

Non-dopaminergic neurons are spared from PQ-related damage in mice. Silver staining to detect neuronal degeneration, glial activation markers, and selective staining of γ-amino-butyric acid (GABA)-ergic cells from the brains of mice treated systemically with PQ indicated that the neurotoxic effects of PQ are limited to dopaminergic neurons (McCormack et al., 2002) and hippocampal neurons are spared (McCormack et al., 2002, 2005; Czerniczyniec et al., 2012). PQ's preferential ability to cause a loss of DA neurons in the midbrain of mice is one reason why PQ is used to model PD.

**Populations of DAcxergic Neurons that are Unaffected by PQ**

All dopaminergic neurons are not vulnerable to PQ toxicity, however. Using human brain samples from PD patients along with healthy controls, Damier et al. (1999) illustrated that the PD-related loss of DAergic neurons consistently occurs in the SN, while four other areas of the midbrain have no pattern of DAergic neuron loss. McCormack et al. (2006) demonstrated that mouse DAergic neurons in A9 (SN neurons) are reduced in number due to PQ treatment, but nearby A10 neurons (mostly ventral tegmental neurons) are not damaged by PQ. Additionally, these researchers suggested that the amount of calcium binding protein (calbindin)-containing neurons in A10 is five-fold higher than in A9 and the calbindin positive neurons are resistant to PQ toxicity, suggesting a connection between the presence of calbindin and resistance to PQ-induced neurodegeneration (McCormack et al., 2006). Mitra et al. (2011) investigated the effect of PQ on

DAergic cells in the SN, frontal cortex, and hippocampus of mouse brain. The activity of ROS scavenger proteins increased in all three areas in a dose-dependent manner; inflammation occurred in all three areas; and the loss of TH+ neurons was greatest in SN and least in the hippocampus (Mitra et al., 2011). Furthermore, the authors noted that the presence of α-synuclein (as measured by immunoreactivity) increased in the hippocampus and frontal cortex but decreased in the SN (Mitra et al., 2009). PQ's unique ability to selectively target DA-producing neurons in the SNpc makes it an excellent model for reproducing the pathology of PD.

## DA Depletion in Rodent Striatum

Some studies with PQ find loss of dopamine in the mouse striatum with loss of dopaminergic neurons in the SNpc (Li X et al., 2005; Kang et al., 2009). Other studies do not. In rats dosed subcutaneously with PQ daily for 5 days (a subchronic dosing regimen), DA and its metabolites were reduced, but PQ microdialysis perfusion leads to DA overflow as the DAergic terminals are damaged (Shimizu et al., 2003). Ossowska and colleagues (2005) reported an initial 4-8 week increase in DA levels which was gradually decreased to 25-30% below control levels over the next 12-20 weeks in rats dosed with 10 mg/kg ip per week over 4-24 weeks. In mice given oral PQ for 4 months (10 mg/kg per day), DA levels in the striatum increased for a few weeks, but they decreased to half that in control mice (Ren et al., 2009). Mice treated with PQ during development and again as adults had lower levels of DA than controls, while mice treated only as adults showed no change in DA levels (Cory-Slechta et al., 2005). Two other groups reported continuous decreases in DA levels in mice treated with PQ (Li X et al., 2005; Kang et al., 2009). Several other groups have reported that DA levels do not change in mice treated with PQ (Perry et al., 1986; McCormack et al., 2002); and (Breckenridge et al., 2013; Minnema et al., 2014; Smeyne et al., 2016, who also did not find loss of dopaminergic neurons). To explain the preservation of DA levels despite DAergic neuron loss, it has been proposed that there are compensatory mechanisms. By measuring TH activity in mice treated with PQ, McCormack et al. (2002) demonstrated that, at all three ages assessed (6 wks, 5 mos, 18 mos), TH activity was increased suggesting a compensatory increase in DA production. Interestingly, positron emission tomography (PET) data in humans indicated that an increase in DA turnover occurs in the early stages of PD (Sossi et al., 2002).

## Paraquat Exposure also Results in Increased α-Synuclein Aggregation, an Additional Hallmark of the Human Disorder

The conformational change and subsequent aggregation of α-synuclein coincide with development of PD (Braak et al. 2004). Lewy bodies (LB) and Lewy neurites (LN) are intraneuronal inclusions of aggregated α-synuclein and LB & LN are well-known features of PD (Braak et al., 2004). Therefore, studies involving α-synuclein in relation to pesticide exposure are important. Manning-Bog et al. (2002) reported that *in situ* mixing of PQ with α-synuclein upregulates α-synuclein aggregation and C57BL/6 mice treated with PQ had increased amounts of α-synuclein along with higher α-synuclein aggregation than controls. When exposed to PQ, mice overexpressing human α-synuclein and their wild-type (WT) littermates showed similar amounts of SN neuron loss, but the α-synuclein overexpressing mice had increased amounts of α-synuclein aggregation compared to WT (Fernagut et al 2007). Peng et al. (2010) used mice carrying a well-known α-synuclein mutation, A53T, in experiments that demonstrated increased PD-like α-synuclein pathology compared to controls when the mice were exposed to PQ. Mitra et al. (2011) also found that PQ treatment in mice resulted in alpha-synuclein aggregation. Because paraquat exposure causes increased α-synuclein aggregation, an additional hallmark of PD, it is an excellent model to study potential drug therapies.

## Paraquat Toxicity Involves Neuroinflammatory Events

An additional aspect of PQ toxicity is neuroinflammation, which is also a key characteristic of PD in humans (Taetzsch and Block 2013). Microglia, the brain's immune cells, release proinflammatory cytokines and ROS which are toxic to neurons. The rat SN has a higher concentration of microglia than the cortex or hippocampus, and therefore the damage to SN neurons from neuroimflammation is greater there (Kim et al., 2000); the situation is similar for human PD (Taetzsch and Block 2013).

Monocultures of N9 microglia (derived from mice) were used in experiments showing that microglia promote PQ redox cycling via the nitric oxide synthase and NADPH oxidase enzymes (Bonneh-Barkey et al., 2005). Using neuron cultures *in vitro*, and low doses of PQ, Wu et al. (2005) demonstrated that a low PQ dose was not directly toxic to neurons, but when microglia are co-cultured with neurons, the microglia are activated and produce extracellular ROS through redox cycling mediated by NADPH oxidase. Further *in vitro* evidence was gleaned from experiments using primary microglia exposed to iron and PQ in which the production of superoxide radicals was blocked by EUK-189 (a SOD mimetic) (Peng et al., 2009). DA neurons

were more susceptible to death in the presence of microglia than in monocultures (Peng et al., 2009).

Findings of microglial activation contributing to neurotoxicity in *in vitro* studies are supported by *in vivo* studies. In mice, a single 10 mg/kg dose of PQ led to microglial activation which was inhibited if minocycline (an anti-inflammatory) was administered beforehand; while a single PQ dose given after lipopolysaccharide (a compound often used to activate microglia) caused microglial activation (Purisai et al., 2007). For groups of mice administered two PQ doses (10 mg/kg/wk), DA neuron loss was noted in the group which had not been treated with minocycline between the PQ injections, so minocycline prevented the inflammation that would otherwise lead to neurotoxicity (Purisai et al., 2007). This group also demonstrated a role for NADPH oxidase as mice receiving two injections were spared neuron loss if they lacked the functional form of the enzyme (Purisai et al., 2007). Inflammatory priming with lipopolysaccharide was also shown to exacerbate neurotoxicity of PQ when both were delivered to mice via infusion through a cannula (Mangano et al., 2009). Studies employing osmotic minipumps in mice confirmed the increased activation of microglia due to the presence of PQ (Peng et al., 2009). In Swiss albino mice receiving intraperitoneal injections of 10 mg/kg twice weekly for 4 weeks, microglia were activated to a greater extent in the SN compared to the frontal cortex or hippocampus (Mitra et al., 2011). Using a viral mimetic to induce microglial activation, Bobyn et al. (2012) reported TH+ neuron losses of 25-50% when the mimetic was administered before PQ, compared to 10-15% when either the mimetic or PQ were administered alone.

## Paraquat, α-Synuclein, and Other Parkinson's Disease-Related Proteins

In PQ-treated young mice, α-synuclein overexpression, whether human wild type or a human A53T (a familial PD mutation) was found to be neuroprotective, with the overexpressing mice displaying higher levels of the chaperone protein HSP70 colocalized with α-synuclein, suggesting a compensatory up-regulation of HSP70 in response to elevations in α-synuclein expression that protects against subsequent PQ exposure (Manning-Bog et al., 2003). A *Drosophila* model demonstrated that HSP70 indeed protects PQ-exposed neurons (Shukla et al., 2014). Long-term PQ exposure however results in increased α-synuclein aggregation. The roles of α-synuclein in a cell are varied and complex (Bernal-Conde et al., 2020). Recently, Mahul-Mellier et al. (2020) showed that, instead of α-synuclein fibrillization, it is the formation of LBs that ultimately causes neurodegeneration via disruptions in cellular processes including synaptic and mitochondrial function.

There are additional Parkinson-related proteins to consider in terms of PQ toxicity. Detrimental DJ-1 mutations underlie some familial forms of PD and it has also been associated with mitochondria (Bonifati et al., 2004) and several cellular processes including response to oxidative stress (Lev et al., 2006). Investigations carried out by Gollamudi et al. (2012) revealed that PQ exposure in mice affected pathways involving not only DJ-1, but also Parkin and PINK-1 mutations in which are also associated with familial PD. Parkin and PINK-1 are involved in the process of mitophagy (the turnover of defective mitochondria) and their impairment leads to dysfunction of mitochondria and mitochondrial cross-talk with other organelles (reviewed in Sironi et al., 2020). Using transgenic mice in which the PINK-1 gene is partially silenced in adult mice, researchers showed that early exposure to PQ resulted in greater loss of DA neurons suggesting that PQ sensitizes them to age-like reductions in gene expression (Zhou et al., 2011).

## Note on Aging and PD

As humans age, there are changes in DA metabolism, iron accumulation particularly in the SNpc, calcium processing changes, increased mitochondrial DNA mutations, accumulation of neuromelanin, and reductions in processes involved in protein degradation; all of these factors influence SN neuron death however most people do not develop PD (Reeve et al., 2014). Using adult NHP brains, Collier et al. (2017) observed that there was no significant loss of DA SN neurons during normal aging, but the numbers of TH+ neurons do decline suggesting reduced function. These researchers also observed an increase of cytoplasmic α-synuclein, proteasome impairment, decreased lysosome function, and an increase in microglial activity (Collier et al., 2017). Their model, based on this data and additional studies they reviewed, shows that aging and PD have some factors in common: impaired proteostasis, oxidative/nitrosative damage, mitochondrial dysfunction, reduced neurotrophic support, inflammation, reduced biochemical compensation, impaired DNA metabolism, and loss of TH phenotype. Thus, an environmental exposure to PQ can push an aging brain into developing PD.

## Exposure Studies

Starting in 1965 and continuing through 2007, exposure studies regarding paraquat application were done in different parts of the world.[11] In general, the studies evaluated systemic paraquat exposure through the use of urine collections of paraquat workers who were observed in their typical work environments. The studies also tested different types or worker protective equipment

[11] SYNG-PQ-00228608, SYNG-PQ-00693891, SYNG-PQ-01806986, SYNG-PQ-02086519, SYNG-PQ-00022018, SYNG-PQ-00125329, SYNG-PQ-00124055, SYNG-PQ-00125211, SYNG-PQ-00124616.

to determine potential effects on systemic contamination by paraquat. The study results present a consistent pattern over the entire study period. Workers were often directly exposed to paraquat in their "normal" work day and, despite the use by some workers of extensive protective equipment, paraquat was consistently found in a significant percentage of the workers.

## Epidemiology

Epidemiological studies show a greater risk of developing PD with exposure to paraquat. Paraquat pesticide applicators were 2.5 times more likely to develop PD than those who did not. (Tanner et al., 2011). Previously, Liou et al., 1999 also found a dose-response relationship between lifetime cumulative exposure to paraquat and developing PD.

## Conclusion

PQ is used as an experimental model for PD in laboratory animals because it causes selective damage to dopaminergic neurons, redox cycling, oxidative stress, increased α-synuclein aggregation, neuroinflammation, and mitochondrial dysfunction. This pathology is consistent with the etiology of PD. It is my opinion, to a reasonable degree of scientific certainty, that paraquat can cause Parkinson's' disease when it is used as intended.

Signed:___ _____ July 10, 2020

## References and Reliance


Abbott NJ, Patabendige AAK, Dolman DEM, Yusof SR, Begley DJ (2010). Structure and function of the blood-brain barrier. Neurobiology of Disease 37:13-25

Andersen JK and Di Monte DA. (2014) Paraquat and Parkinson's disease (PD). *Encyclopedia of the Neurological Sciences* Vol 3, 792-794

Anselmi L, Toti L, Bove C, Hampton J, Travagli RA (2017). A nigro-vagal pathway controls gastric motility and is affected in a rat model of Parkinsonism. *Gastroenterology* 153:1581-1593

Anselmi L, Bove C, Coleman FH, Le K, Subramanian MP, Venkiteswaran K, Subramanian T, Travagli RA (2018). Ingestion of subthreshold doses of environmental toxins induces ascending Parkinsonism in the rat. *Nature Partner Journals: Parkinson's Disease* 4:30

Baltazar MT, Dinis-Oliveira RJ, de Lourdes Bastos M, TsaTsakis AM, Duarte JA, Carvalho F (2014) Pesticides exposure as etiological factors in Parkinson's disease and other neurodegenerative diseases – a mechanistic approach. *Toxicology Letters* 230:85-103

Barlow BK, Cory-Slechta DA, Richfield EK, Thiruchelvam MJ (2007). The gestational environment and Parkinson's disease: Evidence for neurodevelopmental origins of a neurodegenerative disorder. *Reproductive Toxicology* 23:457-470

Barlow BK, Thiruchelvam MJ, Bennice L, Cory-Slechta DA, Ballatori N, Richfield EK (2003). Increased synaptosomal dopamine content and brain concentration of paraquat produced by selective dithiocarbamates. *Journal of Neurochemistry* 85:1075-1086

Bartlett RM, Holden JE, Nickles RJ, Dhanabalan M, Barbee DL, Barnhart TE, Christian BC, DeJesus, OT (2009). Paraquat is excluded by the blood brain barrier in rhesus macaque: an in vivo PET study. *Brain Res* 1259:74-7

Bastias-Candia S, Di Benedetto M, D'Addario C, Candeletti S, Romualdi P (2013). Combined exposure to agriculture pesticides, paraquat and maneb, induces alterations in the N/OFQ-NOPr and PDYN-KOPr systems in rats: relevance to sporadic Parkinson's disease. *Environmental Toxicology* 30(6):656-663

Bernal-Conde LD, Ramos-Acevedo R, Reyes-Hernandez RA, Balbuena-Olvera AJ, Morales-Moreno ID, Arguero-Sanchez R, Shule B, Guerra-Crespo M (2020). Alpha-synuclein physiology and pathology: a perspective on cellular structures and organelles. *Frontiers in Neuroscience* 13:1399

Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F, (1973). Brain dopamine and the syndromes of Parkinson and Huntington Clinical, morphological and neurochemical correlations. *Journal of the Neurological Sciences*. 20(4):415-455 [abstract]

Billingsley KJ, Bandres-Ciga S, Saez-Atienzar S, Singleton AB. (2018) Genetic risk factors in Parkinson's disease. *Cell and Tissue Research* 373(1):9-20

Bobyn J, Mangano EN, Ghandi A, Nelson E, Moloney K, Clarke M, Hayley S (2012). Viral-toxin interactions and Parkinson's disease: poly(I:C) priming enhanced the neurodegenerative effects of paraquat. *Journal of Neuroinflammation* 9:86

Bonifati V, Oostra BA, Heutink P (2004). Linking DJ-1 to neurodegeneration offers novel insights for understanding the pathogenesis of Parkinson's disease. *Journal of Molecular Medicine* 82:163-174

Bonneh-Barkay D, Reaney SH, Langston WJ, Di Monte DA (2005). Redox cycling of the herbicide paraquat in microglial cultures. *Molecular Brain Research* 134:52-56

Braak H, Rüb U, Gai WP, Del Tredici K (2003). Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *Journal of Neural Transmission* 110:517–536

Braak H, Ghebremedhin E, Rub U, Bratzke H, Del Tredici K. (2004) Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Research* 318:121-134

Breckenridge CB, Sturgess NC, Butt M, Wolf JC, Zadory D, Beck M, Mathews JM, Tisdel MO, Minnema D, Travis KZ, Cook AR, Botham PA, Smith LL (2013) Pharmacokinetic, neurochemical, stereological and neuropathological studies on the potential effects of paraquat in the substantia nigra pars compacta and striatum of male C57BL/6J mice. *Neurotoxicology* 37:1-14

Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HA (1999). Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. *Brain Research* 823:1-10

Brouwer, D.H. et al. Formulation R BIX (A9409AL): Measurement of excreted paraquat in workers following a single day's habitual use in Luzerne (TNO Study Code 6420-03) (June 2007) SYNG-PQ-00022018

Brouwer, D.H. et al. Measurement of excreted paraquat in workers following repeated day's habitual use in vines (TNO Study Plan 6420-02) (May 2007) SYNG-PQ-00125329

Brouwer, D.H. et al. Formulation R BIX (A9409AL): Measurement of excreted paraquat in workers following three consecutive days habitual use in bananas (TNO Study Plan P6420-5) (August 2007) SYNG-PQ-00124055

Brouwer, D.H. et al. Formulation R BIX (A9409AL): Measurement of excreted paraquat in workers following a single day's habitual use in vineyards (TNO Study Plan 6420-01) (May 2007) SYNG-PQ-00125211

Brouwer, D.H. et al. Formulation R BIX (A9409AL): Measurement of excreted paraquat in workers following three consecutive days use in bananas with PPE according to label recommendations (TNO Study Plan P6420-06) (September 2007) SYNG-PQ-00124616

Brown TP, Rumsby PC, Capleton AC, Rushton L, Levy LS (2006). Pesticides and Parkinson's disease – is there a link? *Environmental Health Perspectives* 114(2):156-164

Bus JS, Aust SD, Gibson JE (1976). Paraquat toxicity: Proposed mechanism of action involving lipid peroxidation. *Environmental Health Perspectives* 16:139-146

Castello PR, Drechsel DA, Patel M (2007) Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *J Biol Chem* 282(19):14186-14193

Chang X, Lu W, Dou T, Wang X, Lou D, Sun X, Zhou Z (2013). Paraquat inhibits cell viability via enhanced oxidative stress and apoptosis in human neural progenitor cells. *Chemico-Biological Interactions* 206:248-255

Chester, G. & Woollen, B.H. A study of the occupational exposure of Malaysian Plantation Workers to Paraquat ICI Report No. CTL/P/580 (12/19/80) SYNG-PQ-00693891

Choi HS, An JJ, Kim SY, Lee SH, Kim DW, Yoo KY, Won MH, Kang T-C, Kwon HJ, Kang JH, Cho S-W, Kwon O-S, Park J, Eum WS, Choi SY (2006). PEP-1-SOD fusion protein efficiently protects against paraquat-induced dopaminergic neuron damage in a Parkinson disease mouse model. *Free Radical Biology and Medicine*. 41(7):1058-68

Chui Y-C, Poon G, Law F (1988) Toxicokinetics and bioavailability of paraquat in rats following different routes of administration. *Toxicology and Industrial Health* 4(2):203-219

Collier TJ, Kanaan NM, Kordower JH (2017). Aging and Parkinson's disease: different sides of the same coin? *Movement Disorders* 32(7):983-990

Corasaniti MT, Defilippo R, Rodino P, Nappi G, Nistico G (1991). Evidence that paraquat is able to cross the blood-brain barrier to a different extent in rats of various age. *Functional Neurology* 6:385-391

Cory-Slechta DA, Thiruchelvam M, Barlow BK, Richfield EK (2005). Developmental pesticide models of the Parkinson's disease phenotype. *Environmental Health Perspectives* 113(9):1263-1270

Cristóvão AC, Campos FL, Je G, Esteves M, Guhathakurta S, Yang L, Beal MF, Fonseca BM, Salgado AJ, Queiroz J, Sousa N, Bernardino L, Alves G, Yoon K-S, Kim Y-S (2020). Characterization of a Parkinson's disease rat model using an upgraded paraquat exposure paradigm. *European Journal of Neuroscience* 00:1-14

Czernizyniec A, Lores-Arnaiz S, Bustamante J (2012). Mitochondrial susceptibility in a model of paraquat neurotoxicity. *Free Radical Research* 47(8):614-623

Damier P, Hirsch EC, Agid Y, Graybiel AM (1999). The substantia nigra of the human brain II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. *Brain* 122:1437-1448

Desplats P, Patel P, Kosberg K, Mante M, Patrick C, Rockenstein E, Fujita M, Hashimoto M, Masliah E (2012). Combined exposure to maneb and paraquat alters transcriptional regulations of neurogenesis-related genes in mice models of Parkinson's disease. *Molecular Neurodegeneration* 7:49

Dias V, Junn E, Mouradian MM (2013). The role of oxidative stress in Parkinson's disease. *Journal of Parkinson's Disease* 3:461-491

Dinis-Oliveira RJ, Remião F, Carmo H, Duarte JA, Sánchez Navarro A, Bastos ML, Carvalho F (2006). Paraquat exposure as an etiological factor in Parkinson's disease. *Neurotoxicology* 27:1110-1122

Dou T, Yan M, Wang X, Lu W, Zhao L, Lou D, Wu C, Chang X, Zhou Z (2016). Nrf2/ARE pathway involved in oxidative stress induced by paraquat in human neural progenitor cells. *Oxidative Medicine and Cellular Longevity* 2016:1-8

Fei Q, McCormack AL, Di Monte DA, Ethell DW. (2008) Paraquat neurotoxicity is mediated by a Bak-dependent mechanism. *Journal of Biological Chemistry*. 283(6):3357-64.

Fernagut PO, Hutson CB, Fleming SM, Tetreaut NA, Salcedo J, Masliah E, Chesselet MF (2007). Behavioral and histopathological consequences of paraquat intoxication in mice: effects of α-synuclein overexpression. *Synapse* 61(12):991-1001

Findlay, M.L. et al. Paraquat: Worker exposure during mixing, loading and application of Gramoxone with knapsack sprayers Zeneca Report No. WER004 (4/9/98) SYNG-PQ-02086519

Goldman SM, Kamel F, Ross GW, Bhudhikanok GS, Hoppin JA, Korell M, Marras C, Meng C, Umbach D, Kasten M, Chade AR, Comyns K, Richards MB, Sandler DP, Blair A, Langston JW, Tanner CM (2012). Genetic modification of the association of paraquat and Parkinson's disease. *Mov Disord* 27(13):1652-1658

Gollamudi S, Johri A, Calingasan NY, Yang L, Elemento O, Beal MF (2012). Concordant signaling pathways produced by pesticide exposure in mice correspond to pathways identified in human PD. *PLoS ONE* 7(5):e36191

Gonzalez-Polo RA, Rodriguez-Martin A, Moran JM, Niso M, Soler G, Fuentes JM (2004). Paraquat-induced apoptotic cell death in cerebellar granule cells. *Brain Research* 1011:170-176

Hare DJ, Double KL (2016). Iron and dopamine: a toxic couple. *Brain* 139:1026-1035

Henchcliffe C, Beal MF (2008). Mitochondrial biology and oxidative stress in Parkinson disease pathogenesis. *Nature Clinical Practice Neurology* 4(11):600-609

Hirsch E, Graybiel AM, Agid YA (1988). Melanized dopaminergic neurons are differently susceptible to degeneration in Parkinson's disease. *Nature* 334:345-348

Homer RF, Mees GC, Tomlinson TE (1960). Mode of action of dipyridyl quaternary salts as herbicides. *J Sci Food Agric* 11:309-315

Jiang T, Sun Q, Chen S (2016). Oxidative stress: a major pathogenesis and potential therapeutic target of antioxidant agents in Parkinson's disease and Alzheimer's disease. *Progress in Neurobiology* 147:1-19

Jiao Y, Lu L, Williams RW, Smeyne RJ. (2012) Genetic dissection of strain dependent paraquat-induced neurodegeneration in the substantia nigra pars compacta. *PLoS One* 7(1):e29447.

Kang MJ, Gil SJ, Koh HC (2009) Paraquat induces alternation of the dopamine catabolic pathways and glutathione levels in the substantia nigra of mice. *Toxicology Letters* 188:148-152

Kim W-G, Mohney RP, Wilson B, Jeohn G-H, Liu B, Hong JS (2000). Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. *Journal of Neuroscience* 20(16):6309-6316

Kim S, Kwon S-H, Kam T-I, Panicker H, Karuppagounder SS, Lee S, Lee JH, Kim WR, Kook M, Foss CA, Shen C, Lee H, Kulkarni S, Pasricha PJ, Lee G, Pomper MG, Dawson VL, Dawson TM, Ko HS (2019). Transneuronal propagation of pathologic α-synuclein from the gut to the brain models Parkinson's disease. *Neuron* 103:627-641

Kuter K, Nowak P, Golembiowska K, Ossowska K (2010). Increased reactive oxygen species production in the brain after repeated low-dose pesticide paraquat exposure in rats: a comparison with peripheral tissues. *Neurochemical Research* 35:1121-1130

Khwaja M, McCormack A, McIntosh JM, Di Monte DA, Quik M. (2007) Nicotine partially protects against paraquat-induced nigrostriatal damage in mice; link to alpha6beta2* nAChRs. *J Neurochem* 100(1):180-90

Lee P-C, Bordelon Y, Bronstein J, Ritz B (2012). Traumatic brain injury, paraquat exposure, and their relationship to Parkinson's disease. *Neurology* 79(20):2061-2066

Lei S, Zavala-Flores L, Garcia-Garcia A, Nandakumar R, Huang Y, Madayiputiya N, Stanton RC, Dodds ED, Powers R, Franco R (2014). Alterations in energy/redox metabolism induced by mitochondrial and environmental toxins: a specific role for glucose-6-phosphate-dehydrogenase and the pentose phosphate pathway in paraquat toxicity. *ACS Chemical Biology* 9:2032-2048

Lev N, Ronsevich D, Ickowicz D, Melamed E, Offen D (2006). Role of DJ-1 in Parkinson's disease. *Journal of Molecular Neuroscience* 29:215-225

Li F, Tian X, Zhan X, Wang B, Ding M, Pang H (2017). Clathrin-dependent uptake of paraquat into SH-SY5Y cells and its internalization into different subcellular compartments. *Neurotoxicity Research* 32(2):204-217

Li X, Yin J, Cheng C-M, Sun J-L, Li Z, Wu Y-L (2005). Paraquat induces selective dopaminergic nigrostriatal degeneration in aging C57BL/6 mice. *Chinese Medical Journal* 118(16):1357-1361

Lindquist NG, Larsson BS, Lyden-Sokolowski A (1988). Autoradiography of [$^{14}$C]paraquat or [$^{14}$C]diquat in frogs and mice: accumulation in neuromelanin. *Neurosci Lett* 93:1-6

Litchfield MH, Daniel JW, Longshaw S (1973). The tissue distribution of the bipyridylium herbicides diquat and paraquat in rats and mice. *Toxicology* 1:155-165

Mahul-Mellier A-L, Burtscher J, Maharjan N, Weerens L, Croisier M, Kuttler F, LeLeu M, Knott GW, Lashuel HA (2020). The process of Lewy body formation, rather than simply α-synuclein fibrillization, is one of the major drivers of neurodegeneration. *PNAS* 117(9):4971-4982

Mangano EN, Hayley S (2009). Inflammatory priming of the substantia nigra influences the impact of later paraquat exposure: neuroimmune sensitization of neurodegeneration. *Neurobiology of Aging* 30:1361-1378

Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA (2002) The herbicide paraquat causes up-regulation and aggregation of α-synuclein in mice. *Journal of Biological Chemistry* 277(3):1641-1644

Manning-Bog AB, McCormack AL, Purisai MG, Bolin LM, Di Monte DA (2003). α-Synuclein overexpression protects against paraquat induced neurodegeneration. *The Journal of Neuroscience* 23(8):3095-3099

Marks, L (2007). Investigating reported paraquat-induced neurotoxicity in the Alderley Park C57 black mouse: the neurochemical and pathological effects on the dopaminergic system of 3 weekly injections of 10 mg/kg 1,1'-dimethyl-4,4'bipyridinium (paraquat), XM7229, SYNG-PQ-00492889

Marks, L (2007). Investigating reported paraquat neurotoxicity in the Charles River C57 black mouse: the neurochemical and pathological on the dopaminergic system of 3 weekly injections of 1,1'-dimethyl-4,4'-bipyridinium (paraquat), XM7258/research/report, SYNG-PQ-00116782

Marks, L (2007). Investigating reported paraquat-induced dopaminergic neurotoxicity in the Charles River C57 black mouse: the neurochemical, neuropathological and neurobahvioural effects of increasing the dosing frequency of 1,1'-dimethyl-4,4'bipyridinium (paraquat), XM7371/research/report, SYNG-PQ-00490903

Marks, L (2007). Investigating the time course and reversibility of dopaminergic cell loss in the Charles River C57 mouse following administration of 1,1'-dimethyl-4,4'bipyridinium (paraquat), XM7480, SYNG-PQ-00492785

Marks, L (2007). Investigation of nigrostriatal effects following repeat dose administration of antimycin A, caffeine, N-ethylmaleimide or paracetamol in male Charles River C57 black mice, XM7570, SYNG-PQ-00084920.

McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, Di Monte DA (2002). Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiology of Disease* 10:119-127

McCormack AL, Di Monte DA (2003). Effects of L-dopa and other amino acids against paraquat-induced nigrostriatal degeneration. *Journal of Neurochemistry* 85:82-86

McCormack AL, Di Monte DA, Delfani K, Irwin I, DeLanney LE, Langston WJ, Janson AM (2004). Aging of the nigrostriatal system in the squirrel monkey. *Journal of Comparative Neurology* 471:387-395 [SYNG-PQ-00669432]

McCormack AL, Atienza JG, Johnston LC, Andersen JK, Vu S, Di Monte DA (2005). Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. *Journal of Neurochemistry* 93:1030-1037

McCormack AL, Atienza JG, Langston JW, Di Monte DA (2006). Decreased susceptibility to oxidative stress underlies the resistance of specific dopaminergic cell populations to paraquat-induced degeneration. *Neuroscience* 141:929-937

Meier, D.J. Paraquat: Worker exposure during mixing, loading, and application of Gramoxone Extra to pecans using vehicle-mounted, ground boom equipment
Report Number RR95-019B (4/28/95) SYNG-PQ-01806986

Minnema DJ, Travis KZ, Breckenridge CB, Sturgis NC, Butt M, Wolf JC, Zadory D, Beck MJ, Mathews JM, Tisdel MO, Cook AR, Botham PA, Smith LL (2014). Dietary administration of paraquat for 13 weeks does not result in a loss of dopaminergic neurons in the substantia nigra of C57BL/6J mice. *Regulatory Toxicology and Pharmacology* 68:250-258

Mitra S, Chakrabarti N, Bhattacharyya A (2011). Differential regional expression patterns of α-synuclein, TNFα, and IL-1β; and variable status of dopaminergic neurotoxicity in mouse brain after paraquat treatment. *Journal of Neuroinflammation* 8:163

Musgrove RE, Helwig M, Bae E-J, Aboutalebi H, Lee S-J, Ulusoy A, Di Monte DA (2019) Oxidative stress in vagal neurons promotes parkinsonian pathology and intercellular α-synuclein transfer. *The Journal of Clinical Investigation* 129(9):3738-3753

Naylor JL, Widdowson PS, Simpson MG, Farnworth M, Ellis MK, Lock EA (1995) Further evidence that the blood/brain barrier impedes paraquat entry into the brain. *Human and Experimental Toxicology* 14:587-594

O'Leary KT, Parameswaran N, Johnston LC, McIntosh JM, Di Monte DA, Quik M (2008). Paraquat exposure reduces nicotinic receptor-invoked dopamine release in monkey striatum. *Journal of Pharmacology and Experimental Therapeutics* 327(1):124-129

Ossowska K, Wardas J, Smialowska M, Kuter K, Lenda T, Wieronska JM, Zieba B, Nowak P, Dabrowska J, Bortel A, Kwiecinski A, Wolfarth S (2005). A slowly developing dysfunction of dopaminergic nigrostriatal neurons induced by long-term paraquat administration in rats: an animal model of preclinical stages of Parkinson's disease? *European Journal of Neuroscience* 22:1294-1304

Paolillo N, Piccirilli S, Giardina E, Rispoli V, Colica C, Nistico S (2011). Effects of paraquat and capsaicin on the expression of genes related to inflammatory, immune responses and cell death in immortalized human HaCat keratinocytes. *International Journal of Immunpathology and Pharmacology* 24(4):861-868

Paraquat Information Center: www.paraquat.com/en/use (Accessed 3/9/2020, © Paraquat Information Center on behalf of Syngenta Crop Protection AG)

Parkinson's Foundation web site www.parkinson.org/understanding-parkinsons/causes/genetics (Accessed 3/2/2020)

Peng J, Andersen JK (2003). The role of c-Jun N-terminal kinase (JNK) in Parkinson's disease. *IUBMB Life* 55(4-5):267-271

Peng J, Mao XO, Stevenson FF, Hsu M, Andersen JK (2004). The herbicide paraquat induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. *Journal of Biological Chemistry* 279(31):32626-32632

Peng J, Oo ML, Andersen JK. (2010) Synergistic effects of environmental risk factors and gene mutations in Parkinson's disease accelerate age-related neurodegeneration. *Journal of Neurochemistry* 115(6):1363-1373

Peng J, Peng L, Stevenson FF, Doctrow SR, Andersen JK (2007). Iron and paraquat as synergistic environmental factors in sporadic Parkinson's disease accelerate age-related neurodegeneration. *Journal of Neuroscience* 27(26):6914-6922

Peng J, Stevenson FF, Doctrow SR, Andersen JK (2005). Superoxide dismutase/catalase mimetics are neuroprotective against selective paraquat-mediated dopaminergic neuron death in the substantia nigra. *Journal of Biological Chemistry* 280(32):29194-29198

Peng J, Stevenson FF, Oo ML, Andersen JK (2009). Iron-enhanced paraquat-mediated dopaminergic cell death due to increased oxidative stress as a consequence of microglial activation. *Free Radical Biology & Medicine* 46(2):312-320

Perry TL, Yong VW, Wall RA, Jones K (1986). Paraquat and two endogenous analogues of the neurotoxic substance N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine do not damage dopaminergic nigrostriatal neurons in the mouse. *Neuroscience Letters* 69:285–289

Pezzoli G and Cereda E (2013). Exposure to pesticides or solvents and risk of Parkinson's disease. *Neurology* 2013 80:2035-2041

Prasad K, Winnik B, Thiruchelvam MJ, Buckley B, Mirochnitchenko O, Richfield EK (2007). Prolonged toxicokinetics and toxicodynamics of paraquat in mouse brain. *Environmental Health Perspectives* 115:1448-1553

Prasad K, Tarasewicz E, Mathew J, Ohman Strickland PA, Buckley B, Richardson JR, Richfield EK (2009). Toxicokinetics and toxicodynamics of paraquat accumulation in mouse brain. *Experimental Neurology* 215:358-367

Purisai MG, McCormack AL, Cumine S, Li J, Isla MZ, Di Monte DA (2007). Microglial activation as a priming event leading to paraquat-induced dopaminergic dell degeneration. *Neurobiology Disease* 25(2):392-400

Ramachandiran S, Hansen JM, Jones DP, Richardson JR, Miller GW (2007). Divergent mechanisms of paraquat, MPP+, and rotenone toxicity: oxidation of thioredoxin and caspase-3 activation. *Toxicol Sci* 95(1):163-171

Rao J (2007). Neurochemistry of Parkinson's disease. *Handbook of Clinical Neurology* Vol. 3, 153-204

Rappold PM, Cui M, Chesser AS, Tibbett J, Grima JC, Duan L, Sen N, Javitch JA, Tieu K (2011). Paraquat neurotoxicity is mediated by the dopamine transporter and organic cation transporter-3. *PNAS* 108(51): 20766–20771

Ray, WJ (2011). Analysis of brain samples from paraquat-exposed squirrel monkeys for residues of paraquat, SYNG-PQ-00044965.

Reeve A, Simcox E, Turnbull E (2014). Ageing and Parkinson's disease: Why is advancing age the biggest risk factor? *Ageing Research Reviews* 14:19-30

Ren J-P, Zhao Y-W, Sun X-J (2009). Toxic influence of chronic oral administration of paraquat on nigrostriatal dopaminergic neurons in C57BL/6 mice. *Chinese Medical Journal* 122(19):2366-2371

Richardson JR, Quan Y, Sherer TB, Greenamyre JT, Miller GW (2005). Paraquat Neurotoxicity is Distinct from that of MPTP and Rotenone. *Toxicological Sciences* 88(1):193-201

Richter F, Gabby L, McDowell KA, Mulligan CK, De La Rosa K, Sioshansi PC, Mortazavi F, Cely I, Ackerson LC, Tsan L, Murphy NP, Maidment NT, Chesselet M-F (2017). Effects of decreased dopamine transporter levels on nigrostriatal neurons and paraquat/maneb toxicity in mice. *Neurobiology of Aging* 51:54-66

Rose MS, Lock EA, Smith LL, Wyatt I. (1976) Paraquat accumulation: tissue and species specificity. *Biochem Pharmacol* 25:419-423

Shimizu K, Ohtaki K, Matsubara K, Aoyama K, Uezono T, Saito O, Suno M, Ogawa K, Hayase N, Kimura K, Shiono H (2001) Carrier-mediated processes in blood-brain barrier penetration and neural uptake of paraquat. *Brain Research* 906:135-142

Shimizu K, Matsubara K, Ohtaki K, Fujimaru S, Saito O, Shiono H (2003) Paraquat induces long-lasting dopamine overflow through the excitotoxic pathway in the striatum of freely moving rats. *Brain Research* 976:243–252

Shimizu K, Matsubara K, Ohtaki K, Shiono H (2003). Paraquat leads to dopaminergic neural vulnerability in organotypic micbrain cultures. *Neuroscience Research* 46:523-532

Shukla AK, Pragya P, Chaouhan HS, Tiwari AK, Patel DK, Abdin MZ, Chowdhuri DK (2014). Heat shock protein-70 (Hsp-70) suppresses paraquat-induced neurodegeneration by inhibiting JNK and caspase-3 activation in *Drosophila* model of Parkinson's disease. *PLoS ONE* 9(6):e98886

Sironi L, Restelli LM, Tolnay M, Neutzner A, Frank S (2020). Dysregulated interorganellar crosstalk of mitochondria in the pathogenesis of Parkinson's disease. *Cells* 9:233

Smeyne RJ, Breckenridge CB, Beck M, Jiao Y, Butt MT, Wolf JC, Zadory D, Minnema DJ, Sturgess NC, Travis KZ, Cook AR, Smith LL, Botham PA (2016). Assessment of the effects of MPTP and paraquat on dopaminergic neurons and microglia in the substantia nigra pars compacta of C57BL/6 mice. *PLoS ONE* 11(10):e0164094

Somayajulu-Nitu M, Sandhu JK, Cohen J, Sikorska M, Sridhar TS, Matei A, Borowy-Borowski H, Pandey S (2009). Paraquat induces oxidative stress, neuronal loss in substantia nigra region and parkinsonism in adult rats: neuroprotection and amelioration of symptoms by water-soluble formulation of Coenzyme Q10. *BMC Neuroscience* 10:88

Sossi V, de la Fuente-Fernández R, Holden JE, Doudet DJ, McKenzie J, Stoessl AJ, Ruth TJ (2002). Increase in dopamine turnover occurs early in Parkinson's disease: evidence from a new modeling approach to PET $^{18}$F-fluorodopa data. *Journal of Cerebral Blood Flow & Metabolism* 22:232-239

Sturgess N, Travis K (2009). Preliminary verbal findings from experimental studies with paraquat and MPTP in non-human primates (Squirrel monkeys) April 10, 2009, SYNG-PQ-02601797

Stykel MG, Humphries K, Kirby MP, Czaniecki C, Wang T, Ryan T, Bamm V, Ryan SD (2018). Nitration of microtubules blocks axonal mitochondrial transport in a human pluripotent stem cell model of Parkinson's disease. *The FASEB Journal* 32:1-15

Subramaniam Sr, Chesselet M-F (2013). Mitochondrial dysfunction and oxidative stress in Parkinson's disease. *Progress in Neurobiology* 0:17-32

Swan A.A.B. Exposure of spray operators to paraquat, Brit. J. Indust. Med. 26, 322-329, 1969 SYNG-PQ-00228608

Taetzsch T, Block ML (2013). Pesticides, microglial NOX2, and Parkinson's disease. *Journal of Biochemical and Molecular Toxicology* 27(2):137-149

Tamano H, Nishio R, Morioka H, Furuhata R, Komata Y, Takeda A. (2019). Paraquat as an environmental risk factor in Parkinson's disease accelerates age-related degeneration via rapid influx of extracellular Zn2+ into nigral dopaminergic neurons. *Mol Neurobiol* 56:7789-7799

Tanner CM, Ottman R, Goldman SM, Ellenberg J, Chan P, Mayeux R, Langston JW. (1999) Parkinson disease in twins: an etiologic study. *Journal of the American Medical Association* 128(4):341-346

Trist BG, Hare BJ, Double KL (2019). Oxidative stress in the aging substantia nigra and the etiology of Parkinson's disease. *Aging Cell* 18:e13031

Uversky VN, Li J, Fink AL. (2001) Pesticides directly accelerate the rate of α-synuclein fibril formation: a possible factor in Parkinson's disease. *FEBS Letters* 500:105-108

Wang A, Costello S, Cockburn M, Zhang X, Bronstein J, Ritz B (2011). Parkinson's disease risk from ambient exposure to pesticides. *European Journal of Epidemiology* 26:547-555

Widdowson PS, Farnworth MJ, Simpson MG, Lock EA (1996a). Influence of age on the passage of paraquat through the blood-brain barrier in rats: a distribution and pathological examination. *Human and Experimental Toxicology* 15:231-236

Widdowson PS, Farnworth MJ, Upton R, Simpson MG (1996b) No changes in behavior, nigro-striatal system neurochemistry or neuronal cell death following toxic multiple oral paraquat administration to rats. *Human and Experimental Toxicology* 15:583-591

Wu X-F, Block ML, Zhang W, Qin L, Wilson B, Zhang W-Q, Veronesi B, Hong J-S (2005). The role of microglia in paraquat-induced dopaminergic toxicity. *Antioxidants & Redox Signaling* 7(5,6):654-661

Yang W, Tiffany-Castiglioni E (2005). The bipyridyl herbicide paraquat produces oxidative stress-mediated toxicity in human neuroblastoma SH-SY5Y cells: relevance to the dopaminergic pathogenesis. *Journal of Toxicology and Environmental Health A* 68(22):1939-1961

Yin L, Lu L, Prasad K, Richfield EK, Unger EL, Xu J, Jones BL (2011). Genetic-based differential susceptibility to paraquat neurotoxicity in mice. *Neurotoxicology and Teratology* 33:415-421

Zhou H, Huang C, Tong J, Xia X-G (2011). Early exposure to paraquat sensitizes dopaminergic neurons to subsequent silencing of PINK1 gene expression in mice. *International Journal of Biological Sciences* 7(8):1180-1187