Can methylphenidate rescue locomotor symptoms in a *Drosophila* model of Parkinson's disease?

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide (Lew, 2007), yet the current therapies are mainly symptomatic. Though the cause of the disease is largely unknown, the pathological manifestations are characterized by a degeneration of dopaminergic neurons (DANs) in the nucleus accumbens, as well as the presence of alpha-synuclein aggregates commonly known as Lewy bodies. These can ultimately cause aberrant calcium signalling in the brain. The production of reactive oxygen species (ROS) because of mitochondrial dysfunction is also believed to play an important role in PD. Clinical symptoms in humans include rigidity, gait and resting tremor though there are also non-motor symptoms such as sleep disturbances. (Beitz, 2014)

Methylphenidate, a commonly used drug to treat ADHD, blocks the reuptake of dopamine (DA) from the dopamine transporter (DAT), leading to an accumulation in the synaptic cleft and ultimately altered signalling in the postsynaptic neurons. Ultimately, the action of MPH depends on the dosage and method of administration. At high doses (10-20 mg kg^{-1}), effects include oxidative stress ultimately leading to neurodegeneration, similar to METH (methamphetamine) action. (Li et al., 2021). However, at much lower doses (for example 1 mM), a neuroprotective effect seems to exist (Ludolph et al., 2006), perhaps partially through the AMPKa signalling pathway (Li et al., 2021). This possible neuroprotective action is reinforced by the fact effects on locomotion of L-DOPA (one of the current treatments for PD) have been enhanced when used together with methylphenidate (MPH) in both rat and human models (Camicioli et al., 2001), (Nutt et al., 2004). Furthermore, when used in a 6-hydroxydopamine model of neurodegeneration in rats, MPH rescued both behavioural and neurological symptoms (Fleming et al., 2005).

Drosophila, a useful model organism with a short generation time (10 days), was used to investigate whether methylphenidate rescues fly locomotion in a specific model of Parkinson's disease that can later be extended to mammalian and human models- around 75% of genes in humans have an ortholog in flies. (Szabo and Tofaris, 1948) The specificity of genetic manipulation in the fly, and the large range of mutants commercially available, makes it an ideal organism for investigating both the changes in gene expression, and its behavioural manifestations. Furthermore, the well-established Gal4-UAS system allows for specificity of gene expression. A cell-specific promoter the 'driver' expresses the Gal4 protein, which activates the upstream activating sequence and allows for expression of the required gene.

There are many different models of PD available and widely used in Drosophila. These include SNCA, LRRK2, PINK1 and Parkin. The SNCA (NG_011851) gene originates in humans and point mutations (p.A30P or p.A53T) result in the disease phenotype. This mutated gene is inserted into the Drosophila genome, allowing for endogenous expression and aggregation of the alpha synuclein protein (Rocha et al., 2018). This model recapitulates features of PD such as locomotor impairment, adult onset neurodegeneration (loss of DA neurons) and aggregates of alpha synuclein in the brain. (Xiong and Yu, 2018) In this experiment, the SNCA gene was expressed in the fly selectively using an R58E02 driver. The R58E02 labels cells in the PAM cluster, which are important for locomotion (Liu et al., 2012). This model (SNCA-UAS X R58E02-Gal4) has been already well established and has been shown to cause locomotor defects in flies. (Riemensperger et al., 2013)

In this experiment, some key genes (ddc, Pu, AMPKa, SNF4y, 7B2 and Rtnl1) involved in dopamine synthesis and neuroprotection by MPH were tested and quantified. These are discussed below, respectively. Dopa decarboxylase (ddc) is involved in the synthesis for dopa decarboxylase, an enzyme that participates in the synthesis of dopamine by its decarboxylation of L-DOPA (Figure 1). Punch (Pu) also mediates a step in the dopamine synthesis pathway. More specifically, it synthesizes of BH4, a cofactor of tyrosine hydroxylase, which mediates the conversion of tyrosine to L-DOPA.

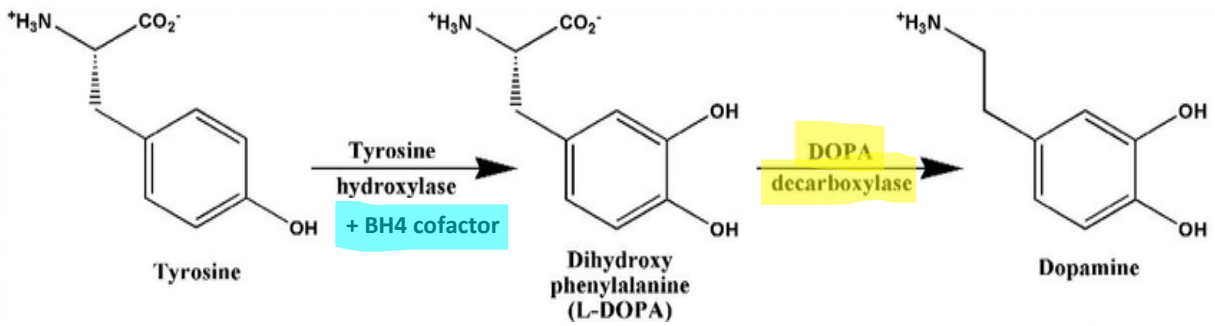

Figure 1: Part of the dopamine synthesis pathway **(Suryawashini et al., 2020)**

The AMPKa and SNF4y genes both encode a subunits of the AMPK complex. As stated before, the possible neuroprotective action of MPH may be, in part, mediated by the AMPK signalling pathway. 7B2 aggregates with alpha synuclein, so its expression (or lack thereof) is a useful marker for neurodegeneration. Finally, the Rtnl1 gene may help to maintain Ca^{2+} homeostasis (potentially opposing the effect of aberrant Ca^{2+} signalling caused by alpha synuclein).

Materials and Methods

Fly stocks

w;;Hsap-SNCA stock from Bloomington 8146; Canton-S stock from Chip Quinn.

Control genotype: w;;HsapSNCA (female)/wild type (male); Experimental genotype: w;;HsapSNCA (female)/w;+;R58E02-GAL4 (male). Virgin females were anaesthetized and collected using CO_2 before being exposed to males with the desired genotype. Adult flies were removed from the vial 3-4 days after introduction before the offspring hatched. Stock bottles were maintained in 75ml bottles and were kept in an incubator at 25℃ at 60% humidity with a 12 hr day/night cycle. Stocks were flipped every 4 days into new bottles. Vials with 4ml food were used for short-term maintenance of stocks.

Food preparation

Flies were kept on 'yellow food for memory' containing agar, yeast, sucrose, cornmeal, water and nipagin. To make the MPH-laced food, 0.1838 g MPH (233.3 g mol^{-1}) powder was diluted in 1.970 ml water, and aliquots of this were taken (0.5 ml, 0.25 ml, 0.05 ml) to create solutions of 1 mM, 0.5 mM, and 0.1 mM respectively. This was added to the food medium after cooking.

RT-qPCR

Primers were designed using Biotechnology Information's Primer-BLAST (Primer designing tool, 2022) and ordered from Sigma Aldrich. Two housekeeping (HK) genes were used to 'normalize' results, as they have been proven to be stable under environmental stressors.

Gene Name	Sense	Antisense
AMPKα	GGTGGCCGTCAAGATACTCA	ACGGCGTCGATATGACCTGG
SNF4Ay	ACAAATCGCCAAATGCGTCC	TTGTGCAGCACGCTCCG
Pu	GGTCGTAGAGGGAGTCCACA	AACACGCCCAGCATAGTTGA
ddc	TCTGGAGAATATACGCGAAAGG	CACTTCTCCGGCTTCTG
Rtnl1	TCTCGCTGTTTACCTTGCCC	GATGGCCACTCGGATCTTGT
7B2	CCGGATATCCTGTGATGCCC	GAAACGTCGTGTATGCTCGC
SdhA (HK)	TTCGCGAGGATGAATACGAT	CACGAGAGCGTGTGCTTG
Gapdh1 (HK)	AAAAAGCTCCGGGAAAAGG	AATTCCGATCTTCGACATGG

1-7 day old mixed-sex flies were placed on food with no MPH or with 1 mM MPH for 1 day, with 20 per vial. Flies were then placed into vials and flash-frozen using liquid nitrogen. The vials were shaken to decapitate the flies. The heads were counted and used for RNA extraction, which was conducted according to manufacturer's specifications using the Monarch RNA Extraction Kit®. The RNA concentration and purity were then analysed using a nanodrop spectrometer. The collected RNA was the converted into cDNA using the appliedbiosystems cDNA Reverse Transcription Kit®. The samples were prepared for qPCR and loaded onto a 96-welled plate using the qPCRBIO SyGreen Blue Mix Lo-ROX Kit®, following manufacturer's instructions.

Climbing Assay

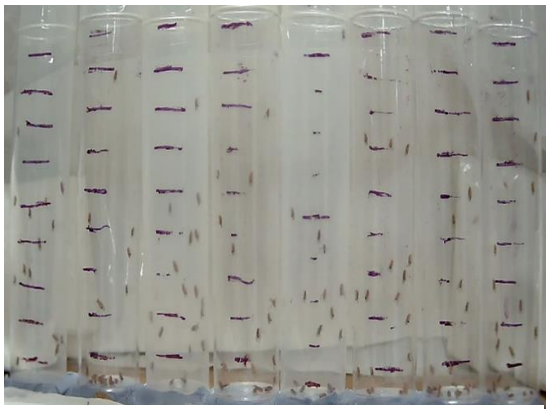

Figure 2: The experimental set-up for the climbing assay.

Flies were collected 10-15 days after eclosion using ice. 20 mixed-sex flies were then transferred temporarily to new vials and left to recover for 45 min- 1 hr. These flies were tested (day 0) and then transferred to food vials with the respective concentrations of the drug.

During testing, the vials were tapped to ensure all flies were at the bottom of the vial and a climbing index (CI) was calculated as follows to allow for quantification of results:

$$\frac{\text{number of flies over 9 cm in 5 s} - \text{number of flies over 1 cm in 1 } s}{\text{Total alive flies} - \text{number of flies over 1 cm in 1 } s}$$

This procedure was repeated on days 1-4 and 7. The total number of repeats was 6 groups of 20 flies per drug concentration and genotype.

Statistics

Samples were analysed using a two tailed t-test, either paired on unpaired depending on the data type. A value of $p < 0.05$ was taken as significant. All statistical analysis conducted using the Graphpad online calculator (Graphpad., 2021).

Results

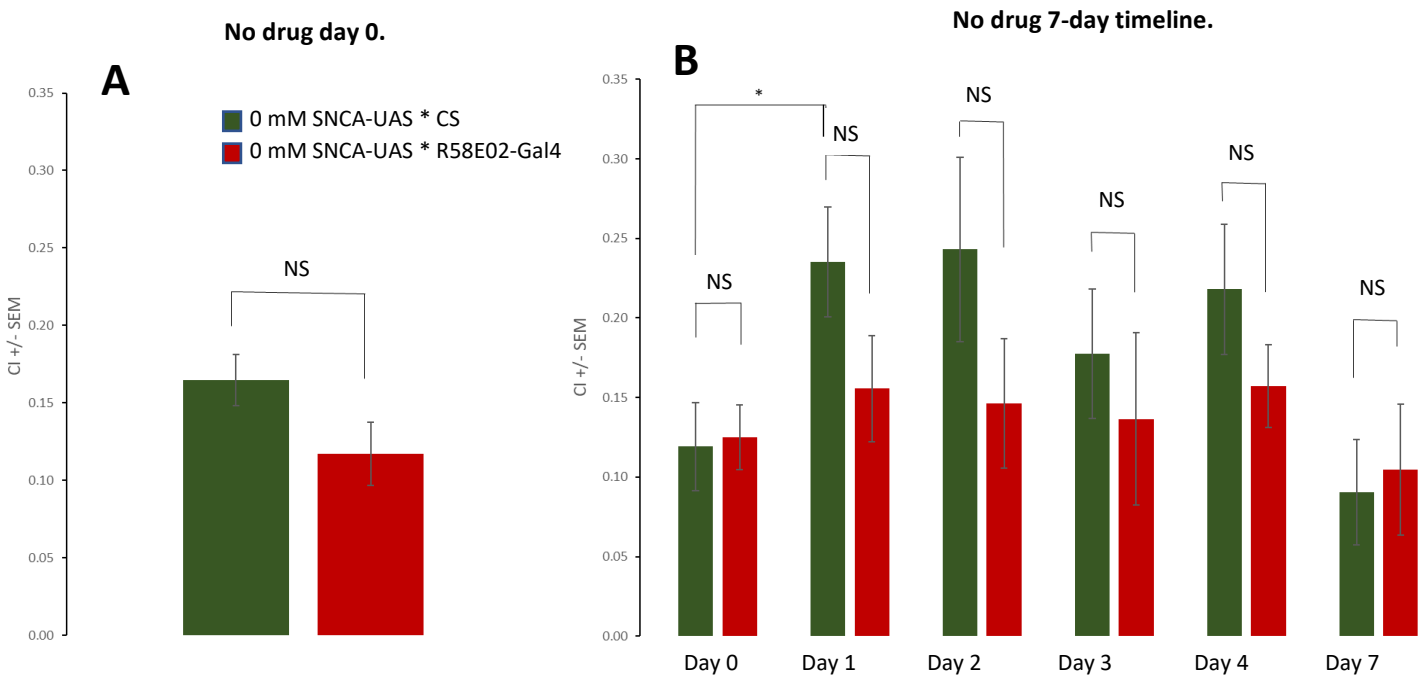

Figure 3: Climbing Index of flies with no drug. A: The climbing of flies at 0 days at 0 mM. B: A 7-day timeline of the climbing of flies with no drug. NS = $p>0.05$; * = $p<0.05$. Unpaired t test or paired t test, two tailed (see methods).

Throughout the 7-day testing period, the climbing differences between flies expressing alpha synuclein, and control flies were not significant (Figure 3, A and B). The climbing significantly increased in the control flies on transition from day 0 to day 1, perhaps because on day 0 the flies had not fully recovered from the counting procedure on ice.

On administration of 0.1 mM or 0.5 mM MPH, the CI (climbing index) was not significantly different to flies administered 0 mM MPH throughout the 7-day testing period in SNCA-UAS * CS flies (Figure 4, A). On administration of 1 mM MPH, the CI was not significantly different to flies administered 0 mM MPH on day 0. However, on days 1 and 2, the CI of flies administered 1 mM MPH was significantly lower than controls. There was no significant difference on days 3 and 4. On day 7, the CI of flies administered 1 mM MPH was significantly higher than controls.

On administration of 0.1 mM, 0.5 mM or 1 mM MPH, the CI was not significantly different to flies administered 0 mM MPH throughout the 7-day testing period in SNCA-UAS * R58E02-Gal4 flies (Figure 4, B). In terms of qCPR results (Figure 5), there were no significant differences in gene expression between the SNCA-UAS * CS flies on 1 mM MPH, the SNCA-UAS * R58E02-Gal4 flies on 0 Mm MPH and the SNCA-UAS * R58E02-Gal4 flies on 1 mM MPH. Perhaps this reinforces the behavioural differences, in which the climbing index of the controls was not significantly different from the climbing index of the alpha synuclein expressing flies.

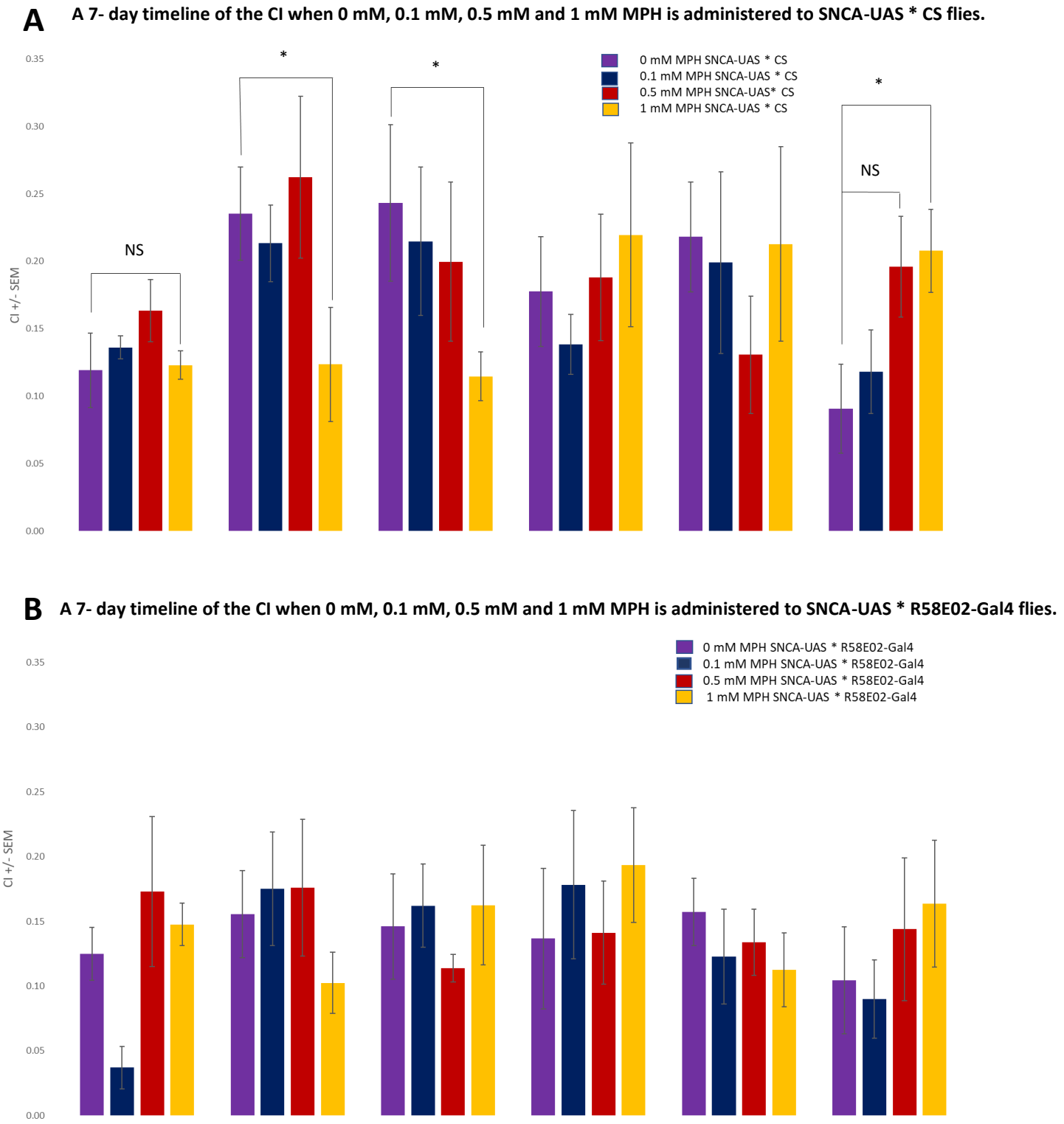

Figure 4: Climbing Index of flies administered MPH over a 7-day period. A: A 7-day timeline of the climbing of SCNA-UAS * CS (wild-type) flies on drug concentrations of 0 mM, 0.1 mM, 0.5 mM or 1 mM. B: A 7-day timeline of the climbing of SNCA-UAS * R58E02-Gal4 flies on drug concentrations of 0 mM, 0.1 mM, 0.5 mM or 1 mM. NS = $p>0.05$; * = $p<0.05$. Unpaired t test two tailed (see methods).

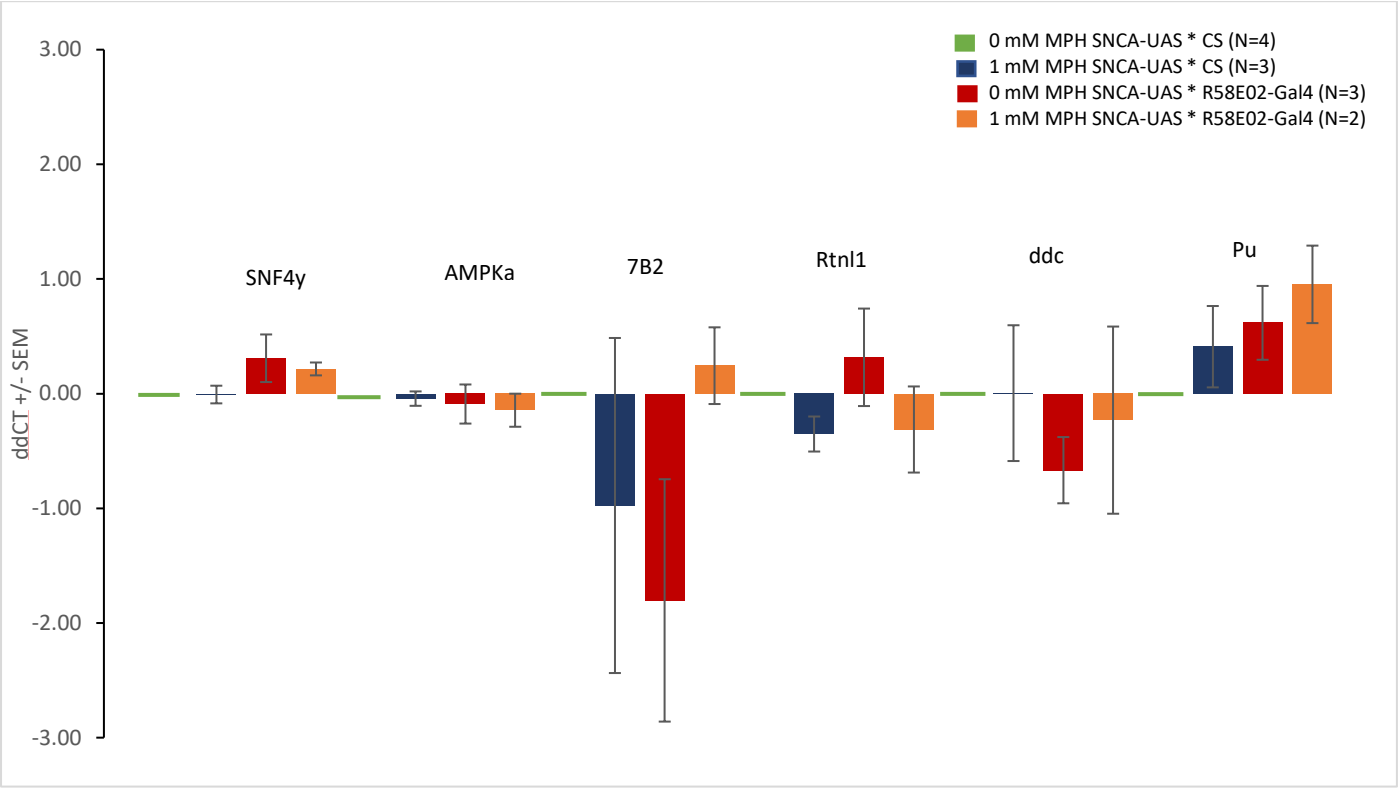

Figure 5: qPCR results of the different genes (SNF4y, AMPKa, 7B2, Rtnl1, ddc and Pu respectively) tested.

Discussion

There are many reasons why the SNCA-UAS * R58E02-Gal4 model failed to demonstrate significantly different climbing defects relative to control. The most obvious is that perhaps not the entire fly population was expressing the alpha-synuclein protein due to experimental error. During the qPCR analysis, it would have been useful to determine this using primers for the SNCA gene. Another reason is that the flies were too young to detect a climbing defect. Although the SNCA gene is expressed from the larval stages of the fly, it may take time for the alpha synuclein to develop and aggregate. As discussed above (Xiong and Yu, 2018) describe how DA loss in the alpha-synuclein model of PD is adult onset, so it is reasonable to extrapolate that los of motor ability in flies using this model is also adult-onset. Interestingly, (Ordonez, Lee and Feany, 2018) have developed a model that produces early-onset locomotor defects by using the Q system to alter gene expression. All in all, most obvious solutions include using flies that are slightly older 10 days, using a slightly longer testing timeline, and making sure the SNCA gene is being expressed using RT-qPCR.

Interestingly, in CS flies methylphenidate did not seem to have an obvious effect in young flies at concentrations of 0.1 mM and 0.5 mM. It does seem, however, that an effect at 1 mM starts to appear. The locomotion may initially be reduced (days 1 and 2) relative to control because the taste is initially not appetitive. At day 7 in control flies, the locomotion of flies on 1 mM is increased compared to those at 0 mM. Perhaps once a 'taste' for the drug has been acquired, the flies consume it which results in the increased climbing index. Since methylphenidate is a psychostimulant, it is logical to conclude that it may increase the climbing abilities of wild type flies. (Kanno et al., 2021) found that, in METH, flies do seem to initially prefer METH at 1mM, but at day 4 this preference decreases. It would be interesting to conduct a simple feeding assay and determine the eating behaviours of flies on food laced with MPH, and how this correlates with their locomotion. This may be able to explain the initial decrease at days 1-2, and the final increase at day 7. It would be useful to repeat the assay experiment at 1 mM MPH over a longer time-span to more definitively determine what differences in climbing MPH has on SNCA-UAS*CS (wt) flies.

The qPCR results were quite variable, and the lack of significance between control and alpha-synuclein-expressing flies at 1 mM may, like the previous behavioural experiments, indicate a genotyping issue. However, it would be interesting to look more closely at the expression patterns of 7B2, Rtnl1, and potentially ddc and Pu. As mentioned

before, it would also be useful to include an analysis for the expression pattern of alpha-synuclein to make sure it is being expressed in the SNCA-UAS*R58E02-Gal4 flies.

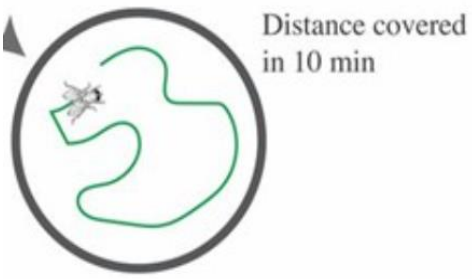

Figure 5: An alternative locomotion assay in flies. (Rhode et al., 2019)

Ultimately, the next steps would be to repeat this assay and optimize for drug concentration and timings at which the disease phenotype is most pronounced. It would also be interesting to repeat the procedure using a different locomotion assay such as ones described in (Rhode et al., 2019) and (Scaplen et al., 2019), as well as different models of Parkinson's. If the neuroprotective effect of MPH, both on a gene expression and behavioural level, rescues PD symptoms in some way, it would then be interesting to optimize for a different route/ timing of drug administration. For example, to mimic the possible treatment in PD, it would perhaps be useful to administer MPH for only a given time before testing locomotion to determine the optimum timings for drug delivery.

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