Neuropharmacology 236 (2023) 109573

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Kanamycin treatment in the pre-symptomatic stage of a *Drosophila* PD model prevents the onset of non-motor alterations

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ARTICLE INFO

Handling Editor: J.F. Cryan

Keywords:
Parkinson's disease
Drosophila
Presymptomatic stage
Microbiota
Pink1
Aging

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor alterations, which is preceded by a prodromal stage where non-motor symptoms are observed. Over recent years, it has become evident that this disorder involves other organs that communicate with the brain like the gut. Importantly, the microbial community that lives in the gut plays a key role in this communication, the so-called microbiota-gut-brain axis. Alterations in this axis have been associated to several disorders including PD.

Here we proposed that the gut microbiota is different in the presymptomatic stage of a *Drosophila* model for PD, the $Pink1^{B9}$ mutant fly, as compared to that observed in control animals. Our results show this is the case: there is basal dysbiosis in mutant animals evidenced by substantial difference in the composition of midgut microbiota in 8–9 days old $Pink1^{B9}$ mutant flies as compared with control animals. Further, we fed young adult control and mutant flies kanamycin and analyzed motor and non-motor behavioral parameters in these animals. Data show that kanamycin treatment induces the recovery of some of the non-motor parameters altered in the pre-motor stage of the PD fly model, while there is no substantial change in locomotor parameters recorded at this stage. On the other hand, our results show that feeding young animals the antibiotic, results in a long-lasting improvement of locomotion in control flies.

Our data support that manipulations of gut microbiota in young animals could have beneficial effects on PD progression and age-dependent motor impairments.

This article is part of the Special Issue on "Microbiome & the Brain: Mechanisms & Maladies".

1. Introduction

PD is the second most common neurodegenerative disease. Although its main feature is motor impairment, non-motor symptoms that precede motor alterations are also described. PD highly affects the elder population; actually, aging is associated with an increase in PD incidence, and therefore, it is considered that people with a PD diagnosis belong to a group with double vulnerability: old age and the disease itself. This disorder is characterized by the loss of dopaminergic neurons whose cell bodies are located in the Substantia nigra pars compacta (SNpc) and project their axons to the striatum in the basal ganglia in the brain. The decrease in movement, gait impairment, postural instability, rigidity, slowness, and tremor, among other problems, are explained by the striatal deficit of dopamine caused by the loss of dopaminergic neurons (Peterson and Horak, 2016). These are the classical motor PD symptoms and are observed late in the progression of the disease, at the point of no

Abbreviations: PD, Parkinson's disease; CI, Centrophobism Index; PI, Preference Index; Bz, Benzaldehyde; SNpc, Substantia Nigra pars compacta; ASV, Amplicon Sequence Variant; GYC, glucose, yeast and calcium supplemented culture medium; MRS, De Man, Rogosa and Sharpe culture medium; MDS, multidimensional scaling analysis.

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https://doi.org/10.1016/j.neuropharm.2023.109573
Received 4 May 2022; Received in revised form 2 May 2023; Accepted 4 May 2023
Available online 15 May 2023

return when most of dopaminergic neurons in SNpc have been lost (Kalia and Lang, 2015).

The discovery of physiological hallmarks before the onset of motor impairment in the period that is known as prodromal PD, is crucial in the attempt to obtain an early diagnosis of the disease and hopefully to modify its progression. In this regard, it has been reported that non-motor symptoms associated with PD could be evident even 20 years before the onset of motor impairments. These include anxiety, fatigue, sensory alterations, autonomic and gastrointestinal dysfunction, and sleep disorders (Kalia and Lang, 2015). Unfortunately, individuals may be unaware of these symptoms and of their potential relevance for an early PD diagnosis. The physiological bases underlying the non-motor symptoms are just beginning to be studied. In this regard, gastrointestinal symptoms in PD, which include swallowing problems, delays in gastric emptying and constipation, have gained attention in recent years due to the influence that the gut could have on brain diseases (Deidda and Biazzo, 2021; Loughman et al., 2021; Stocchi and Torti, 2017; Tsafaras and Baekelandt, 2022).

The microbiota-gut-brain axis refers to the bidirectional communication established between the brain and the gut, where bacterial communities reside. It has been suggested that the impairment of intestinal motility could affect the gut microbiota, which has been previously associated with general good health (Cryan et al., 2019; Morais et al., 2021). Regarding PD, around 50% of patients exhibit gastrointestinal disorders. Interestingly, α-Synuclein aggregates, a classical marker of PD, are present in the submucosal and myenteric plexuses of the enteric nervous system before they are detected in the brain (Cox and Weiner, 2018; Dinan and Cryan, 2017). Moreover, most of the PD patients exhibit reduced intestinal barrier function that may increase their exposure to microbial metabolites (Forsyth et al., 2011; Menozzi et al., 2021; Wang et al., 2021). Changes in the microbiota as well as alterations in microbial function and production of metabolites have been detected in PD patients (Boertien et al., 2019; Scheperjans et al., 2015). Thus, it has been proposed that the gut could be the starting point in PD progression, although this is an issue still under debate.

In the same way, whether changes in the intestinal microbiota contributes to the progression of neurodegenerative diseases, including PD, remains undetermined. However, an interesting observation is that some of the phenotypes related to PD are very similar to those observed in murine models of gut dysbiosis (Diaz Heijtz et al., 2011). The demonstration of the contribution of the gut and its microbiota to PD progression could open the opportunity to explore new therapeutics targets and ways to modify the course of the disease before the point of no return.

In the present work we used a well-known *Drosophila* model for PD, the *Pink1*^{B9} mutant (Park et al., 2006), to advance on the characterization of the pre-motor stage of the disease, and begin closing the knowledge gap regarding the possible contribution of microbiota to PD-associated phenotypes. We have previously reported the progression of neurochemical and behavioral changes in this PD model, that leads to a full parkinsonian phenotype by when adult animals reach the third week of age (Molina-Mateo et al., 2017). We and others have reported that before the onset of motor deficits, it is possible to describe non-motor phenotypes, such as alterations in olfactory responses, circadian rhythms, and impaired learning and memory (Julienne et al., 2017; Molina-Mateo et al., 2017; Park et al., 2006). Here we compared and found differences in the microbiota of *Pink1*^{B9} mutant vs control animals in the presymptomatic phase of this PD model. We treated control and mutant animals with the antibiotic kanamycin in order to kill most gut bacteria over time windows associated to presymptomatic stages in the PD model. Our data demonstrate that this manipulation affect non-motor behavioral phenotypes associated to the fly PD model.

2. Methods

2.1. Bioethical and biosafety issues

All experimental procedures were approved by the Bioethical and Biosafety Committee of the Pontificia Universidad Católica de Chile, and were conducted in accordance with the guidelines of the National Committee for Scientific and Technological Research, ANID, and the Servicio Agrícola y Ganadero de Chile, SAG.

2.2. Flies

All experiments were carried out with male flies due to the fact that the mutation in the *Pink1* gene (a deletion) is in the X chromosome. Thus, we used hemizygous male mutant flies as the experimental group; control animals were also male flies. The *Pink1*^{B9} mutant strain (w*, PINK1^{B9}/FM7i,P{w^{+mC} = ActGFP}JMR3) and the genetic control strain, *w*^{1118}, were obtained from the Bloomington *Drosophila* Stock Center (lines # 34,749 and 5905, respectively). Animals were raised at 19 °C on a 12/12 h light/dark cycle and maintained on a standard yeast meal diet. All data shown were obtained from experiments carried out with animals obtained from at least three independent cohorts.

2.3. Antibiotic treatment

Kanamycin, a broad-spectrum antibiotic able to kill Gram-positive, Gram-negative, mycoplasma, and bacterial pathogens (White and Knight, 1958), was used following a protocol modified from Wu et al. (2017). Briefly, flies were fed with fresh regular food supplemented or not with Kanamycin (final concentration 0.5 mM), from day 1 post-eclosion until day 15–16 as adult animals. The food was prepared weekly and stored in the lab cold room at 4 °C. Flies were moved to new vials containing fresh food every 2 days.

2.4. Lifespan and chronic exposure to antibiotic

Newly eclosed flies were raised in standard food (control) or kanamycin (0.5 mM) supplemented food (experimental), in groups of 20 flies. The total number of flies per condition was 200 animals. Flies were changed every 48 h to a new vial containing standard or kanamycin-supplemented food, respectively. The number of dead flies was registered daily until there were no flies alive. Assays were performed at 25 °C. For data analysis, results were expressed as percent survival rates.

2.5. Video recording, centrophobism index, and chemotaxis assay

For behavioral assays, we used a setup previously described that allows us to assess motor and non-motor behavioral features (Hidalgo et al., 2017, 2021; Molina-Mateo et al., 2017). In brief, single flies were placed in a circular arena (39 mm diameter, 2 mm high), with two pieces of cotton placed in opposite sides. Fly behavior was recorded in this arrangement for 3 min at room temperature. Afterward, one of the pieces of cotton was soaked with 100 μl of Benzaldehyde (Bz, 1% v/v in water), while the other was soaked with 100 μl of distilled water (control side). Fly behavior was recorded for another 3 min, indicated from here on as the "Bz" experimental group. All video recordings were analyzed offline, using the Buridan tracker software (Colomb et al., 2012).

In addition, heat maps that reflect the position of flies in absence and presence of Bz were generated. These maps were processed with ImageJ to calculate Performance Index (PI), a value that reflects the innate aversion of flies to Bz. To do this, the heat map was divided into two halves and PI was calculated according to the following formula:

$$PI = \frac{AUC_{H2O} - AUC_{Bz}}{AUC_{H2O} + AUC_{Bz}}$$

where AUC_{H2O} corresponds to the area under the curve of the time spent by the fly in the half of the arena closer to water, while AUC_{Bz} represents the area under the curve of the time spent by the animal in the half of the arena closer to Bz.

Out of the video recordings, it is also possible to calculate centrophobism. For doing this, the circular arena is divided into two concentric circular areas of equal surface, a smaller, inner circle and an outer ring. The software records the amount of time flies spent in each subdivision. Centrophobism is calculated using the following formula:

$$CI = \frac{TE - TI}{TI + TE}$$

where CI is the centrophobism index, TI is the time that the fly spends in the internal area and TE is the time that flies spend in the external area. Therefore, an index of 1 means that the fly spent the entire experiment in the outer area, −1 is obtained if the animal spends the entire experiment in the center and 0 denotes an equal distribution between outside and inside areas.

2.6. Analysis of behavioral data

All behavioral data was analyzed using One-way ANOVA or Two-way ANOVA, followed by Tukey's post-hoc test. Also, we performed the study of the median survival time by Mantel-Cox test. These studies were carried out in Prism software (GraphPad Prism 8).

2.7. Analysis of Drosophila gut microbiota

2.7.1. Fly preparation

To avoid contamination of gut samples with bacteria from the cuticle, flies were rinsed in three different solutions in the following order: sodium hypochlorite 2.5% (twice, 1 min each time), Ethanol 70% (twice, 1 min each time) and sterile water (twice, 1 min each time). Intestines from 40 male 8–9 days old flies were pooled and that was considered one sample (n = 1). Each experimental groups consisted originally of n > 20 samples per strain, obtained from independent groups of flies.

2.7.2. DNA extraction

Intestines were dissected out in sterile conditions and placed in a 0.5 ml tube containing sterile water (180 mL). Lysis buffer and solid glass beads (1 mm diameter) were added to the tube to homogenize the material by bead beating (three pulses, 20 s each). Then, phenol/chloroform extraction was used to obtain DNA. The quality and quantity of the extracted nucleic acids were checked by Qubit Fluorometric Quantification and by inspection after separation in a 1% agarose gel. Samples were kept at −20 °C before being sent to NOVOGENE (USA), where metabarcoding library construction and sequencing were performed, after carrying out a PCR of the bacteria 16S rRNA gene hypervariable regions V3 and V4. Analysis of DNA at our lab and then at NOVOGENE determined that some of the samples prepared were not of the quality required for further analysis. In spite of this, we ended up with n > 11 per genotype. Libraries were generated in Illumina platform to generate 250bp paired-end raw reads.

2.7.3. Analysis of 16S rRNA hypervariable regions V3 and V4

The raw tag sequences were processed using QIIME2 package v.2018.11. 16S rRNA gene raw sequences were demultiplexed with the q2-demux implemented in the QIIME2 pipeline (Bokulich et al., 2018; Bolyen et al., 2019). Demultiplexed reads were then trimmed, quality-filtered and merged using DADA2 (Callahan et al., 2016) to obtain amplicon sequencing variants (ASVs). The 16S rRNA ASVs taxonomy was assigned with q2-feature-classifier using the SILVA132 database (Quast et al., 2013) with the "classify consensus vsearch" method (Rognes et al., 2016). Finally, taxonomically annotated ASVs that belong to "Mitochondria", "Chloroplast", "Eukaryota", and Wolbachia sequences were removed from the subsequent bacterial and archaeal community analysis. To determine the beta diversity between samples a MDS (PCoA) analysis was performed with the weighted Unifrac distances metric (Lozupone et al., 2007) calculated with the ASVs sequences. To construct the final dendrogram, the Ward2 algorithm (Murtagh and Legendre, 2014), implemented in the vegan packages (Oksanen et al., 2019) in R, was used. Subsequently, silhouette validation criterium was used to select the optimal number of clusters (Rousseeuw, 1987).

About 30% of BDSC stocks are positive for *Wolbachia* (Clark et al., 2005). Importantly, *Wolbachia* presence in *Drosophila* stocks affects the gut microbiome, which could be considered a confounding factor in our study on gut microbiota composition (Simhadri et al., 2017). PCR carried out on samples obtained from control w^{1118} and mutant $Pink1^{B9}$ flies in our lab supports that both strains are positive for *Wolbachia* (not shown). Thus, it is not likely that the presence of *Wolbachia* could play a differential effect on our results on gut microbiota composition in the two strains studied.

2.8. Midgut microbiota culture

Four intestines of each condition (control or kanamycin-treated flies) were disaggregated in plates containing enriched culture medium: MRS (0.125 g/ml universal peptone, 0.075 g/ml yeast extract, 0.20 g/ml glucose, 0.02 g/ml dipotassium phosphate, 0.02 g/ml ammonium citrate, 0.05 g/ml sodium acetate, 0.001 g/ml magnesium sulphate, 5 μg/ml manganous sulphate and 0.12 g/ml agar) or GYC (0.5 g/ml Glucose, 0.03 g/ml yeast extract, 0.015 g/ml calcium carbonate and 0.06 g/ml agar). The plates were kept in an oven for up to 72 h at a temperature of 31 °C, under normoxic conditions. The qualitative results were obtained by taking pictures of bacteria colonies with a Leica Camera and microscope.

3. Results

3.1. Characterization of gut microbiota in young flies

To investigate whether there is a difference in gut microbiota in the pre-motor stage of the fly PD model as compared to control animals, we performed 16S rRNA gene (16S iTag V3-4 paired-end amplicons) sequencing of 8–9 days old $Pink1^{B9}$ and w^{1118} flies. The analysis by taxa (Fig. 1) describes the relative abundance of bacteria in each genotype from phylum to family. The results obtained from the microbiota analysis indicate that the w^{1118} strain is characterized by Proteobacteria and Cyanobacteria, which represents almost 50% and 25% of the phylum, respectively (Fig. 1A). On the other hand, the $Pink1^{B9}$ strain is characterized by Proteobacteria, Actinobacteria, and Cyanobacteria, although unlike the control strain, the Proteobacteria phylum reaches almost 70% of the total bacterial composition while Actinobacteria and Cyanobacteria represent about 10% each (Fig. 1B). Thus, in both - control and PD flies - the proteobacteria phylum was the most abundant, although at a different proportion (Fig. 1). At the class level, this trend is maintained: Alphaproteobacteria and Cyanobacteria classes represent about 50% and 25%, respectively in w^{1118} (Fig. 1A). In $Pink1^{B9}$ flies, Alphaproteobacteria are also the most prevalent bacterial class, reaching almost 70% of the total composition, while Actinobacteria and Cyanobacteria classes constitute about 10% each (Fig. 1B). The most detailed analysis we carried out is at the family level. In w^{1118} the most abundant families are Acetobacterareae (20%) and Sphingomonadaceae (25%), which belong to Proteobacteria and Nostocaceae family (25%), which belongs to Cyanobacteria. However, in the $Pink1^{B9}$ strain Acetobacteraceae represents about 40% of the family bacterial composition (two times what is detected in control animals), followed by Sphinomonadaceae (20%) and Nostocaceae (10%). Additionally, $Pink^{B9}$ and w^{1118} present 10.7% and 3.1% of Amplicon Sequence Variant (ASV) unique for each genotype

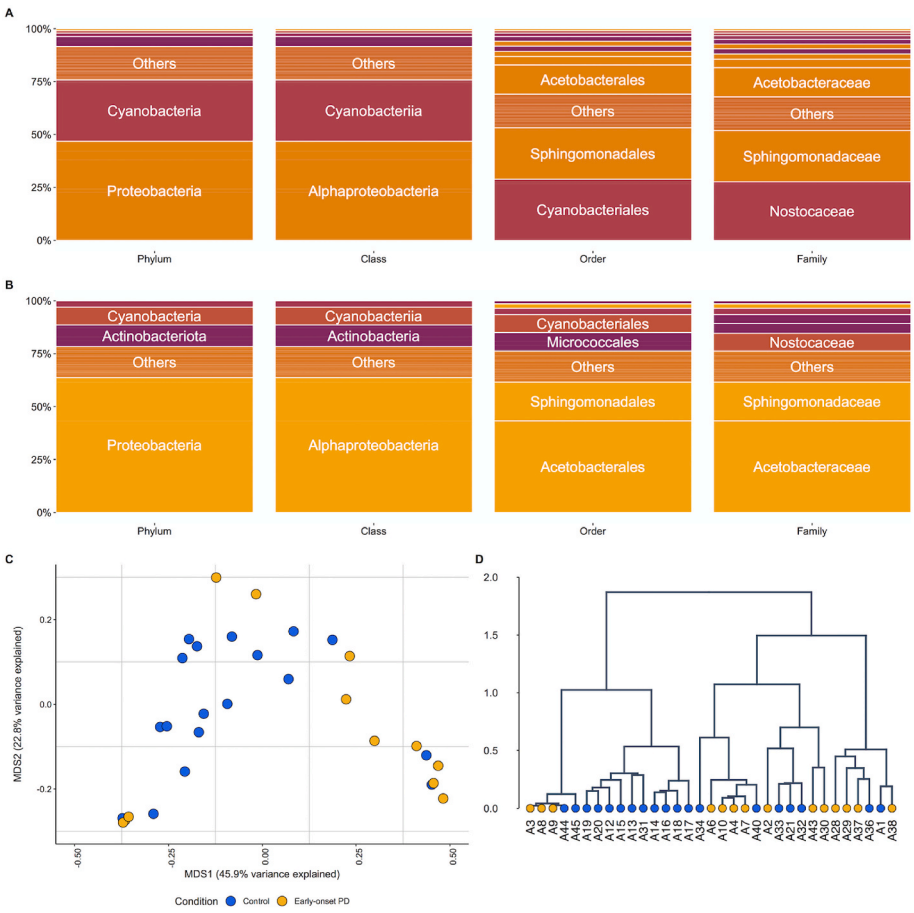

Fig. 1. Characterization of microbiota from PD flies model in the pre-motor stage. 16S rRNA v3-v4 sequencing from *Pink1*^{B9} and *w*^{1118} flies 8–9 days post-eclosion were analyzed to evaluate differences between intestinal microbiota in both genotypes. The diagram shows the taxa analysis of (A) *w*^{1118} and (B) *Pink1*^{B9}. (C) β diversity of the microbiota composition was shown as Principal co-ordinates analysis based on unweighted UniFrac distances. (D) Weighted Unifarc distances clustering of the microbiota from control and the PD *Drosophila* model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively, while they share 56.3% of ASVs (Supplementary Fig. S1).

Beta diversity analysis between samples by PCoA weighted Unifrac distances, a MDS study (Fig. 1C), shows in general differences in the PD model flies (yellow dots) when compared to control flies (blue dots). This supports the idea that there is a difference in microbiota in both strains. The analysis shows that two components are able to explain about 77% of the variability between groups (Fig. 1C). This suggests that other factors play a significant role in explaining the variability between control and mutant strains. The final dendrogram, which was computed using the weighted Unifrac distances approach, reveals two distinct clusters where blue dots are closely related and separated from most yellow dots detected (Fig. 1D). Actually, most blue dots are found concentrated in one branch of the dendrogram, while yellow dots are found in several clusters. Given the presence of several outliers that could obscure our understanding of the data obtained, we finally performed PERMANOVA on the weighted Unifrac distances analysis, versus the strains of the flies or sample clusters. This analysis shows a low but significant association between the fly strains and the samples diversity (PERMANOVA Df = 1, R2 = 0.086; p = 0.0299). Overall, the statistical analysis presented here supports that the composition of gut microbiota is complex, and affected by various factors.

All these results indicate important differences in intestinal microbiota between genotypes, at the time window studied. Since this is a critical time window in the progression of PD-associated features in this animal model (Molina-Mateo et al., 2017), we asked whether a perturbation in intestinal microbiota can modify the evolution of PD-associated features in the *Pink1*^{B9} genotype. To do this, we fed young flies with the antibiotic kanamycin.

3.2. *Kanamycin effect on young flies*

3.2.1. *Survival assay*

Before beginning to assess the effect of kanamycin on fly behavior, it was evaluated the effect of this antibiotic on fly survival in the two genotypes. For this, flies were maintained in fly food supplemented with kanamycin (0.5 mM). Results show no effect of the antibiotic on lifespan in control or mutant PD flies (Fig. 2B and C). It is already known that there is a difference in lifespan between the *Pink1*^{B9} mutant and the *w*^{1118} control fly (Liu et al., 2020). Therefore, as an internal control, we confirmed that *Pink1*^{B9} flies exhibit a shorter lifespan than *w*^{1118} control flies (Fig. 2A). Results show a median lifespan of 7 and 9 weeks respectively.

3.2.2. *Effect of chronic kanamycin treatment in Drosophila midgut*

It has been reported that the intestines of flies contain both Gram-negative bacteria such as Acetobacteraceae and Gram-positive such as *Lactobacillus* spp. (Bost et al., 2018). Data obtained here show that control and *Pink1*^{B9} flies contain mainly Gram-negative bacteria like Acetobacteraceae, Sphinomonadaceae and Nostocaceae (Fig. 1).

It was carried out a general evaluation of the effect of a chronic kanamycin treatment on intestinal microbiota. To monitor the effect of the antibiotic, *Drosophila* midgut was dissected and then we evaluated bacterial growth from this tissue in MRS or GYC agar plates. Flies were separated into four groups according to genotype (*w*^{1118} or *Pink1*^{B9}) and diet (standard food, or standard food + antibiotic).

Results show that no bacterial colonies are obtained when agar plates are seeded with gut samples of flies maintained under chronic kanamycin treatment, regardless of the genotype (Supplementary Fig. S2). No growth of colonies was observed either when we extend the chronic

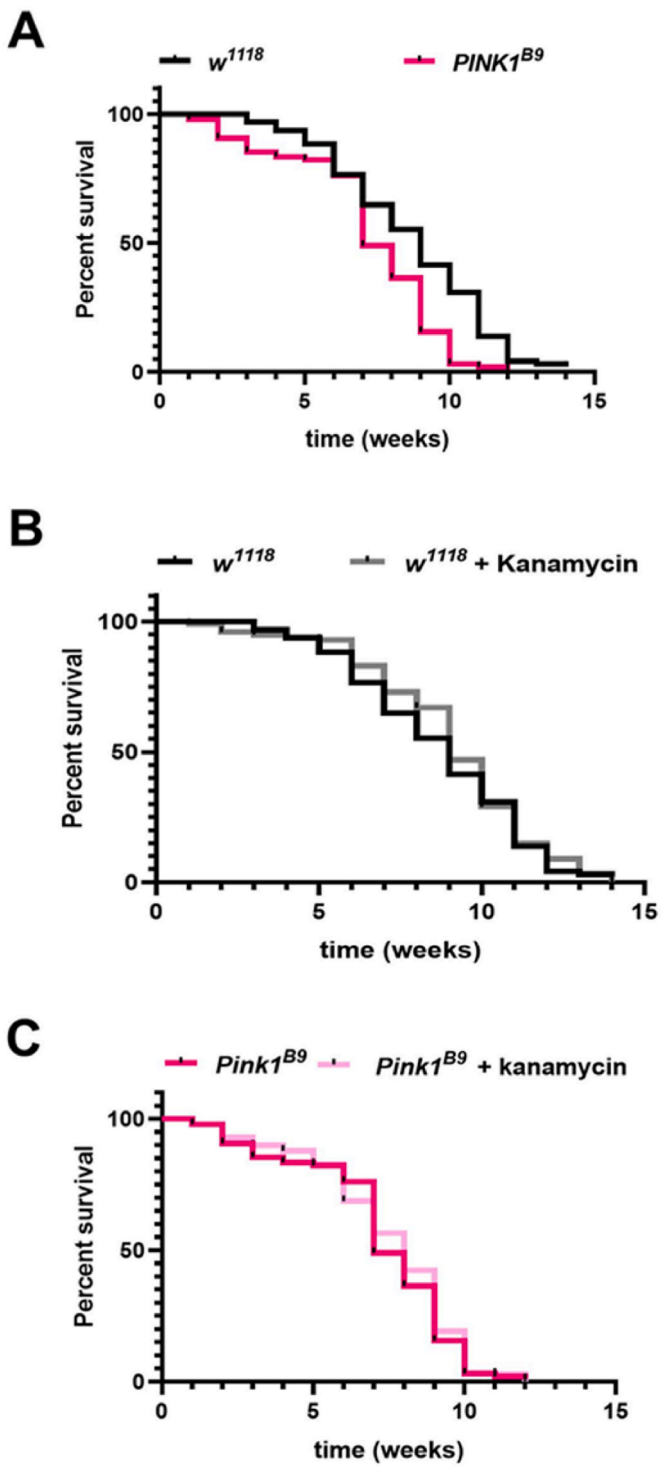

Fig. 2. Effects of Kanamycin treatment on lifespan in the genotypes. Lifespan in (A) w^{1118} v/s $Pink1^{B9}$ mutant flies; (B) w^{1118} exposed or not to the antibiotic, and (C) $Pink1^{B9}$ mutant strain when exposed or not to the antibiotic. Data are presented as % survival rates. Mantel-Cox test show differences between genotypes in (A) ($p < 0{,}0001$; $n = 100$ flies), while no significant differences were observed when evaluating the effect of antibiotic on each genotype (B or C; $p > 0.05$; $n = 100$ flies in each experimental group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment for up to 15 days (Supplementary Fig. S3). These data suggest that the kanamycin treatment is effective at eliminating most bacteria in the fly gut.

3.3. Behavioral characterization of Drosophila $Pink1^{B9}$ mutants treated with kanamycin

One of the hallmarks of PD is the detection of non-motor behavioral phenotypes 10 or more years before the onset of any deficit in locomotion (Kalia and Lang, 2015). Anxiety, depression, constipation, sleep behavior disorder and hyposmia are some of the symptoms associated with the prodromal phase of PD (Elbaz, 2016).

3.3.1. Kanamycin treatment reverts the deficit in naïve aversive olfactory responses observed in $Pink1^{B9}$

Olfactory impairment, which includes anosmia or hyposmia, is present in more than 70% of PD cases in the early premotor stage (Schapira et al., 2017). We have previously begun the characterization of features associated with the presymptomatic stage of the $Pink1^{B9}$ model of PD (Molina-Mateo et al., 2017). Thus, we described a decreased olfactory response and also several neurochemical changes in dopaminergic components, early after fly eclosion. Here we studied olfactory responses in the pre-motor stage of the PD fly model, while flies are being treated with kanamycin. We recorded the PI, as a parameter to assess olfactory responses in animals under the different experimental conditions.

We observed the expected impairment in olfactory response in young mutant $Pink1^{B9}$ flies as compared to w^{1118} animals in control conditions, that is, in absence of the antibiotic [$F(1, 78) = 12.22$; $P = 0.0008$; Fig. 3A]. This is consistent with what we previously reported (Molina-Mateo et al., 2017). Kanamycin treatment for 3 days post eclosion improved the olfactory response measured in mutant flies [$F(1, 77) = 14.52$; $P = 0.0003$ Fig. 3A], while did not modify the aversive response recorded in w^{1118} flies. Thus, the difference between genotypes in control conditions is not observed when animals are fed the antibiotic. As expected, in the next time window we did not detect a difference in olfactory response between strains in control conditions (Molina-Mateo et al., 2017). Interestingly, the mutant flies treated with kanamycin for 8–9 days, exhibit an increased olfactory response that reaches a magnitude similar to that recorded in younger control animals [$F(1, 77) = 14.52$; $P = 0.0003$ Fig. 3B]. At a longer time window, (15-16 days-old flies) it is observed that the difference between control and mutant genotypes [$F(1, 78) = 12.22$; $P = 0.038$ Fig. 3C] is not longer observed after kanamycin feeding (Fig. 3C). Overall, these results support that the kanamycin treatment affects olfactory response measured in the PD fly model.

3.3.2. Anxiety-like behavior as a hallmark of the pre-motor stage in $Pink1^{B9}$

Anxiety is one of the most common non-locomotor symptoms of PD that highly affect the quality of life in PD patients (Ray and Agarwal, 2020). Anxiety is highly associated with the serotonergic system, and in flies it can be studied as centrophobism (Hidalgo et al., 2017; Mohammad et al., 2016).

Since this is a behavioral feature that has not been thoroughly assessed in PD *Drosophila* models previously, we first studied it in control conditions. Our results support an effect of the age factor on centrophobism that depends on the genotype. In particular, it is recorded a decrease in the centrophobism index as control animals age [$F(2, 57) = 5058$ $P = 0.0095$, Fig. 4], while it is observed an increase in centrophobism in $Pink1^{B9}$ mutant flies 15–16 days post eclosion [$F(2, 58) = 16{,}11$; $P < 0.0001$, Fig. 4]. Thus, results suggest that both $Pink1^{B9}$ and control flies exhibit changes in this behavior over aging, although in opposite directions. When comparing the centrophobism index between genotypes at particular time windows, it is possible to describe differences over time [$F(2, 115) = 16.24$; $P < 0.0001$, Fig. 4], which are

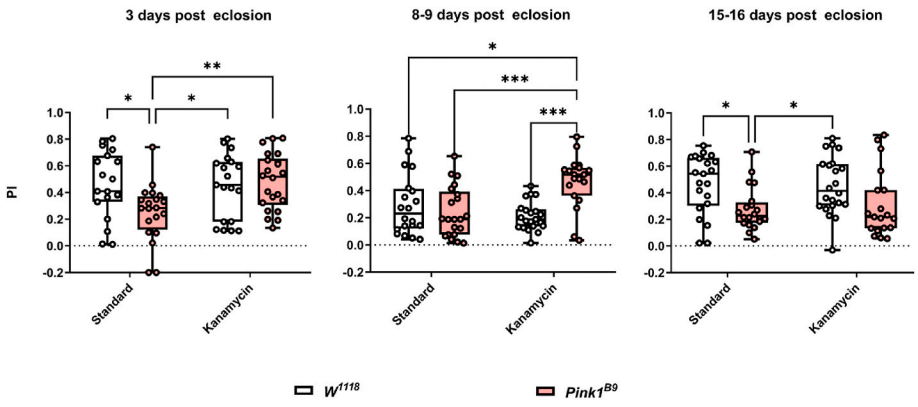

Fig. 3. Kanamycin treatment reverts olfactory deficits in *Pink1*^{B9} mutant animals. Mutant *Pink1*^{B9} and control (*w*^{1118}) flies were fed for (A) 3 (B) 8–9 and (C) 15–16 days kanamycin 0.5 mM. Then, they were exposed to an aversive odorant, Bz (1%, 3 min) in an arena, and behavior was video recorded. Response to this odorant stimulus was measured as preference index (P.I.). Video recordings were analyzed offline using the Buridan tracker software. Results presented as box and whiskers graph, whiskers indicate min and max values recorded; data obtained from n = 19–22 independent animals per experimental group; two-way ANOVA followed by Tukey post-hoc test, *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

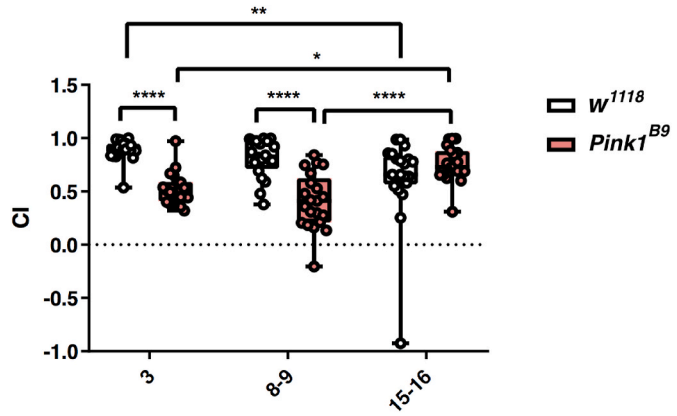

Fig. 4. Change in centrophobism, an anxiety-like behavior in flies, over aging. Centrophobism is measured as explained in Methods, and expressed as Centrophobism Index, at 3, 8–9, and 15–16 days after eclosion. Results presented as box and whiskers graph, whiskers indicate min and max values recorded of n = 19–22 flies per experimental condition. *, **, **** represent $p < 0.05$, $p < 0.01$ and $p < 0.0001$, two-way ANOVA with Tukey post-test when comparing indicated experimental groups.

significant at the first and second time windows, with the *Pink1*^{B9} mutant animals exhibiting a lower index as compared with control flies. No statistical differences are observed between genotypes at the final time window (Fig. 4). While in Molina-Mateo et al. (2017) it was reported olfactory dysfunction in the presymptomatic period in *Pink1*^{B9} flies, the present study advances in the characterization of this animal model for PD. Here we show for the first time a progressive increase in the anxiety-like behavior in mutant flies as a non-locomotor early-stage behavioral feature, similar to what is reported in prodromal phases of PD in humans (Broen et al., 2016; Elbaz, 2016; Schapira et al., 2017).

3.3.3. *Antibiotic treatment reverts the centrophobism phenotype in Pink1*^{B9}

We then assessed whether the antibiotic treatment affects centrophobism in the PD model (Fig. 5). In this set of results we have included the data presented in Fig. 4 to better show changes induced by the antibiotic treatment. We have done so considering that all these experiments were carried out in parallel. Results show that the antibiotic treatment eliminates the differences between *w*^{1118} and *Pink1*^{B9} strains, although this seems to be explained by a differential effect of kanamycin on the control and mutant genotypes. Kanamycin treatment reduces centrophobism in control flies (specially at the first time window), so that the difference observed between genotypes in centrophobism in control conditions, is not longer recorded in presence of the antibiotic (Fig. 5). The difference recorded in centrophobism between genotypes in control conditions at the second time window, is not observed when strains are exposed to kanamycin which seems to be explained by an increase in this behavioral feature in mutant animals (Fig. 5). The extended treatment with kanamycin for 15–16 days post eclosion does not have any effect on the anxiety-like behavior in control and mutant strains (Fig. 5).

3.3.4. *Locomotor activity in pre-symptomatic stages of the PD model*

Both centrophobism and the olfactory response depend on the ability of flies to execute motor programs. We decided to assess whether the results presented above on non-motor parameters could be accounted for alterations in motor output at the experimental conditions used in this work.

First, data obtained show no differences in motor parameters in

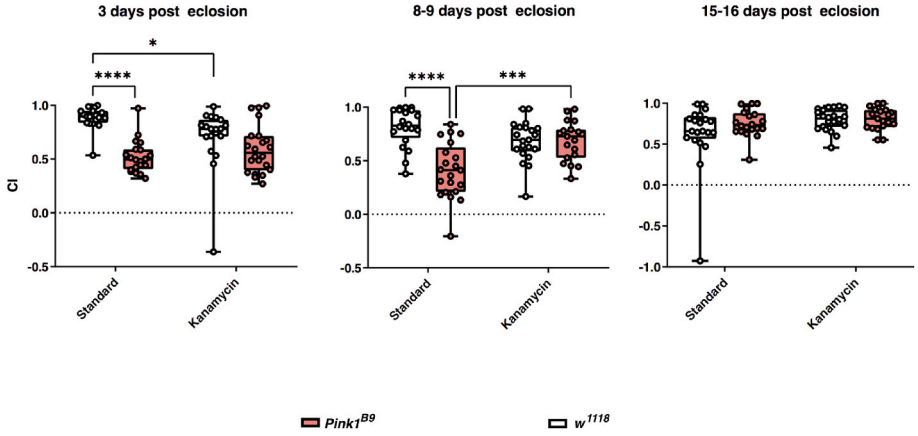

Fig. 5. Kanamycin treatment affects anxiety-like behavior in both mutant and control strains. The innate centrophobism behavior of flies was recorded for 3 min. Videos were analyzed offline using the Buridan tracker software. The centrophobism index represents the time that the fly spends avoiding the center (Hidalgo et al., 2017). Results presented as box and whiskers graph, whiskers indicate min and max values recorded from n = 19–22 flies; two-way ANOVA followed by Tukey post-test. *, ***; **** indicate $p < 0.05$; $p < 0.005$ and $p < 0.001$ respectively when comparing data from experimental groups identified.

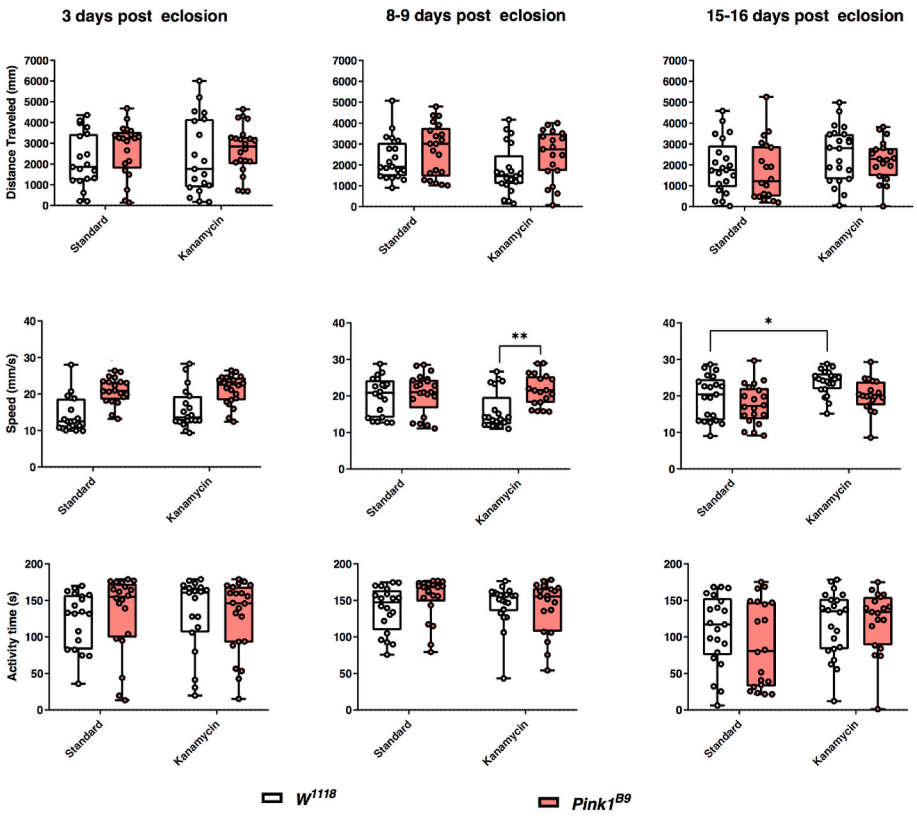

Fig. 6. No differences are detected in control or mutant flies at the premotor phases of the PD model, regardless of antibiotic treatment. Flies of the two genotypes (w^{1118} and $Pink^{B9}$ mutant flies) were fed with kanamycin or standard food. Innate motor behavior of single male mutant or control flies was recorded at different time points. Video recordings were analyzed offline using the Buridan tracker software. Motor parameters studied included Distance traveled (top panels), Speed (center panels) and Activity time (bottom panels). These parameters were assessed in flies 3, 8–9 and 15–16 days post eclosion (left, center and right panels, respectively). Results presented as box and whiskers graph, whiskers indicate min and max values recorded from n = 19–22 flies per experimental group. Two-way ANOVA followed by Tukey post-test indicates * and **$p < 0.05$ and $p < 0.01$ respectively, between experimental groups identified.

$Pink1^{B9}$ mutant flies as compared with age-matched w^{1118} animals, in control conditions (Fig. 6). This is similar to what has been previously reported (Molina-Mateo et al., 2017), and agrees with the idea that these time windows correspond to a stage in the $Pink1^{B9}$ PD fly model where no motor alterations are observed in mutant animals. Moreover, when studied the effect of kanamycin treatment on locomotion in flies, results show changes in few motor parameters; e.g. speed: control 15–16 days old flies fed kanamycin show increased speed as compared to control flies that did not receive the antibiotic [Fig. 6 $F(1, 76) = 8.663$; $P = 0.0043$]. At the previous time window, there is also a significant difference in speed of movement between strains. No other differences are observed (Fig. 6). Thus, these results show that no major changes in locomotion are observed in these time windows that correspond to the pre-symptomatic motor stage in the $Pink1^{B9}$ PD fly model as compared to control animals. Importantly, the kanamycin treatment does not strongly affect locomotion in these genotypes, either.

3.3.5. *Kanamycin treatment in early adulthood increases locomotor activity in older control and mutant flies*

One of the hallmarks of PD is the deficit in motor behavior, which is age-related. We decided to study parameters that reflect the execution of motor programs in old flies (29–30 days post eclosion) (Fig. 7A), which

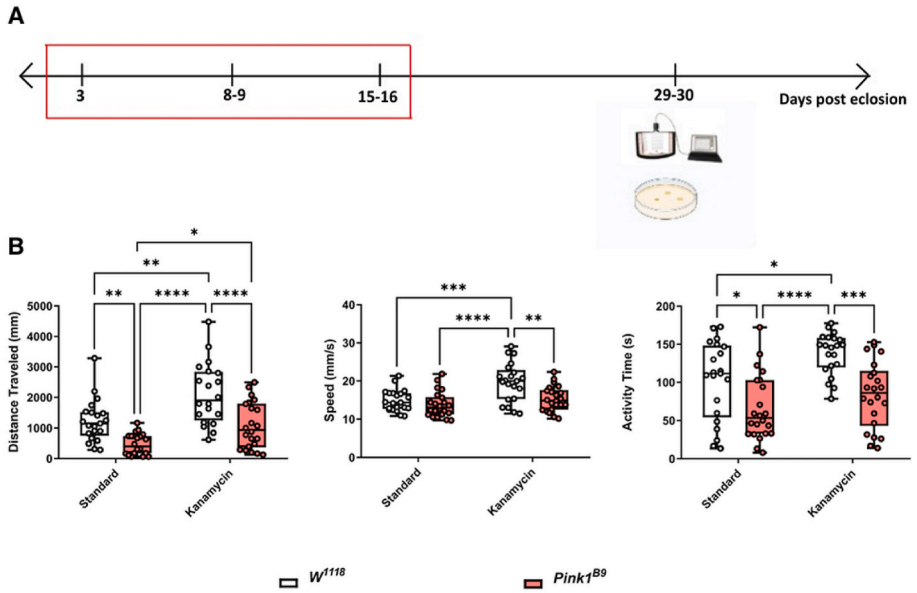

Fig. 7. Kanamycin treatment in early adulthood increases locomotor activity in old control and $Pink1^{B9}$ mutant flies. (A) General protocol for experiment. Flies of the two genotypes were fed with standard food or standard food supplemented with Kanamycin for 16 days since eclosion. After 16 days flies were fed with standard food. Innate behavior of single flies was recorded at 29–30 days post eclosion. Videos were analyzed offline using the Buridan tracker software. Results of different motor parameters were compared between genotypes and experimental conditions. (B) Motor parameters assessed: Distance traveled (left panel), Speed (center panel), and Activity time (right panel). Results presented as box and whiskers graph, whiskers indicate min and max values recorded. *; **; *** and **** indicates $p < 0.05$; $p < 0.01$; $p < 0.005$ and $p < 0.0001$ respectively, when comparing data from control flies and $Pink1^{B9}$ mutant animals; two-way ANOVA followed by Tukey post-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is a time window at which alterations in locomotion are evident in mutant flies (Molina-Mateo et al., 2017). Results were obtained from animals in control conditions (maintained in normal fly food) or in animals where kanamycin treatment was carried out from days 1–16 post eclosion.

As it has been reported, it is observed a decrease in locomotor parameters in *Pink1*^{B9} mutant flies as compared to age-matched control flies, in regular food (Fig. 7B). This is consistent with the fact that the *Pink1*^{B9} is a PD model.

Regarding the kanamycin treatment, the results show an increase in all locomotor parameters in old control animals fed the antibiotic as compared to age-matched control flies maintained in normal food (Fig. 7B). It is also recorded an increase in the parameter distance traveled in old *Pink1*^{B9} flies fed the antibiotic as compared to what is measured in age-matched mutant flies not receiving the antibiotic (Fig. 7A). Activity time and speed are parameters not affected by the antibiotic treatment in mutant animals.

3.3.6. Two weeks after stopping kanamycin treatment it is possible to observe a repopulation of the Drosophila gut

To obtain more information about *Drosophila* microbiota by two weeks after the suspension of kanamycin treatment, we inoculated tissue samples from the intestines of 29–30 day old flies on agar plates. Intestines were dissected out from animals at the four conditions previously mentioned (two genotypes and two treatments). In the case of kanamycin-treated animals, flies were fed with antibiotic-supplemented food for up to 15 days and then kept in regular food. The control condition is maintaining flies in regular food until the moment when experiment is carried out. Then, 29–30 days old flies were sacrificed to dissect out the intestines.

As expected, after stopping kanamycin treatment there is repopulation of gut microbiota evidenced by the observation of bacterial colonies in agar plates regardless the experimental conditions studied (Supplementary Fig. S4).

4. Discussion

PD is a neurodegenerative disorder for which we do not understand its causes. However, we know that the earlier the diagnosis, the better the prognosis for PD patients. That is the reason several research groups are studying the prodromal phase of PD in order to gain insights on the cellular and molecular mechanisms that underlie the disease but also to propose new therapeutic interventions that could slow down or even halt its progression.

On the other hand, it has been recently suggested that even though PD is a brain disease, its progression could be associated to the gut physiology. This idea is supported by several evidences, including the fact that it is possible to find α-synuclein aggregates in the gut early in the progression of the disease (Chen et al., 2018). Interestingly, it has not been thoroughly studied the occurrence of changes in gut microbiota in the prodromal stage of PD. It is also an open question whether modifying gut microbiota in the presymptomatic stage of PD could affect the manifestations associated with this disease. Here we advanced on these issues and provided further support to the idea that affecting gut microbiota affects some of the behavioral phenotypes associated with the pre-motor stage of a well-studied animal PD model, the *Pink1*^{B9} mutant fly.

The *Pink1*^{B9} mutant fly has been extensively studied as an animal model for PD, due to its behavioral characteristics and well-studied studied brain neurochemistry. It has been described that this mutant exhibits several behavioral impairments as it ages, including olfactory dysfunction and changes in the circadian rhythm as young adults, and later, impaired movement (Julienne et al., 2017; Molina-Mateo et al., 2017). These features make the *Pink1*^{B9} *Drosophila* strain a very good model for the study of PD progression, and particularly, the events occurring at the presymptomatic stage. Additionally, *Drosophila melanogaster* is a very useful model for the study of the gut-brain axis because its intestinal tract shares several characteristics with vertebrate animals at the molecular and cellular levels. For instance, the fly intestine exhibits the same types of transporters, receptors, and aminergic innervation as compared to mammals (Apidianakis and Rahme, 2011). Importantly, there is one study that relates the progression of neurodegenerative diseases with the gut-brain axis: it has been demonstrated that intestinal dysbiosis aggravates the progression of phenotypes associated to a *Drosophila* model for Alzheimer's disease (Wu et al., 2017). These antecedents support that the fruit fly can be used as an animal model to assess whether manipulations in the gut and its microbiota affect the progression of phenotypes in fly models for neurodegenerative diseases, including PD.

4.1. Characterization of young flies gut microbiota

A key first step for the study and characterization of midgut microbiota in animals or humans is to identify its bacterial composition and the relative abundance of bacteria species. Taxa assignation performed here is not able to discriminate bacterial species but it is able to identify bacteria at the family level, which is very informative. This is explained by the fact that only one segment of the 16S rRNA gene was used for our analysis (Knight et al., 2018). In addition, in our work we used flies 8–9 days post eclosion since at this age it is possible to observe clear evidence of non-motor symptoms before the onset of motor impairments.

While microbiota constantly interacts with the environment, it is highly dynamic. Thus, it can modify and be modified by several factors including stress and inflammation (Adak and Khan, 2019; Vandenplas et al., 2020), both present in pathologies like PD. Considering this, we predicted that gut microbiota would be different in *Pink1*^{B9} and *w*^{1118} flies, which was corroborated by bacterial 16S rRNA gene analysis. Beta diversity analysis suggests that the midgut microbiota of *Pink1*^{B9} and *w*^{1118} can be differentiated, based on the abundance and phylogenetic diversity of families of bacteria. Permanova analysis of the microbiota sequencing results supports this idea. This means that the two fly strains exhibit different midgut microbiota composition in this particular time window at the pre-motor PD stage (Fig. 1C–D). Supporting this data, while mutant flies and control flies share ASVs, they also exhibit some that are exclusive (Supplementary Fig. S1), suggesting substantial differences in the microbiota composition between genotypes. In the same way, Xu et al. (2020) described differences in diversity of microbiota species between control and *Pink1*^{B9} flies at 20 days post eclosion, which is by the time when it is described the onset of movement impairment in this PD animal model (Molina-Mateo et al., 2017). Our results are also consistent with data obtained in a different fly model for PD (*park*^{25}), where microbiota analysis was carried out from whole animals (Parker-Character et al., 2021). Although the material to start with and the procedure for analysis are different, the results are consistent in that they show differential composition of microbiota in control and the PD models. Thus, several studies are coherent in showing differences in microbiota composition in fly PD models as compared to their controls. Future studies should also take into account the compositional nature of microbiome datasets, as discussed elsewhere.

In particular, Proteobacteria was the most abundant in both genotypes, although their relative proportion was higher in *w*^{1118} compared to mutant flies; as it has been already noted, this is the most common phylum for wild-type flies (Broderick and Lemaitre, 2012). The most radical differences observed in this study were found in relative abundance at the family level in the phylum Proteobacteria. In *Pink1*^{B9} flies, Acetobacteraceae dominated with almost 50% of the total microbial community, while its abundance does not exceed 25% in control flies (Fig. 1A). The Acetobacteraceae family includes *Acetobacter pomorum* and *Acetobacter pasteurianus*, which are described as the most dominant strains in fruit fly strains at laboratories worldwide (Chiang et al., 2022; Wong et al., 2013). Interestingly, inhibiting the growth of *Acetobacter pomorum* results in the rescue of motor and neuronal defects in the

Pink1-null fly mutant, as recently shown Xu et al. (2020).

Overall our results and previous evidence support the idea that at premotor stages there are differences in intestinal microbiota composition and abundance of bacterial species, which could be considered a condition of gut basal dysbiosis. This characterization is already very interesting, but future studies should assess whether the differences in gut microbiota composition at premotor stages could be associated with the progression of motor phenotypes associated with this PD fly model.

4.2. Chronic kanamycin treatment as a tool to affect gut microbiota

Flies were exposed to a chronic kanamycin treatment in order to kill most bacteria in the gut of flies. There are no reports regarding the eventual noxious effects of this antibiotic on invertebrate animals. Due to this, it was first evaluated whether kanamycin could affect the survival of both control and *Pink1*^{B9} flies. Kanamycin did not have any effect on the survival of flies, suggesting it is non-toxic at the concentration used in this work (Fig. 2B and C).

Kanamycin is able to act on different types of microorganisms including gram negative, gram positive and mycoplasma. Even more, it could be used to kill intestinal fly pathogens like *P. entomophila* (Jin et al., 2017). We showed that feeding flies kanamycin apparently eliminates bacteria from the midgut in both *Pink1*^{B9} and control flies (Supplementary Fig. S2 and Supplementary Fig. S3). On the other hand, stopping antibiotic treatment for two weeks is enough for recovering bacteria in the midgut (Supplementary Fig. S4). All these data suggest that kanamycin is a safe, non-toxic, antibiotic with the potential to affect gut microbiota.

4.3. Behavioral characterization of premotor stages in the Pink1^{B9} Drosophila model for PD

Impairment in the execution of motor programs is the most well-known symptom of PD. Nonetheless, the molecular mechanism associated with non-motor symptoms involved in the progression of PD remains largely unexplored. Hyposmia is a promising biomarker of the premotor stage of PD; however, it is a poorly understood phenomena (Sui et al., 2019). This symptom is highly represented in PD cases in the prodromal stage. Although some investigators report that impaired olfaction is independent of the disease stage, others describe that it is associated with disease severity, rapid progression and dementia (Kalia and Lang, 2015; Schapira et al., 2017). Some studies have suggested that olfactory testing could be useful to diagnose PD in the premotor stage, but confirmation with additional studies would still be necessary (Reichmann, 2017). Moreover, fluctuations in anxiety levels are understood as a symptom of several neurological and neurodegenerative human diseases. Particularly, it has been shown that the average prevalence of an anxiety disorder in people with a PD diagnosis is 31% or more depending on the criteria utilized to measure this behavioral feature (Broen et al., 2016; Marinus et al., 2002). It is poorly understood why an important proportion of PD patients exhibit anxiety disorder or the reason anxiety is so prominent in premotor stages in people that will receive a PD diagnosis later in life (Kalia and Lang, 2015; Schapira et al., 2017; Thobois et al., 2017). Research in animal models aimed at studying premotor stages of PD should provide new insights on these issues and the value of early alterations in olfaction and anxiety as tools for a timely PD diagnosis.

Considering this, here we evaluated olfactory responses and also centrophobism, a proxy to anxiety, in the *Pink1*^{B9} mutant at different time windows. These are two behavioral features associated with non-motor symptoms in presymptomatic PD. Hyposmia has been already reported in early, non-motor stages of several PD models in flies, including the *Pink1*^{B9} mutant (Chen et al., 2014b; De Rose et al., 2016; Molina-Mateo et al., 2017). On the contrary, little information exists on centrophobism in fly models for PD. In addition, we recorded activity time, speed and distance traveled, three motor parameters that helped us to sustain that we were working at premotor stages in the PD model.

4.4. Kanamycin treatment restores anxiety levels in pre-motor stages

Anxiety is a pre-existent disorder in several cases of PD, including patients with autosomal-recessive early-onset Parkinsonism due to *Pink1* mutations (Ephraty et al., 2007; Ricciardi et al., 2014). Here, we described for the first time that as compared with control animals which exhibit centrophobism, young *Pink1*^{B9} mutant flies exhibit decreased anxiety-like behavior. Interestingly, our data also show that as mutant flies age, it is possible to detect an increase in centrophobism. On the contrary, control animals reduced their anxious behavior over aging (Fig. 4). Our findings support increased anxiety behavior in aging *Pink1*^{B9}, which is similar to the description of increased centrophobism in the α-synuclein fly mutant model for PD, as these animals age (Chen et al., 2014a). These results are also similar to those observed in rat models for early-onset PD (*Pink1*−/−), where animals begin exhibiting changes in anxious behavior as they age (Marquis et al., 2020). Thus, the increase in centrophobism in aging *Pink1*^{B9} flies is consistent with what has been described in other PD models and also similar to what has been reported in a percentage of people at prodromal PD stages: anxious behavior becomes evident by ~10 years before a diagnosis.

The differences in centrophobism observed between control and mutant animals disappear after antibiotic treatment (Fig. 5). This seems to be explained by differential effects of kanamycin on the two fly strains. Some bacteria (Lactobacillus and Bifidobacterium) influence brain neurotransmitter levels (Skolnick and Greig, 2019; Strandwitz, 2018). Thus, it is possible that the dramatic alteration in the composition of the gut microbiome in flies triggered by the antibiotic induces changes in neurotransmitters that could underlie effects on centrophobism. On the other hand, it is known that anxiety and depressive disorders are characterized by a lower abundance of short-chain fatty acid (SCFA) produced by gut bacteria. The net production of these metabolites depends on the synthesis and metabolism of specific bacteria phylum including proteobacteria (Simpson et al., 2021). Interestingly, differences in the relative abundance of this bacteria phylum were found in *Pink1*^{B9} mutant animals as compared to control flies (Fig. 1A–B). Thus, future studies could be directed to assess whether differences in gut composition between fly genotypes or eventual dynamic changes in gut microbiota composition over time contribute to the behavioral outputs here described.

4.5. Kanamycin treatment recovers innate olfactory discrimination in the early stages of the PD model

It has been reported olfactory dysfunction in PD patients. Importantly, asymptomatic heterozygotic carriers of mutations in the *Pink1* gene also exhibit olfactory dysfunctions, suggesting an underlying preclinical process (Eggers et al., 2010; Ferraris et al., 2009). Several articles support the idea that gut, oral, and/or nasal dysbiosis could be a factor on taste and smell impairment in PD patients (Pereira et al., 2017; Shen, 2020). Studies proposed that alterations in nasal microbiome is part of the cause for smell deficiency in PD, although data is not conclusive (Melis et al., 2021).

Our results show that two days or one week of kanamycin treatment restores olfactory behavior in the *Pink1*^{B9} PD model to levels observed in control animals (Fig. 3). In flies, intestine microbiota modulates aversive olfactory responses by regulation of the octopamine pathway in the gut. This is an aminergic neurotransmitter that is considered the physiological orthologue of noradrenaline in flies, and which is related to attention, stress, and fight or flight responses (O'Donnell et al., 2020). On the other hand, gut dysbiosis changes food preference in *Drosophila melanogaster*, a behavior that relies on smell (Qiao et al., 2019). This suggests that gut microbiota and the sense of smell are closely related. However, the exact mechanism underlying this interaction remains unknown.

Our results show that olfactory responses are not affected by

kanamycin treatment in control animals, but they are increased in *Pink1*^{B9} flies fed the antibiotic to the levels recorded in control animals (Fig. 3). This would suggest that the basal dysbiosis we report in *Pink1*^{B9} flies inhibits olfactory responses in flies, and that the kanamycin treatment restores this behavior. It is a possibility that dysbiosis can induce changes in the physiology of flies, that involve the modification of circuits related to the perception and learning about odorants, as it has been previously proposed (Sayin et al., 2018). In this sense, the kanamycin treatment would restore fly physiology in *Pink1*^{B9} flies to control conditions, thus increasing olfactory responses to levels recorded in control flies. More experiments are needed to test this hypothesis. Additionally, the length of the kanamycin treatment could cause some antibiotic resistance or microbial adaptation at the last time window studied which would explain why we did not detect any effect of the antibiotic over olfactory discrimination (Fig. 3).

Future experiments should be aimed at assessing whether centrophobism and olfactory responses are associated to one or few bacteria species, which could inform on future treatments directed to easy these non-motor PD symptoms and hopefully slow-down disease progression.

As it is possible to suggest that a change in fly locomotion could explain the performance of flies in the non-motor assays here described, we also studied locomotion in the animals of interest at premotor stages. We detected no changes in locomotion that could affect the interpretation of the results obtained in non-motor behaviors (Fig. 6).

4.6. Kanamycin treatment in young animals results in increased locomotor activity in aged control flies

There is evidence about the increase in locomotor activity in germ-free vertebrate and invertebrate animals (Diaz Heijtz et al., 2011; Schretter et al., 2018). Since our results support that the kanamycin treatment kills most of bacteria in the gut, we were expecting that young flies fed the antibiotic could exhibit changes in motor output. However, we did not find strong evidences that support that kanamycin treatment affects locomotion in young animals, regardless of the genotype studied. Though, our results evidenced that kanamycin treatment in young control animals improves the ability of flies to move when they become older flies, even weeks after the antibiotic treatment has ended (Fig. 7). This supports the idea that elimination of gut microbiota in young animals influences brain functioning at long time periods, particularly the execution of motor programs. Since older control and mutant animals exhibit reduced locomotion over aging (Molina-Mateo et al., 2017), our data would help propose a very radical idea: kanamycin treatment is protective against the reduction in locomotion caused by aging. Future studies should corroborate these results and advance on possible mechanisms.

This work advances our understanding on the premotor stage in the *Pink1*^{B9} *Drosophila melanogaster* PD model, while it characterizes the microbiota of *Pink1*^{B9} at 8–9 days post eclosion as compared to control animals. These new insights point out a temporal window where it would be possible to modulate the progression of different behavioral features of PD models. In this regard, our data support that kanamycin treatment at the premotor stage was successful at recovering olfactory and anxiety-like behaviors. Surprisingly, a two-weeks treatment with the antibiotic induces long-term effects: control 29–30 days old flies that were treated with kanamycin during their early life exhibit increased motor performance. All this data supports the idea that modification of gut microbiota could be used as a non-invasive treatment for some of the behavioral symptoms observed in PD and also as an approach to protect from the age-dependent reduction in locomotion.

Funding

This work was supported by FONDECYT Regular grants 1141233 and 1231556 (JMC), 1191424 (CO) and Center of Genome Regulation (CGR) ANID/FONDAP/15200002 (BD). DMM, RVZ, SH and JTL were suported by ANID Doctoral fellowships (folio Nos. 21170543, 21160214, 21161611 and 21171048, respectively). DMM was also supported by Programa de apoyo a tesis doctoral, Facultad de Medicina, Pontificia Universidad Católica de Chile.

Data availability

Data will be made available on request.

Acknowledgements

We thank all members of the Diez laboratory for helping with molecular biology determinations and of the Campusano laboratory for comments, suggestions, and ideas. We also thank Dr. Luis Castañeda for initial help on this project. We would also like to acknowledge and thank the work of reviewers and editors on comments and ideas on our manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2023.109573.

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