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Inhibition of *foxo* and *minibrain* in Dopaminergic Neurons Can Model Aspects of Parkinson Disease in *Drosophila melanogaster*

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Abstract

Symptoms of Parkinson Disease (PD), the second most common neurodegenerative disease, emerge due to degeneration of dopaminergic neurons. Recently, a genome wide study revealed a role for a *foxo* transcription factor in PD. In the model organism *Drosophila melanogaster*, we have attempted 1) to inhibit the sole *Drosophila* homologue of *foxo* through the directed expression of a stable inducible RNAi transgene and 2) to indirectly increase *foxo* transcription activity through the inhibition of the kinase *minibrain* (*mnb*), a *foxo* transcriptional inhibitor. To evaluate the lifetime consequences upon the flies, longevity assays and locomotion over time assays were conducted. The inhibition of *foxo* by *foxo-RNAi* decreases life span significantly when expressed under the control of *Tyrosine Hydroxylase-Gal4* (*TH-Gal4*). The targeted expression of *mnb-RNAi*, in the dopaminergic neurons, with an expected loss of suppression of *foxo* transcriptional activity, results in a significant loss of climbing ability. Thus alteration of *foxo* activity, both by RNA-inhibition and by down-regulation of an inhibitor of *foxo*, *minibrain*, produces novel *Drosophila* models of Parkinson Disease.

Keywords

Drosophila melanogaster*, Model of Parkinson Disease, *foxo*, *minibrain

1. Introduction

Parkinson Disease (PD), only surpassed by Alzheimer Disease, is the second most common human neurodege-

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nerative disease and the most prevalent neurodegenerative movement disorder [1]. Pathologically, PD is characterized by the loss of dopaminergic (DA) neurons in the ventral mesencephalic *substantia nigra pars compacta* and, usually, by the formation of Lewy bodies (aggregation of proteins including *α*-synuclein) in the neurons of ventral midbrain and some other regions such as the prefrontal cortex. The associated impairments often include resting tremor, rigidity, bradykinesia and postural instability. Inherited forms (autosomal-dominant and autosomal-recessive) of the disease account for 5% to 15% of all PD cases. Recently, variations in the *foxO*1 gene among others, have been implicated in PD [2]. As part of a widening investigation of the genetic basis of PD, the potential role of *foxo* in PD has become of interest.

The forkhead box subfamily "o", foxo, is one of the larger family of *forkhead* genes that encode a class of winged helix-turn-helix proteins which act as transcription factors that control homeostasis in response to external influences including variation in growth factor availability and various stresses (for review see [3]). There are 4 mammalian *foxo* members (*foxO*1, *foxO*3, *foxO*4 and *foxO*6 [4], one homologue in *C. elegans* (*daf*-16) [5] and one homologue in *Drosophila melanogaster* (*foxo*) [6]. The behaviour of the *foxo* proteins is modified through various post-transcriptional modifications such as acetylation, ubiquitination and phosphorylation. The akt kinase can phosphorylate *foxo* to exclude the transcription factor from the nucleus [7]. Another kinase, dual-specificity tyrosine-phosphorylation regulated kinase 1a, Dyrk1a can also phosphorylate *foxo* [8]. Dyrk1a is located within the Down Syndrome Critical Region of human chromosome 21 and its *Drosophila* homologue, *minibrain* (*mnb*), is well conserved [9] [10]. Dyrk1a/mnb can phosphorylate *foxo* to sequester it from the nucleus to suppress transcriptional activity.

Drosophila melanogaster has been proven to be an excellent organism in which to model Parkinson Disease (for reviews see [11] [12]). The first fly model of PD was established through the directed expression of human alpha-synuclein and subsequent loss of dopaminergic neurons [13]. Subsequently, a number of genes implicated in PD have been manipulated in this model including Parkin/PARK2 [14] [15] and Pink1/PARK6 [16]-[18]. As the role of *foxo* proteins seemed to be very well conserved and considering the potential role in PD, these experiments were undertaken in the *Drosophila* model system to take a closer look at *foxo* modulation in the modeling PD.

2. Materials and Methods

2.1. Drosophila Culture and Stocks

Dr. J. Hirsh (University of Virginia) provided the *ddc-Gal*4 (*dopa decarboxylase-Gal*4$^{HL4.36}$) transgenic line. The *TH-Gal*4 (*Tyrosine Hydroxylase-Gal*4^{3}/*ple-Gal*4^{3}; BDSC-8848); *UAS-lacZ* (*UAS-lacZ*$^{4\text{-}2\text{-}1}$; BDSC-1776) and *UAS-mnb-RNAi* ($y^{1}sc\ v^{1}$; *P*{*y v*; *TRiP.GL*00104/*mnb*^{GL00104}}*attP*2; BDSC-35222) lines were obtained from the Bloomington Drosophila Stock Center at Indiana University. The *UAS-foxo-RNAi* (*P*{*KK*108485/*foxo*^{KK108485}} *VIE*-260*B*; VDRC-106097) was obtained from the Vienna Drosophila Resource Center. All crosses were performed using standard techniques and cultured on standard cornmeal-yeast-molasses-agar media at 25˚C. Directed expression of transgenes in dopaminergic neurons was achieved by crossing males of the responding lines to *TH-Gal*4 and *ddc-Gal*4 females and males of each critical class were assayed.

2.2. Longevity Assay

Longevity assays were performed on critical class males collected under carbon dioxide every 24 hours. Due to the well-established variation in ageing females, only males were analyzed. Approximately 200 male flies were introduced into fresh food without anaesthesia three times a week and kept in numbers of no more than 20 per vial to prevent overcrowding. As they aged, flies were monitored for viability until all perished [19]. Results were analyzed with GraphPad Prism 5. For survival results, the statistical test of Mantel-Cox was carried out.

2.3. Locomotor Assay

A standard climbing assay every 6 or 7 days, beginning at day 6 or 7, during the lifespan of the critical class males was carried out by following a standard protocol [20]. For the locomotor assay the results were analyzed by GraphPad Prism 5 with an unpaired t-test was carried out to detect any significant differences between means of groups and the slopes of the curves with non-overlapping 95% CI were considered significantly different.

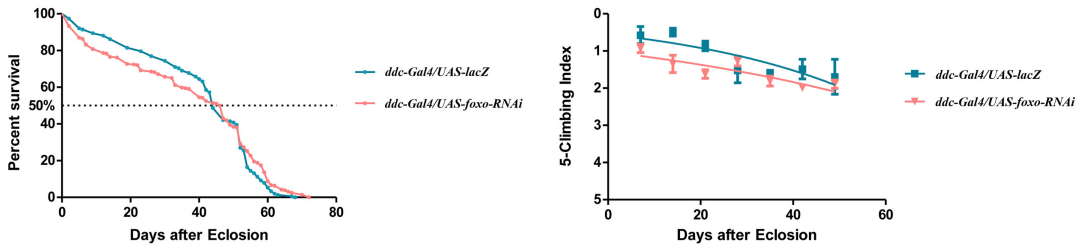
3. Results

3.1. *foxo-RNAi* Expression under the Direction of *ddc-Gal4* Does Not Alter Lifespan or Climbing Ability

To inhibit the expression of *foxo*, the inducible transgene *UAS-foxo-RNAi* was placed under the control of the *ddc-Gal4* transgene. As a control, the benign responder gene *UAS-lacZ* resulted in a fairly standard sub-optimal longevity response with a median lifespan of approximately 42 days when directed by *ddc-Gal4*. When *foxo-RNAi* was expressed under the control of the *ddc-Gal4* transgene, it did not alter the median lifespan nor the overall longevity characteristics compared to the control (**Figure 1(a)**). Likewise, the measurement of the loss of locomotor function as monitored by the evaluation of climbing ability over time was not altered when *foxo-RNAi* was directed by *ddc-Gal4* (**Figure 1(b)**).

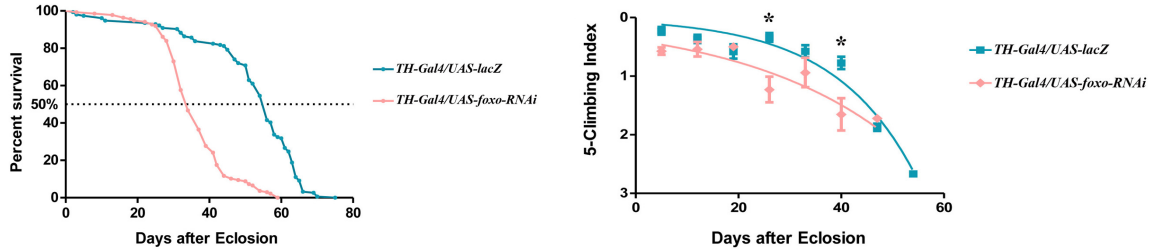
3.2. The Expression of *foxo-RNAi* Directed by *TH-Gal4* Reduces Lifespan Greatly but Alters Climbing Ability over Time Slightly

The inhibition of *foxo* through expression of the *UAS-foxo-RNAi* in dopaminergic neurons under the control of the *TH-Gal4* transgene reduced lifespan significantly but resulted in a slight alteration in climbing ability over time. The median lifespan of *TH-Gal4/UAS-lacZ* males was measured to be 58 days whereas the median length of life for the *TH-Gal4/UAS-foxo-RNAi* male flies was 38 days (**Figure 2(a)**). In analysis of climbing over time, a pair-wise comparison of climbing ability overtime for the two genotypes (**Figure 2(b)**) shows that their climbing ability was different at days 26 and 40.



(a) (b)

Figure 1. The expression of *foxo-RNAi* under the control of *ddc-Gal4* does not alter lifespan or climbing ability in *D. melanogaster* males at 25˚C. (a) Longevity assays were performed on *ddc-Gal4/UAS-lacZ* and *ddc-Gal4/UAS-foxo-RNAi* critical male flies (initial n = 200 for both) and the pairwise comparison does not reveal any significant difference. Both genotypes displayed a median lifespan of 42 days; (b) Critical class males of the two genotypes (*ddc-Gal4/UAS-lacZ* and *ddc-Gal4/UAS-foxo-RNAi*) were analyzed by a locomotion assay over time (initial n = 70 was to reduced to a minimum of 5 through lethality) and non-linear regression curve was fitted to best demonstrate the climbing over time pattern. The 95% CI slopes overlap indicating any difference caused is likely due to chance.



(a) (b)

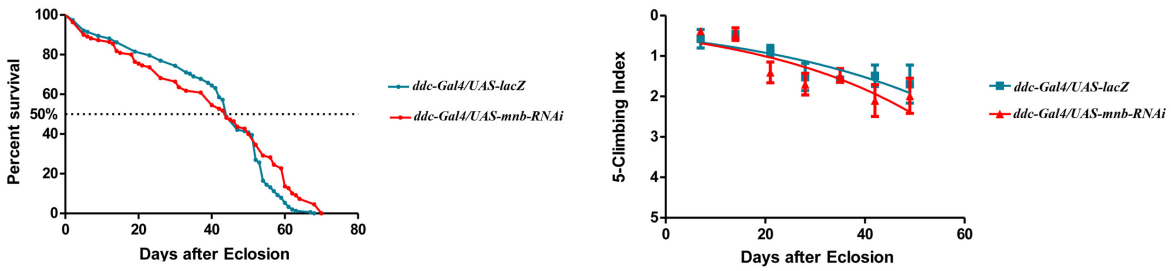
Figure 2. The expression of *foxo-RNAi* directed by *TH-Gal4* reduces lifespan greatly and climbing ability slightly in *D. melanogaster* males at 25˚C. (a) The *foxo-RNAi* transgene induced in dopaminergic neurons under the control of *TH-Gal4* transgene (*TH-Gal4/UAS-foxo-RNAi*) reduces lifespan of critical class males significantly compared to the control (*TH-Gal4/UAS-lacZ*) as supported by Mantel-Cox test results (initial n = 200 for both); (b) Comparison of climbing ability over time for *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-foxo-RNAi* shows that the pattern of their climbing ability is slightly different, notably at days 26 and 40 (initial n = 70 was to reduced to a minimum of 5 through lethality). Error bars represent standard error of mean and an asterisk indicates a significant difference.

3.3. Expression of *mnb-RNAi* Directed by *ddc-Gal*4 Does Not Alter Lifespan or Climbing Ability

The *ddc-Gal*4 transgene was used to direct the expression of the interfering *mnb-RNAi* transgene in the dopaminergic neurons, along with serotonergic neurons and other cells. The *UAS-lacZ* benign responding transgenics as the control, and *ddc-Gal*4/*mnb-RNAi* flies have median lifespans of 42 days and both genotypes (*ddc-gal4/UAS-lacZ* and *ddc-Gal*4/*UAS-mnb-RNAi*) live equally well up to approximately day 70 (**Figure 3(a)**). The Mantel-Cox statistical test did not reveal any significant difference in their survival pattern. Non-linear regression curves of climbing ability over time for the two genotypes, *ddc-Gal*4/*UAS-lacZ* compared to *ddc-Gal*4/*UAS-mnb-RNAi*, reveal little difference (**Figure 3(b)**). Results of statistical analysis of this measurement of locomotion over time did not reveal any significant early loss of climbing ability.

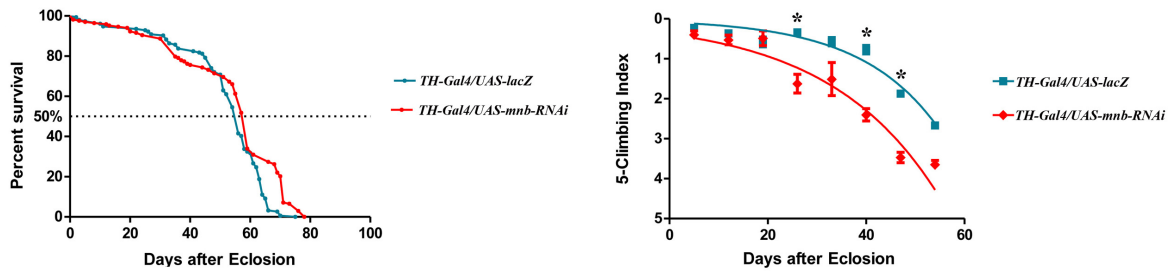
3.4. Expression of *mnb-RNAi* Directed by *TH-Gal*4 Significantly Diminishes Climbing Ability over Time

With the expression of the inhibitory *UAS-mnb-RNAi* under the directed control of the *TH-Gal*4 transgene to the dopaminergic neurons, a very significant decrease in climbing ability over time was observed (**Figure 4(a)**). Analysis of ageing of these flies shows that there was no significant difference in lifespan of the two genotypes, and, therefore, inhibition of *mnb* does not alter greatly the survival of the flies. The median lifespan of flies expressing *TH-Gal*4 directing the expression of the control responding transgene *UAS-lacZ* or the *UAS-mnb-RNAi* both produced a median life span of 58 days with some alive until day 78. However, the climbing ability over time, as illustrated in **Figure 4(b)**, demonstrates a significant loss when *mnb-RNAi* is expressed in dopaminergic neurons directed by *TH-Gal*4. Pairwise, day-to-day comparison of climbing ability over time for two genotypes



(a) (b)

Figure 3. The expression of *mnb-RNAi* directed by *ddc-Gal*4 did not alter lifespan or climbing ability in *D. melanogaster* raised at 25˚C. (a) Longevity assay were performed on *ddc-Gal*4/*UAS-lacZ* and *ddc-Gal*4/*UAS-mnb-RNAi* critical male flies (initial n = 200 for both) and do not reveal significant differences in lifespan when analysed by Mantel-Cox to detect significant differences in survival pattern. Both genotypes gave a median lifespan of 42 days; (b) The expression of *mnb-RNAi* directed by *ddc-Gal*4 did not change climbing ability over time compared to the *ddc-Gal*4/*UAS-lacZ* control (initial n = 70 was to reduced to a minimum of 5 through lethality).



(a) (b)

Figure 4. The expression of *mnb-RNAi* in dopaminergic neurons using *TH-Gal*4 transgene does not alter lifespan but decreases climbing ability significantly in *D. melanogaster* raised at 25˚C. (a) Lifespan was not reduced or increased when *mnb-RNAi* is expressed under the control of *TH-Gal*4 transgene (initial n = 200); (b) Climbing ability was significantly reduced at days 26, 40 and 47 when *mnb-RNAi* was induced in dopaminergic neurons by the *TH-Gal*4 transgene (initial n value = 70). Error bars represent standard error of mean and asterisk indicates significant difference.

of *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-mnb-RNAi* flies revealed that the decline in climbing ability in day 26, 40 and 47 is significant.

4. Discussion

In this study, the *foxo-RNAi* transgene was utilized to directly decrease *foxo* expression and the *mnb-RNAi* transgene to induce a slight, indirect elevation in *foxo* activity, among other effects. The expression of *foxo-RNAi* in dopaminergic neurons gives two distinct results: 1) expression of *foxo-RNAi* directed by *ddc-Gal*4 does not alter life span or climbing ability compared to the control; 2) expression of *foxo-RNAi* under the control of *TH-Gal*4 decreases lifespan significantly but does not alter the locomotion of the surviving flies greatly over time. Reduced life span of *TH-Gal4/foxo-RNAi* flies points to a protective role for *foxo* against organismal death. This greatly reduced viability may model severe aspects of early onset PD in Drosophila although the survivors maintain the ability to move.

Expression of the *mnb-RNAi* transgene in dopaminergic neurons exhibits in two very different results: 1) directed by *ddc-Gal4/mnb-RNAi* expression does not alter lifespan or longevity compared to that *UAS-lacZ* control (**Figure 3**) and 2) expression of *mnb-RNAi* directed by the *TH-Gal*4 transgene, climbing ability was lost over time but the lifespan was not greatly changed (**Figure 4**). The difference in expression presented by the two transgenes may account for the observed difference in results; the *ddc-Gal*4 transgene directs the expression of Gal4 and hence the gene under the control of *UAS* element differently than that of the *TH-Gal*4 transgene. The dopa decarboxylase enzyme is synthesized in the 150 dopamine and serotonin neurons, in a subset of glial cells and in the most hypodermal cells whereas tyrosine hydroxylase is produced in dopamine synthesizing cells [21] [22]. An alternative difference may be that the expression of *Gal*4 may not be as robust under *ddc-Gal*4 control compared to the *TH-Gal*4 transgene. The significant decrease in locomotor activity in these flies may reflect the slight elevation of *foxo* activity caused by inhibition of *mnb* in dopaminergic neurons. If so, it is consistent with the results the recent genome wide study [2] indicating a role for a *foxo* in P.D. Finally, the inhibition of *mnb* under the control of *TH-Gal*4 has produced a novel model of Parkinson Disease in Drosophila.

5. Conclusion

In conclusion, the inhibition of *foxo* through directed RNA-inhibition directed by *TH-Gal*4 in the dopaminergic neurons can significantly reduce lifespan in *Drosophila melanogaster*. Most importantly, the inhibition of *mnb*, which encodes a kinase that negatively regulates *foxo* transcriptional activity in the dopaminergic neurons can model aspects of Parkinson Disease in flies by a significant premature reduction in locomotor ability over time.

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Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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