HHS Public Access

Author manuscript

Brain Res. Author manuscript; available in PMC 2025 January 01.

Published in final edited form as:

Brain Res. 2024 January 01; 1822: 148641. doi:10.1016/j.brainres.2023.148641.

Activation of Cyclic AMP signaling pathway in dopaminergic neurons rescues locomotion defects in a *Drosophila* larval model of Parkinson's disease

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Abstract

Parkinson's disease (PD) is a neurodegenerative disease showing uncontrollable motor symptoms that are primarily caused by the progressive loss of dopaminergic neurons in the brain. Currently no treatment exists to prevent PD progression. Therefore, discovery of new neuroprotective strategies still has great potential to benefit PD patients. A handful of studies show that activation of cAMP pathways is neuroprotective against PD progression. However, the neuroprotective role of this signaling cascade specifically in DA neurons has not been explored. In this study, fruit fly *Drosophila melanogaster* was used because of its sophisticated and powerful genetic approaches, especially with related to cAMP signaling pathway. We have investigated molecular mechanisms of neuroprotection in a fly larval model of PD by administering an environmental PD toxin rotenone. Increased cAMP signaling in the dunce mutant fly carrying defects in phosphodiesterase (PDE) gene, is neuroprotective against rotenone-induced locomotion deficits. Furthermore, the neuroprotective role of cAMP signaling specifically in DA neurons has been studied as it has not been explored. By using transgenic flies expressing designer receptors exclusively activated by designer drugs (DREADDs), we have shown that an increase of cAMP levels in DA neurons rescues rotenone-induced locomotion deficits. We also showed that this neuroprotection is mediated by activation of Gαs and PKA-C1 subunits. The results provide novel findings that expand our knowledge of neuroprotective mechanisms in DA neurons affecting PD progression, which could contribute to the development of new therapeutic treatments against PD. An important future study will explore downstream targets of cAMP-PKA signaling.

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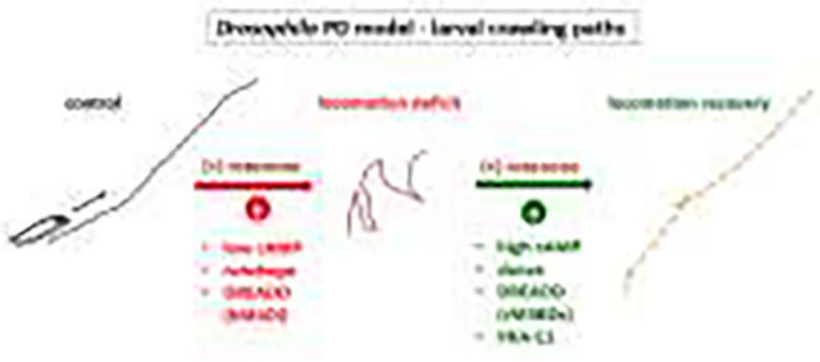
Authorship contribution statement

Conceptualization, S.I. and D.L.; Methodology, S.I.; Validation, S.I., R.A.C and D.L. Formal Analysis, S.I.; Investigation, S.I. and D.L.; Resources, S.I. and D.L.; Data Curation, S.I.; Writing – Original Draft Preparation, S.I.; Writing – Review & Editing, S.I., R.A.C. and D.L.; Visualization, S.I. and D.L.; Supervision, D.L. and R.A.C.; Project Administration, D.L.; Funding Acquisition, D.L. and R.A.C.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical Abstract



Keywords

Drosophila larval locomotion; rotenone; dunce; rutabaga; DREADD; PKA-C1

1. Introduction

Parkinson's Disease (PD) is the second most prevalent neurodegenerative disease after Alzheimer's disease (de Lau et al, 2006; Lamptey et al, 2022), and is characterized by several motor symptoms in patients such as tremors, rigidity, bradykinesia and postural abnormality (Beal, 2001; Poewe et al, 2017). Degeneration of dopaminergic neurons (DA) in substantia nigra pars compacta (SNpc) (Giguère et al, 2018) is directly associated with PD. Genetic models are established by using mutations in certain genes related to PD to produce the same motor symptoms. Those PD models carry mutated human genes found in the inherited forms of PD including α-Synuclein (α-Syn), PINK1, Parkin, DJ-1 and LRRK2 (Dawson et al, 2010; Panicker et al, 2021). In addition, epidemiological studies show that exposure to pesticides has increased prevalence of sporadic PD among people in rural regions (Goldman, 2014). Exposure to the pesticide rotenone causes nigral DA neurodegeneration, and results in development of both motor impairments and formation of protein aggregates called Lewy bodies (Beal, 2001; Panov et al, 2005; Goldman, 2014). Currently, there is no cure for PD, and thus it warrants the development of new therapeutic strategies.

In both postmortem PD brain tissues and PD animal models, protein kinase A (PKA) signaling dysregulation has been discovered (Howells et al, 2000; Dagda et al, 2015; Alharbi et al, 2022; Cao et al, 2022), strongly suggesting that activation of this pathway can be neuroprotective. Indeed, a handful of studies show activation of cAMP pathways is neuroprotective in PD (Dagda et al, 2015). However, manipulation of this cascade specifically in DA neurons to examine neuroprotection is missing.

Intracellular cyclic AMP (cAMP) levels can be regulated by either its synthesis via adenylate cyclase or degradation through a group of cyclic nucleotide 3', 5'-phosphodiesterases (Morales-Garcia et al, 2014; Lee, 2015). An elevation of intracellular cAMP levels stimulates PKA. This stimulation follows binding of cAMP molecules to

regulatory subunits of PKA (PKA-R), which causes a conformational change leading to release of active catalytic subunits (PKA-C). These active catalytic subunits phosphorylate other proteins, including cAMP response element-binding protein (CREB) (Mayr & Montminy, 2011; Lee, 2015).

The fruit fly *Drosophila melanogaster* became an excellent model to study human disease because gene sequence and function are highly conserved between flies, rats, and humans (Nitta and Sugie, 2022). Drosophila 3rd instar larva has a simple but intact dopaminergic system that can control locomotion. Larval locomotion can be easily quantifiable. It also showed age-dependent PD symptoms (Varga et al, 2014). Therefore, *Drosophila* larva can be an excellent PD model to study the role of cAMP signaling in neuroprotection. Indeed, all molecular constituents of cAMP signaling are conserved in the fruit fly, such as adenylate cyclase (AC), phosphodiesterase (PDE), protein kinase A (PKA) and cAMP response element-binding protein (CREB) (Lee, 2015). Among these genes, dunce and rutabaga are the most well studied. Dunce (dnc) encodes for phosphodiesterase (PDE), which breaks down cAMP (Dudai et al, 1976), and rutabaga (rut) encodes for adenylate cyclase (AC), which synthesizes cAMP from ATP (Livingstone et al, 1984). dnc and rut mutant flies are available allowing determination of the consequences of altering intracellular cAMP levels on locomotion defects caused by a PD toxin rotenone. Furthermore, a powerful chemogenetic tool called designer receptors exclusively activated by designer drugs (DREADDs) is available in flies to manipulate intracellular cAMP levels (Becnel et al, 2013; Majeed et al, 2013; Garbe etal, 2016; Tian, 2021). DREADDs are altered G protein-coupled receptors that have lost their affinity to acetylcholine but gained the ability to be fully activated by Clozapine-N-oxide (CNO). The advantage of this technique includes dose-dependent control of cAMP signaling pathways in specific neurons. Therefore, DREADD allows us to regulate intracellular cAMP levels temporally and spatially by applying various doses of the otherwise inert chemical CNO (Becnel et al, 2013). In this study, we have investigated molecular mechanisms of neuroprotection against rotenone toxicity in a *Drosophila* larval model of PD by using mutant and transgenic flies expressing DREADDs.

2. Results

2.1. Effect of rotenone on *Drosophila* larval locomotion

In this study, we have investigated mechanisms underlying neuroprotection using a model of *Drosophila* 3rd instar larvae administered with an environmental Parkinson's disease (PD) toxin rotenone. Three locomotion parameters relevant to PD were examined: speed, angular velocity and pause time that are useful behavioral parameters evaluating bradykinesia, walking difficulties, and freezing of gait, respectively. 3rd instar larvae (92–96 hours) administered 10μM rotenone showed a significant decrease in locomotion speed, an increase in angular velocity and pause time compared to control (Fig. 1). Further, we tested whether a PD gene α-Synuclein (α-Syn) causes similar behavioral defects to confirm that both genetic and environmental PD factors result in the same behavioral phenotype. Expression of the mutant form of human α-Syn (A53T) in larval brain dopaminergic (DA) neurons was achieved by using DA specific driver, TH-Gal4 (Friggi-Grelin et al, 2003). α-Syn (A53T)

expressing larvae showed reduced locomotion speed compared to wild-type larvae, and their crawling pattern is different from wild-type larvae that have a straight crawling path (Fig. 1). In addition, α-Syn (A53T) expressing larvae spent more time pausing and turning around than wild-type larvae.

Rotenone treated larvae and α-Syn (A53T) expressing larvae showed similar changes in crawling pattern, locomotion speed, angular velocity and pause time. Therefore, locomotion defects caused by a genetic factor (α-Syn) and an environmental factor (rotenone) are similar, and both could be reliably used as PD models to test therapeutic effects of new drugs for the disease (Varga et al, 2014; Blosser et al, 2020).

2.2. The rescue effect of cAMP-PKA signaling on rotenone-treated larva

Since dysregulation of cAMP-PKA signaling has been implied in PD (Dagda & Banerjee, 2015), we wanted to examine the role of cAMP-PKA signaling in neuroprotection. cAMP-PKA pathway has been intensively studied in *Drosophila* (Lee, 2015). Two well-known learning and memory genes in the *Drosophila* cAMP signaling pathway are dunce (dnc) and rutabaga (rut). Here, we used dnc1 mutant larvae as dunce encodes phosphodiesterase gene (PDE), which breaks down cytoplasmic cAMP. This mutation presumably increases cytoplasmic cAMP levels (Lee & O'Dowd, 1999; Ganguly & Lee, 2013; Lee, 2015). Dnc1 mutant larvae showed locomotion parameters similar to wild-type larvae. Interestingly, dnc1 mutant larvae administrated 10μM rotenone for 92–96 hours showed rescue of locomotion speed defects when compared to wild-type larvae treated with rotenone. Dnc1 mutant larvae administered 10μM rotenone had pausing time and turning rates that were reduced compared to wild-type larvae treated with rotenone. However, the differences were not significant (Fig. 2). These results demonstrate that the toxic effect of rotenone was reduced in dnc1 mutant larvae with elevated cAMP levels.

Next, we wanted to examine the effect of lower levels of cAMP on rotenone toxicity. In order to accomplish this, a fly mutant rut1 was used. Rutabaga (rut) encodes the adenylate cyclase gene (AC), a cAMP synthesizing enzyme (Lee & O'Dowd, 2000; Ganguly & Lee, 2013; Lee, 2015). Interestingly, all three locomotion parameters of rut1 mutant larvae are significantly different from wild-type larvae. Mutants had lower locomotion speed, higher angular velocity and pause time. The actions of rut1 were similar to rotenone-treated wild-type larvae (compare Fig.1 with Fig. 3), suggesting that, at least in part, rotenone toxicity may be resulting from lower intracellular cAMP levels.

2.3. CNO has no effect on wild-type larvae

The above results from dnc1 mutant larval behaviors suggest that increased cAMP signaling can counter rotenone toxicity and thus, elevated cAMP levels could play a role in neuroprotection. However, dnc1 and rut1 are preferentially expressed in mushroom body neurons which are known to have an important role in olfactory learning and memory in *Drosophila* (Lee & O'Dowd, 2000; Keene & Waddell, 2007). Therefore, it is unknown if these actions against rotenone toxicity are mediated from DA neurons or other neurons. To explore the neuroprotective role of cAMP pathways in DA neurons, we used a chemogenetic approach. Designer receptors exclusively activated by designer drugs (DREADDs) are

modified muscarinic G protein-coupled receptors that do not have affinity for acetylcholine, but instead have affinity for a biologically inert chemical, Clozapine-N-oxide (CNO) (Lee et al, 2014). Activation of Gαs-coupled rM3BCs with CNO positively modulates cAMP levels in a dose-dependent manner while stimulation of Gαi-coupled hM4Di with CNO decreases cAMP levels in a dose-dependent manner (Becnel et al, 2013).

As a first step, we determined if CNO has any actions on wild-type fly larval behavior. Wild-type larvae were grown to 3rd instar larvae in food with either 100μM CNO or without CNO. We detected no developmental delays related to CNO-treated group compared to no CNO-treated group. To test the effect of CNO on larval locomotion, we used the same locomotion assays. No significant differences were found between CNO-treated group in larval locomotion speed, angular velocity and pause time compared to control group (Fig. 4).

2.4. DREADD activation in DA neurons modulates sensitivity to rotenone

To further explore the neuroprotective role of cAMP in a drug treatment context, we expressed Gαs-coupled rM3BD receptor specifically in DA neurons using a DA driver TH-Gal4. F1 progeny 3rd instar larvae of a cross line between UAS- rM3BD and TH-Gal4 were grown with drugs (rot and/or CNO). Concomitant treatment with CNO (100μM) and rotenone (10μM) rescued the rotenone-induced defects in locomotion speed in the 3rd instar larvae, but their angular velocity and pause time were not rescued (Fig. 5). To examine the effect of inhibitory of Gαi-coupled hM4Di receptor on rotenone toxicity, we expressed Gαi-coupled hM4Di receptor in DA neurons. F1 progeny 3rd instar larvae of a cross between UAS- hM4Di and TH-Gal4 were grown with drugs, as described above. Concomitant treatment of CNO and rotenone decreased the locomotion speed in the 3rd instar larvae. No significant differences were found in angular velocity and pause time (Fig. 6).

2.5. PKA-C1 expression protects larvae from rotenone toxicity.

As PKA is the main target of cAMP in *Drosophila* (Kiger et al, 1999), we explored whether PKA activation plays a neuroprotective role against rotenone toxicity without altering intracellular cAMP levels. PKA consists of two subunits; cAMP-binding unit PKA-R which dissociates from catalytic subunit (PKA-C) upon binding to cAMP, leaving PKA-C active to phosphorylate its substrates (Kalderon & Rubin, 1988). Three types of catalytic subunits of PKA (PKA-C1-C3) have been identified in *Drosophila* (Chen et al, 2022). PKA-C1 is the most well characterized whereas the functions of PKA-C2 and PKA-C3 are much less understood (Cassar et al, 2018). We, therefore, expressed PKA-C1 in DA neurons by crossing UAS-PKA-C1 with TH-Gal4 to provide constant levels of activated catalytic subunits in DA neurons of larval CNS. These larvae showed similar speed, angular velocity and pause time compared to F1 progeny from WT crossed with TH-Gal4 (Fig. 7). Furthermore, F1 progeny of fly larvae expressing PKA-C1 catalytic subunit in DA neurons that were grown with rotenone, did not show the characteristic locomotor deficits seen with rotenone toxicity (compare Fig. 1 & Fig. 7).

3. Discussion

Our current work uses a *Drosophila* larval model to study pathology of Parkinson's disease (PD) and potential neuroprotective mechanisms. We show that an environmental PD toxin rotenone caused locomotion deficits, but activation of cAMP signaling specifically in dopaminergic (DA) neurons can reduce rotenone toxicity. The major finding of this study is that activation of the cAMP-PKA signaling pathway in DA neurons is neuroprotective in a rotenone-induced PD-like model.

The fruit fly *Drosophila melanogaster* has been an excellent model organism to study neurodegenerative diseases due to the availability of various genetic tools that allows researchers to rapidly and easily generate transgenic flies (Lu & Vogel, 2009; Nitta & Sugie, 2022). We use *Drosophila* as a model organism to develop PD-like symptoms to study PD pathology and possible neuroprotective mechanisms. To model a disease like PD, it would be necessary to have behavioral defects since PD is a movement disorder. Our larval locomotion system has been used to evaluate larval crawling speed, pausing time, and turning rate. These altered behaviors mimic movement difficulties in PD patients. We have developed a PD model using *Drosophila* larvae, demonstrating locomotion deficits and DA degeneration in their brains (Varga et al, 2014). Also, our lab has shown that L-DOPA, the current standard of treatment for PD patients, alleviates motor impairment in this model (Blosser et al, 2020). Therefore, this larval PD-like model mimics human PD symptoms.

It was previously reported that rotenone reproduces PD associated pathology in rodents including Lewy body-like structures that is not achieved by other PD toxins such as MPTP (Perier et al, 2003). We show larvae grown in 10μM rotenone as well as larvae expressing α-Syn (A53T) have significant defects in their locomotion. These are likely due to dopamine deficiency. We previously have demonstrated that the number of DA neurons is reduced over time in rotenone-treated as well as α-Syn (A53T) larval brains using confocal immunochemistry (Varga et al, 2014).

We show that modulation of cAMP pathways is involved in neuroprotection by exploring the actions of intracellular cAMP elevated in DA neurons in our 3rd instar larval models. Our data show that inhibition of PDE (*dnc1* mutation) results in neuroprotection in *Drosophila* larvae treated with rotenone. This suggests that higher levels of cAMP as a result of PDE gene defects is neuroprotective. However, the *dunce* mutation is not restricted to DA neurons. We examined whether modulation of cAMP signaling pathways specifically in DA neurons can affect rotenone-mediated behavioral defects as well as DA neurodegeneration. Indeed, we show that the activation of Gαs coupled receptors selectively expressed in DA neurons rescues motor deficits (Fig. 5) and protects DA neurons against rotenone toxicity in *Drosophila* neuronal cultures at 9 DIV (Ismael, Baitamouni & Lee, in preparation). Conversely, activation of Gαi coupled receptors, inhibits adenylate cyclase leading to decreased cAMP levels and worsens the movement defects of larvae that are treated with rotenone. This might be due to DA degeneration not only by rotenone but also by lower levels of cAMP in surviving DA neurons. In addition, primary neuronal culture shows that the activation of Gαi coupled receptors increased DA neurodegeneration (Ismael, Baitamouni & Lee, in preparation). The results from our experiments with modulation of

cAMP pathways in PD-like models using *Drosophila* larvae supports the idea that reduction of cAMP is involved in the mechanism of rotenone toxicity and up regulation of this pathway hinders the toxic effects of rotenone within DA neurons.

Recently, several studies have shown that dysregulation of cAMP signaling pathways contributes to PD etiology (Dagda & Das Banerjee, 2015). One study showed that mRNA levels of BDNF, a PKA-regulated gene, is greatly reduced in the substantia nigra of PD patients compared to control patients (Howells et al, 2000). Another study used 6-OHDA to model PD with B65 neuronal cells. They showed BDNF and Bcl2 mRNA levels are reduced following decreased PKA signaling and cAMP treatment rescued the 6-OHDA induced cell death (Chalovich et al, 2006). Furthermore, the contribution of PKA signaling dysregulation to PD etiology might be via altered mitochondrial function, as mitochondrial malfunction and fragmentation are found in various *in vitro* and *in vivo* models of PD (Dagda & Das Banerjee, 2015). In a familial form of PD, cAMP treatment and transient expression of outer mitochondrial membrane (OMM) targeted PKA catalytic subunit that was constitutively active, improved mitochondrial interconnectivity, while OMM-targeted PKA inhibitor induced mitochondrial fragmentation prior to cell death (Merrill et al, 2011). Hwang et al (2014) demonstrated that expression of hUCP2, which is the mitochondrial membrane transport protein human uncoupling protein 2 in flies attenuated rotenone-induced mitochondrial fragmentation, and this neuroprotective effect was possibly by elevation of intracellular cAMP. Conversely, PKA-inhibitor eliminated the mitochondrial integrity improvement by hUCP2 expression (Hwang et al, 2014). Our results show activation of cAMP signaling pathways specifically in DA neurons can protect against rotenone-induced locomotion defects. Future studies will explore those above targets altered by cAMP-PKA dysregulation to identify molecular underpinnings of neuroprotection mechanisms mediated by cAMP in *Drosophila* model of PD.

An important translational question related to our study is how we manipulate cAMP signaling in a specific subset of neurons. DREADD itself can be a valuable tool to meet this need as shown in our study. Using DREADD, cAMP signaling can be regulated in dopaminergic neurons as well as its duration of action (e.g., acute versus chronic).

In summary, we have investigated molecular mechanisms of neuroprotection against rotenone toxicity in a *Drosophila* larval model of PD. We found that modulating cAMP signaling pathways specifically in DA neurons protect against rotenone-induced locomotion deficits by using mutants showing defects in cAMP signaling and transgenic fruit flies expressing DREADDs. We also showed that this neuroprotection is mediated by activation of Gαs and PKA-C1 subunits. Our new findings will help to expand our knowledge of neuroprotective mechanisms against PD pathology, which will hopefully contribute to the development of new therapeutic treatments for PD.

4. Experimental Procedure

4.1 Fly strains:

Flies were kept in a standard cornmeal agar medium with 0.4% propionic acid at 25°C in a 12 h light/dark cycle. Cantonized white eye stock w1118 were used as wild type

from Bloomington *Drosophila* stock center (BDSC). For cAMP experiments, dnc1 and rut1 from BDSC were used. For DREADD experiments, UAS-rM3BCs and UAS-hM4Di were received from Dr. Robin Cooper (University of Kentucky), originated from Dr. Charles Nicholas at LSU health Science Center (Becnel et al, 2013). The tyrosine hydroxylase line TH-Gal4 was gift from Dr. Serge Birman at PSL Research University, Paris, France (Friggi-Grelin et al, 2003). UAS-PKA-C1.Flag was from BDSC and used for PKA-C1 overexpression experiments. Using those fly lines and TH-Gal4 driver, cAMP levels in DA neurons were conditionally controlled. For α-Syn experiments, TH-A53T (UAS-α-Syn A53T; TH-Gal4), a permanent homozygous line, was created in our lab by chromosome swapping. UAS-α-Syn (WT) and UAS-α-Syn (A53T) were kind gifts from Dr. Leo Pallanck at University of Washington, Seattle, USA.

4.2. Administration of drugs:

Rotenone and CNO (Sigma-Aldrich, St. Louis, MO) were dissolved in DMSO as stock solutions and given to flies at the final concentration of 10μM and 100μM, respectively. They were added to melted fly food in the petri dishes (35 mm) before solidifying. Larvae were exposed to rotenone and CNO from the 1st instar for 4 days before locomotion assay. The control food plates had matching amounts of DMSO. In our study, rotenone was prepared and disposed according to its safety data sheet from Sigma-Aldrich.

4.3. Collection of third instar larvae:

In order to obtain 3rd instar larvae (92–96 h after egg-laying), adult flies were transferred into egg collection bottles and capped with egg laying plates (food plates). The bottles were placed upside down and incubated at room temperature for 4 hours. Next, the egg laying plates were removed and incubated at 22 °C.

4.4. Locomotion assay:

As previously described (Varga et al, 2014), larvae were used at 92–96 hours after egg-laying for the locomotion assay. A black stage was made by boiling 0.75g agar in 30 ml distilled water and mixed with 6 drops of India ink (black color). The solution was poured into a 100 mm Falcon petri dish and cooled to solidify. Individual larvae were rinsed of the food and transferred on to the black agar dish. The larvae were allowed to adapt to the new environment for 1 minute. A Miticam3 digital camera (Motic) and Motic Image Plus 2.0 software were used to record their movement for 30 seconds at a speed of 16 frames per second. Then, the video was analyzed with Mtrack2 plug-in via ImageJ (http://valelab.ucsf.edu/ñico/IJplugins/MTrack2.html) to determine the path length. Pause time and angular velocity were quantified from the raw movement data as exported from MTrack2 as previously described (Blosser et al, 2020).

4.5. Statistical Analysis:

Student T-test (unpaired) was used to compare group pairings. Data are shown as mean ± SEM. p values are * <0.05, ** <0.01, *** < 0.001. Each experiment was repeated at least 3 times independently.

Funding:

This work was partially supported by NIH grants AG065925 (DL & RAC) and NS130256 (DL).

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Highlights

- Parkinson's Disease (PD) is a neurodegenerative disease characterized by motor deficits and caused by malfunction of dopamine (DA) signaling.
- Currently no treatment exists to prevent PD progression. Therefore, discovery of new neuroprotective strategies has great potential to benefit PD patients.
- Activation of cAMP pathways is known to be neuroprotective against PD progression. In this study, we have shown that increased cAMP signaling in the dunce mutant fly carrying defects in phosphodiesterase (PDE) gene, is neuroprotective against rotenone-induced locomotion deficits.
- The neuroprotective role of cAMP signaling specifically in DA neurons has been studied as it has not been explored. By using transgenic flies expressing DREADDs, we have shown that an increase of cAMP levels in DA neurons rescues rotenone-induced locomotion deficits.
- We also showed that this neuroprotection is mediated by activation of Gαs and PKA-C1 subunits.
- These results provide novel insights into the development of new therapeutic treatments for PD.

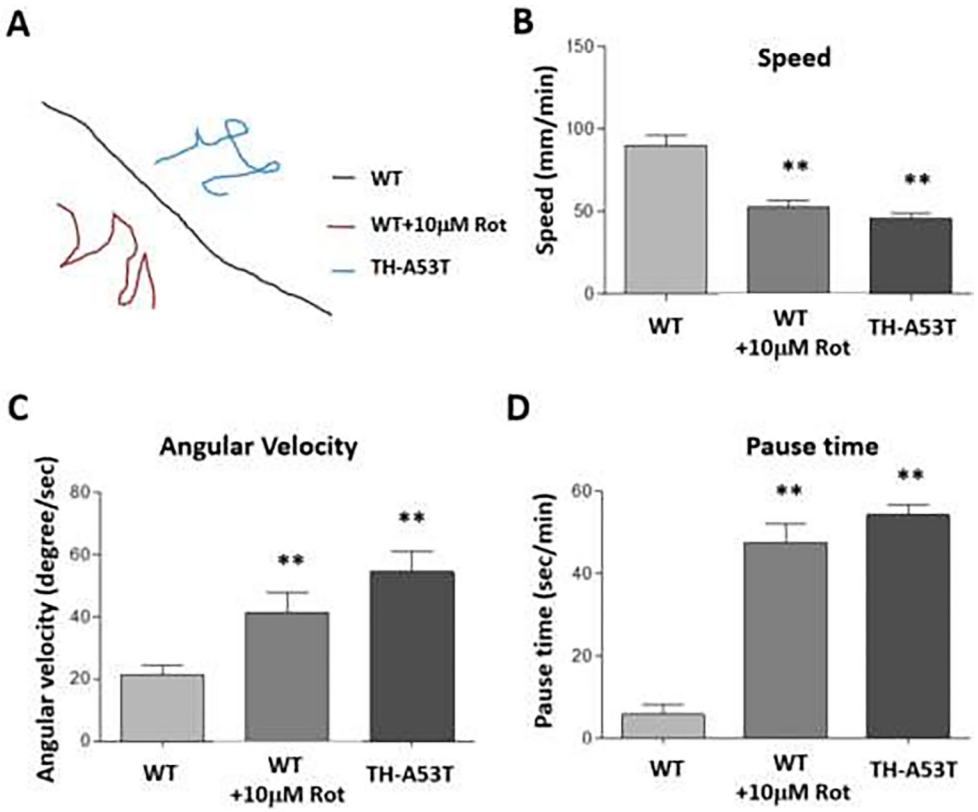

Figure 1. Locomotion defects in *Drosophila* larvae are caused by neurotoxin rotenone and PD gene α-Syn (A53T).

(A) Locomotion assays demonstrate that α-Syn A53T mutant and rotenone-treated larvae have irregular movement patterns compared to wild type (WT). **(B)** Locomotion speed, **(C)** angular velocity, and **(D)** pause time of WT, A53T mutant, and rotenone-treated larvae. UAS- α-Syn (A53T) mutant form was expressed by crossing with a dopaminergic driver TH-Gal4 (TH-A53T). Student's t-test, **p<0.01. Number (n) of larvae examined: WT (18), WT+10μM rotenone (16), TH-A53T (16), all data are from 3 independent experiments.

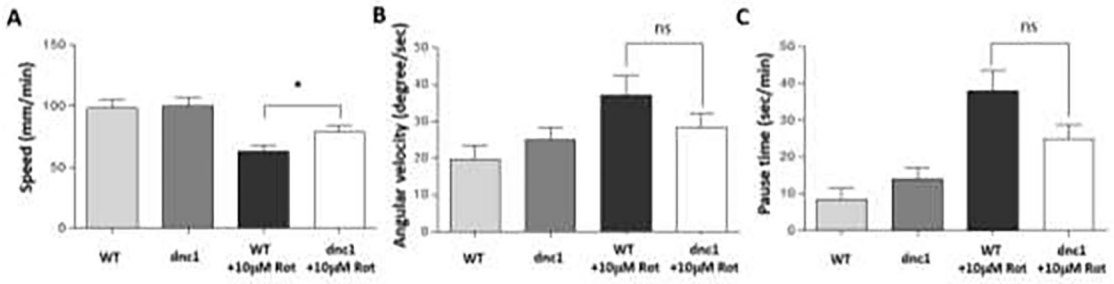

Figure 2. Rescue of rotenone toxicity by dnc1 mutant.

Locomotion assays demonstrate that dnc1 mutant larvae had normal locomotion compared to WT. Rot-treated dnc1 mutant larvae showed reduced locomotion defects including locomotion speed **(A)**, angular velocity **(B)**, and pause time **(C)**. Number (n) of larvae examined: WT (20), dnc1 (16), WT+10μM rotenone (21), dnc1+10μM rotenone (14). Data are from 3 independent experiments. Student's t-test, *$p<0.05$

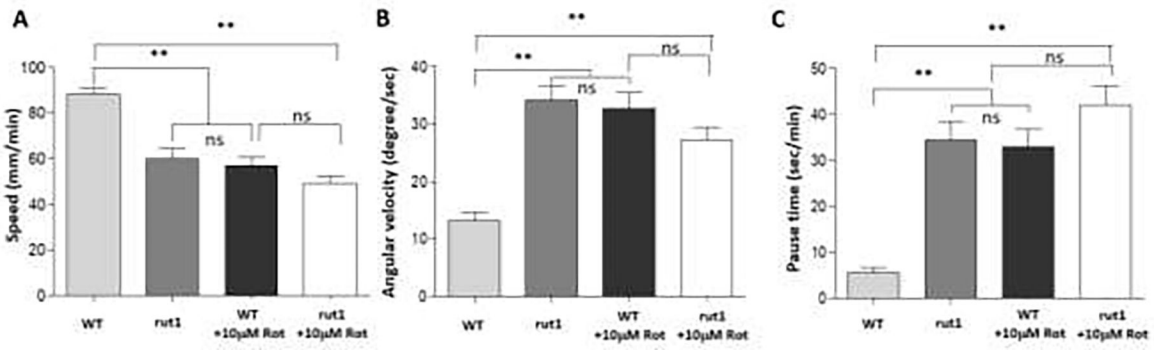

Figure 3. Locomotion defects in *rut1* mutant larvae.

Locomotion assays demonstrate that *rut1* mutant larvae showed locomotion defects compared to WT, and rotenone (rot) had no effect on *rut1* mutant larvae in any of the locomotion parameters including locomotion speed **(A)**, angular velocity **(B)**, and pause time **(C)**. Student's t-test, **p<0.01. Number (n) of larvae examined: WT (14), rut1 (25), WT+10μM rotenone (19), rut1+10μM rotenone (22). Data are from 4 independent experiments.

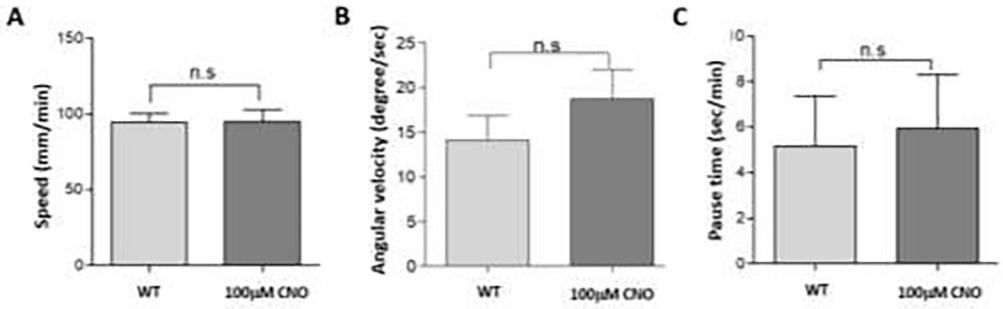

Figure 4. Clozapine-N-oxide (CNO) has no effect on wild-type larval locomotion.

Larvae grown in food with no CNO (Cont, DMSO only) or 100μM CNO. There were no significant differences in larvae locomotion assays with CNO in any of locomotion speed **(A)**, angular velocity **(B)** and pause time **(C)** when compared to control larvae. Number (n) of repeated experiments: Con (22), 100 μM CNO (19). Data are from 3 independent experiments. Student's t-test.

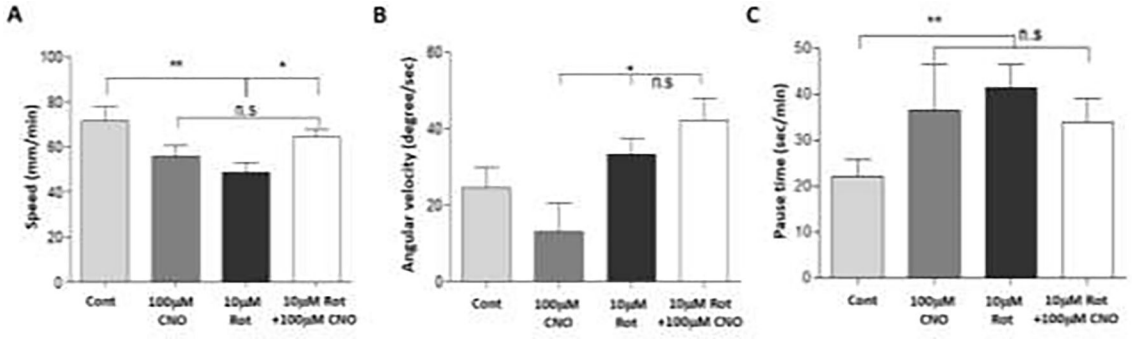

Figure 5. Activation of Gαs-coupled rM3BDs receptor in dopaminergic (DA) neurons reduces rotenone toxicity in *Drosophila* larvae.

3rd instar larvae from a cross between UAS- rM3BDs and TH-Gal4 flies were grown in food with no CNO (Cont, only DMSO), 100µM CNO, rotenone alone or rotenone with 100µM CNO. 100µM CNO with rotenone had significantly higher locomotion speed **(A)** compared to rotenone treated alone. **(B)** Angular velocity and **(C)** Pause time. Student's t-test, *p<0.05. Number (n) of larvae examined: Cont (15), 10µM Rot (17), 10µM Rot + 100 µM CNO (14). Data are from 3 independent experiments.

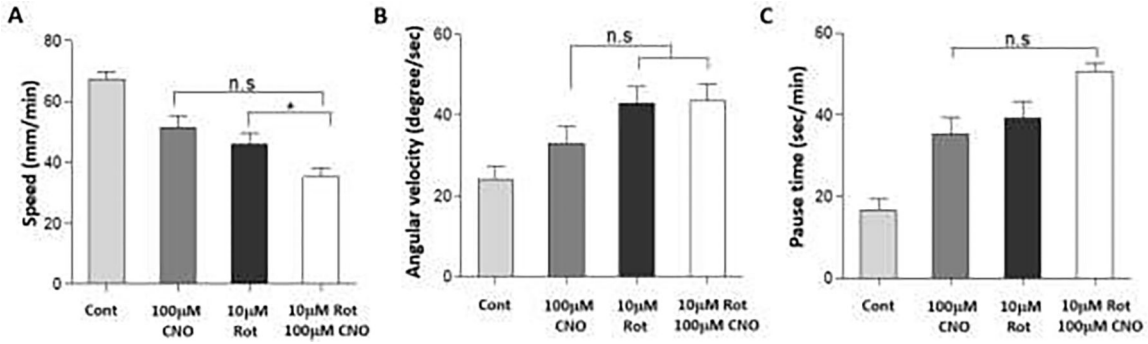

Figure 6. Activation of hM4Di receptor in DA neurons induces rotenone toxicity in *Drosophila* larvae.

3rd instar larvae from F1 progeny of a cross between UAS- hM4Di and TH-Gal4 flies were grown in food with no CNO, 100µM CNO, 10µM rotenone alone or rotenone plus 100µM CNO. 100µM CNO with rotenone had significantly lower locomotion speed **(A)** compared to rotenone treated alone. **(B)** Angular velocity and **(C)** Pause time. Student's t-test, *p=0.05. Number (n) of repeated experiments: Cont (20), 100 µM CNO (18), 10µM Rot (19), 10µM Rot + 100 µM CNO (18). Data are from 3 independent experiments.

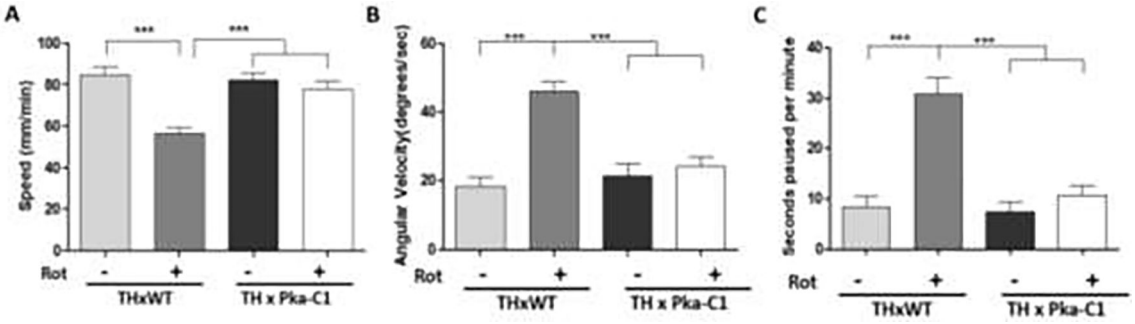

Figure 7. Overexpression of catalytic subunit of PKA in DA neurons protects 3rd instar larvae from rotenone toxicity.

3rd instar larvae from F1 progeny of a cross between WT and TH-Gal4 and another cross between UAS- PKA-C1and TH-Gal4 flies grown in food with either rotenone (10μM) or no rotenone (DMSO only). **(A)** Locomotion speed, **(B)** Angular velocity and **(C)** Pause time. Student's t-test, ***p<0.001. Number (n) of repeated experiments: TH × WT (control) (24), TH × WT (rot) (28), TH × PKA-C1 (Con) (19), TH × PKA-C1 (rot) (22). Data are from 3 independent experiments.