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Drosophila PINK1 and *parkin* loss-of-function mutants display a range of non-motor Parkinson's disease phenotypes

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ABSTRACT

Parkinson's disease (PD) is more commonly associated with its motor symptoms and the related degeneration of dopamine (DA) neurons. However, it is becoming increasingly clear that PD patients also display a wide range of non-motor symptoms, including memory deficits and disruptions of their sleep-wake cycles. These have a large impact on their quality of life, and often precede the onset of motor symptoms, but their etiology is poorly understood. The fruit fly *Drosophila* has already been successfully used to model PD, and has been used extensively to study relevant non-motor behaviours in other contexts, but little attention has yet been paid to modelling non-motor symptoms of PD in this genetically tractable organism. We examined memory performance and circadian rhythms in flies with loss-of-function mutations in two PD genes: *PINK1* and *parkin*. We found learning and memory abnormalities in both mutant genotypes, as well as a weakening of circadian rhythms that is underpinned by electrophysiological changes in clock neurons. Our study paves the way for further work that may help us understand the mechanisms underlying these neglected aspects of PD, thus identifying new targets for treatments to address these non-motor problems specifically and perhaps even to halt disease progression in its prodromal phase.

1. Introduction

Parkinson's disease (PD) is more commonly associated with its debilitating motor symptoms, which include tremor, rigidity and slowness of movement. These symptoms have been linked with the degeneration of dopamine (DA) neurons, and thus treatments for the disease have primarily been developed to treat symptoms by compensating for depleted levels of DA in the brain. However, it is becoming increasingly clear that PD patients also display a wide range of non-motor symptoms that most treatments are not specifically designed to address and may even make worse (Chaudhuri et al., 2006a,b; Langston, 2006). These include problems related to cognition and disruption of the sleep-wake cycle. Cognitive impairments include memory problems and abnormalities related to reinforcement learning, in which DA is known to play an important role (Barone et al., 2011; Frank et al., 2004). Sleep impairments are particularly common, affecting up to two-thirds of PD patients, and include disorders such as insomnia, excessive daytime sleepiness and REM sleep behaviour disorder (RBD) (Barone et al., 2009; Mattis and Sehgal, 2016; Menza et al., 2010).

These aspects of the disease have typically attracted less attention than the hallmark motor symptoms, but there is growing interest in understanding how they arise, as they have a large impact on the quality of life of both patients and their carers, and their appearance can often precede the onset of motor symptoms (Barone et al., 2009). RBD in particular is thought to be a strong predictor of PD and dementia (Iranzo et al., 2013; Schenck et al., 2013). However, the etiology of non-motor symptoms is still poorly understood. It is not yet clear the extent to which they too result directly from the degeneration of DA neurons, as opposed to the dysfunction of other cell types. Matters are further complicated by possible adverse effects of medication and of the different symptoms on one another. For instance, symptoms of depression, which are commonly found in patients, may in turn cause sleep problems themselves, as can taking L-dopa

Abbreviations: DD, Constant darkness; D/NI, Diurnal/nocturnal index; DA, Dopamine; DAM2, *Drosophila* Activity Monitor; l-LNv, Large ventral lateral neurons; LD, 12:12 h light-dark cycle; R_{in}, Membrane input resistance; MCH, 4-methylcyclohexanol; PD, Parkinson's disease; PI, Performance Index; *PINK1*, PTEN-induced putative kinase 1; OCT, 3-octanol; RBD , REM sleep behaviour disorder; RMP, Resting Membrane Potential; RS, Rhythmicity statistic; SFR, Spontaneous firing rate; ZT, Zeitgeber time.

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medication at bed time. The benefits of using a simple, genetically tractable model organism in a controlled environment are clear in the face of such complications.

Although most cases of PD have no identifiable cause, some genetic mutations have been linked to familial cases of the disease, of which many affect genes that have homologs in the fly (Lu and Vogel, 2009). Here, we focus particularly on two genetic fly models of PD, with mutations in genes that are thought to act together in a mitochondrial quality control pathway: *PTEN-induced putative kinase 1* (*PINK1*) and the E3 ubiquitin ligase *parkin*. It is thought that *PINK1* accumulates on the outer membrane of damaged mitochondria, where it activates *parkin*, leading to the ubiquitination of *parkin* targets on the outer mitochondrial membrane. This ultimately results in the recruitment of autophagic machinery to degrade the defective mitochondria (von Stockum et al., 2016). It is thought that mitochondrial quality control may be particularly important in DA neurons, which are susceptible to oxidative stress (Subramaniam and Chesselet, 2013).

Loss-of-function mutations in *PINK1* and *parkin* in humans cause early-onset forms of PD (Kitada et al., 1998; Valente et al., 2004). Few studies have yet explored the impact of specific mutations on non-motor symptoms, but evidence suggests that patients with homozygous *parkin* mutations exhibit the usual range of PD sleep disorders (Limousin et al., 2009). *Drosophila PINK1* and *parkin* loss-of-function mutants exhibit a set of relevant phenotypes such as impaired locomotor activity, reduced longevity, mitochondrial abnormalities, and DA neuron degeneration (Greene et al., 2003; Park et al., 2006; Pesah et al., 2004; Whitworth et al., 2005; Yang et al., 2006). Neurophysiological studies are still in their infancy, but have detected abnormalities in synaptic signalling in larvae (West et al., 2015). Interestingly, rodent loss-of-function models have largely failed to replicate the hallmark symptoms of PD (Dawson et al., 2010).

Drosophila display many behaviours that are pertinent to modelling human disease, which are underlied by simple, tractable neural circuits. Learning and memory has been extensively studied using an olfactory associative learning assay, and DA has been shown to play a crucial role, as it does in mammals (Malik and Hodge, 2014; Tully and Quinn, 1985; Waddell, 2010). The fly has also been central to ongoing chronobiology research. Wild type flies are diurnal and show robust circadian rhythms in their activity in the absence of external time cues, their locomotor activity thus providing a convenient output of their internal clock (Rosato and Kyriacou, 2006). These behavioural fluctuations appear to be partly underpinned by fluctuations in the electrophysiological properties of pacemaker neurons expressing the neuropeptide pigment dispersing factor (PDF), including the large ventral lateral neurons (l-LNvs) (Peschel and Helfrich-Förster, 2011). These thus represent defined neurons in the clock neural circuit that can be recorded from (Buhl et al. 2016; Chen et al. 2015).

Despite these conserved behaviours, little attention has been paid to modelling non-motor symptoms of PD in *Drosophila*, except for two studies using flies expressing mutated form of the human PD-related gene *α-synuclein* throughout their brains. These flies displayed short-term memory deficits after sleep deprivation, as well as abnormal sleep and circadian rhythms (Gajula Balija et al., 2011; Seugnet et al., 2009).

The relative simplicity of the fly brain and its genetic tractability, along with the existence of a number of quantitative assays to study fly behaviour, means there is great untapped potential for studying non-motor symptoms of PD in this model organism. We examined learning and memory performance and circadian rhythms in *parkin*-null and *PINK1*-null flies, seeking to determine if these could model some non-motor aspects of PD as well as the previously-documented motor defects and neurodegeneration. We also performed electrophysiological recordings of l-LNv clock neurons in control and mutant genotypes revealing novel mechanisms of action of these disease-causing genes.

2. Methods

2.1. Fly stocks

Drosophila were raised on cornmeal, molasses and agar medium under standard conditions. The wild type strain used was *CSw* $^{-}$, obtained from Dr Scott Waddell (University of Oxford). $park^{25}$ and $PINK1^{B9}$ null mutants, $PINK1^{RV}$ revertant allele controls and *UAS-PINK1-RNAi* flies were all obtained from Dr. Alex Whitworth (University of Cambridge) (Greene et al., 2003; Park et al., 2006; Yang et al., 2006). *Timeless* (*tim*)-*GAL4* flies (stock 27) were obtained from Dr. Ralf Stanewsky (University of Münster) (Buhl et al., 2016; Chen et al., 2015).

2.2. Learning and memory experiments

To test learning and memory in flies, we used the olfactory-shock aversive conditioning protocol (Malik and Hodge, 2014; Tully and Quinn, 1985). Experiments were conducted at 25 °C and 70% humidity in dim red lighting conditions, using the T-maze apparatus. The odours used were 4-methylcyclohexanol (MCH) and 3-octanol (OCT), dissolved in 10 ml of mineral oil at concentrations of 1:500 and 1:250 respectively. The negative shock reinforcement used for conditioning consisted of 1.5 s pulses of 60 V electric shock, with 3.5 s pauses between shocks.

For training, groups of 30–50 flies were collected into a training tube containing a copper grid covering its inside surface. After an initial resting period of 90 s to acclimatise the flies, the first odour for conditioning was attached to the training tube and was drawn over the flies by a pump. For shock-paired odours, the electric shock was simultaneously administered through the copper grid. The flies were exposed to each odour for 1 min with a 30 s break of fresh air in between.

For memory tests, flies were kept in food vials before being reintroduced to the maze for testing. For testing, the flies were introduced into the central compartment of the T-maze. After a 90 s resting period they were transferred to a decision point from which they were allowed to move freely into the two arms of the maze, each with a different odour attached. They were given 2 min to make their decision, after which time the number of flies in each arm was counted.

After counting the number of flies making a correct decision (moving into the arm away from the shock-paired odour) and the number making a wrong decision, a performance index (PI) was calculated:

$$\mathrm{PI} = (\text{number of correct flies} - \text{number of incorrect flies}) / \text{total number of flies.} \quad (1)$$

A PI of 1 thus indicates 100% avoidance of the shock-paired odour (perfect learning) and a PI of 0 an even split (no learning). To eliminate any effects of odour bias, the assay was always performed with two groups of flies, one shocked with MCH and the other shocked with OCT. The average was then taken of the two scores to give n=1 PI value.

Control experiments were conducted to confirm that any decrements in PI scores were due to a central learning or memory deficit and not to a peripheral defect in odour acuity or shock reactivity. To test for odour acuity flies were given 2 min to decide between an odour at the concentration used for experiments and fresh air in the T-maze. The percentage of flies avoiding the odour was then recorded. Flies that can smell normally typically avoid odours and