Co-expression of *α-synuclein* in *Drosophila* dopaminergic neurons does not affect lifespan reduction resulting from *PI3K* overexpression.

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Introduction

The phosphatidylinositol-3-kinase (PI3K)/Akt pathway is a ubiquitous and evolutionarily conserved signaling cascade that functions in cell growth, survival, proliferation, migration, and metabolism (Endersby and Baker, 2008). PI3K is a lipid kinase that phosphorylates the three position of the inositol ring of phosphatidylinositols and phosphoinositides (Engelman, *et al.*, 2006). A major downstream target of PI3K signaling is the serine-threonine kinase AKT, which, once activated, results in an anti-apoptotic or cell survival signal. Cell culture experiments have demonstrated the rescue of α-synuclein toxicity with activation of the PI3K pathway (Kao, 2009), suggesting that activation of this pathway may have broad protective effects.

In *Drosophila melanogaster*, as in mammals, PI3K plays a role in the conserved insulin signaling pathway, regulating metabolism, growth, and lifespan (Oldham and Hafen, 2003). This pathway is negatively regulated by a PTEN orthologue. Previous experiments show that deletion of *PI3K* in *Drosophila* results in a reduction in cell size and cell number, whereas deletion of *PTEN* leads to an increase in cell size and organ size (Engelman *et al.*, 2006). In contrast, we have previously shown an unexpected reduction in lifespan with overexpression of *PI3K* in the dopaminergic neurons of *Drosophila* (Saunders *et al.*, 2003). Although unexpected, other studies have shown apoptosis with overexpression of *PI3K* (Klippel *et al.*, 1998; Vanhaesebroeck *et al.*, 2001). This suggests a sensitivity in specific cell types may exist, likely sensitivity to the deregulation of the cell cycle.

Given the conflicting role of PI3K in the survival of dopaminergic neurons, we looked at the reproducibility and the possible effect that an additional stressor, in the form of *α-synuclein* overexpression, may have on the previously observed reduced lifespan.

Materials and Methods

Fly stocks and culture: Dr. M. Feany (Harvard Medical School) generously provided *UAS-α-synuclein* flies (Feany and Bender, 2000), Dr. J. Hirsh (University of Virginia) the *Ddc-Gal4* flies (Li *et al.*, 2000), Dr. Sally Leevers (University College, London) the *UAS-PI3K-dp110* (*PI3K*$^{+}$) and *UAS-PI3K-dp110*^{D954A} (*PI3K*^{DN}) flies (Leevers *et al.*, 1996), and the w^{1118} line was provided by Dr. H. Lipshitz (University of Toronto). All flies were raised upon standard cornmeal/yeast/molasses/ agar medium at 25°C in standard plastic shell vials.

Aging assay: Approximately 300 adult males of each genotype were collected under gaseous carbon dioxide and aged upon standard cornmeal/yeast/molasses/agar media, at 25°C, in upright standard plastic shell vials. Flies were maintained in non-crowded conditions with one to twenty individuals per vial. Flies were scored for viability every two days and transferred to fresh media

according to established protocol (Staveley *et al.*, 1990). Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test were used to compare resulting survival curves.

Results and Discussion

Overexpression of *PI3K*$^{+}$ using the *Ddc-Gal4* tissue specific driver showed a dramatic reduction of lifespan, with a median survival of 46 days as compared to 80 days seen in the control (Log-rank and Wilcoxon: $P < 0.0001$) (Figure 1). In contrast, expression of the *PI3K*^{DN} showed a normal life span, median survival of 78 days, as compared to the control (Wilcoxon: $P = 0.4495$). As mentioned previously, these results are in contrast to the typical role of the PI3K signaling pathway, as an initiator of cell growth and proliferation. This is likely due to sensitivity in the dopaminergic cells with respect to deregulation of cell cycle controls. In post-mitotic cells, including those in *Drosophila*, PI3K signaling has been shown to control cell growth (cell size) as opposed to proliferation (Engelman *et al.*, 2006). Cells may be triggered to undergo apoptosis due to failure to progress through cycle (Klippel *et al.*, 1998; Vanhaesebroeck *et al.*, 2001). It also appears that *Drosophila* lifespan is limited by metabolic activity and oxidative damage to the nervous system in particular, opposed to other tissues (Shmookler Reis *et al.*, 2009). Therefore, targeting of *PI3K* expression to these sensitive cells may show an effect that is otherwise unnoticed during expression in other tissues.

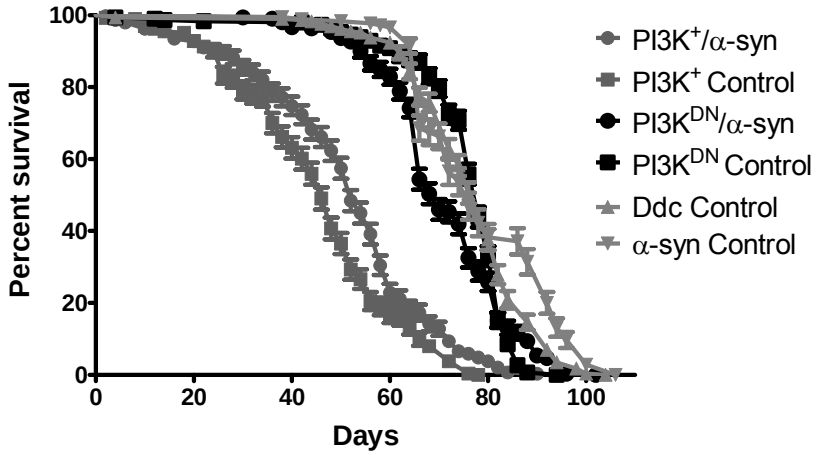

Figure 1. Survival curves of *Drosophila* overexpressing *PI3K*$^{+}$ or *PI3K*^{DN} with or without the co-overexpression of *α-synuclein*, in the dopaminergic neurons, aged on a standard medium. Genotypes are *UAS-PI3K*$^{+}$*/ UAS-α-synuclein; Ddc-Gal4/+* (PI3K^{+}/ α-syn; n = 289), *UAS-PI3K*$^{+}$*/ +; Ddc-Gal4/+* (PI3K^{+} Control; n = 263), *UAS-PI3K*^{DN}*/ UAS-α-synuclein; Ddc-Gal4/+* (PI3K^{DN}/ α-syn; n = 298), *UAS-PI3K*^{DN}*/+; Ddc-Gal4/+* (PI3K^{DN} Control; n = 294), *+/+; Ddc-Gal4/+* (Ddc Control; n = 287), and *UAS-α-synuclein/+; Ddc-Gal4/+* (α-syn Control; n = 175). Error bars indicate standard error of the mean.

The PI3K pathway involves many levels of regulation including various negative feedback mechanisms (Reviewed in Engelman *et al.*, 2006). In addition, activation of downstream transcription factors can have various effects including the activation of protective proteins such as PTEN induced punitive kinase 1 (Pink1) (Mei *et al.*, 2009). *Pink1* has been shown to rescue an *α-synuclein* induced phenotype in *Drosophila* (Todd and Staveley, 2008), presumably through downstream tagging and degradation of α-synuclein. It was not found that this additional cell stressor of α-synuclein overexpression contributes to the PI3K induced reduction in longevity. Overexpression of *α-synuclein* with *PI3K*^{DN} using the *Ddc-Gal4* tissue specific driver does not have a substantial effect on lifespan, median survival 70 days, as compared to overexpression of *PI3K*^{DN}

alone, median survival 78 days (Log-rank: $P = 0.0677$) (Figure 1). Overexpression of of *α-synuclein* with $PI3K^{+}$ shows a slight increase in lifespan, median survival 52 days, compared to overexpression of $PI3K^{+}$ alone, median survival 46 days (Log-rank and Wilcoxon: $P < 0.0001$). This, however, is not rescued to the level of the *α-synuclein* or *Ddc-Gal4* controls (Log-rank and Wilcoxon: $P < 0.0001$). The engagement of protective proteins such as Pink1 during α-synuclein cytotoxicity may not have enough of an effect to increase the *PI3K* phenotype; alternatively, the detrimental effects of *PI3K* may be too severe on its own, or is initiated before *α-synuclein* induced cytotoxicity takes effect.

In conclusion, it appears that, in dopominergic neurons, overexpression of $PI3K^{+}$ results in a severe decrease in lifespan, likely due to the sensitivity of this particular cell type coupled with the inability of these cells to progress through cell cycle, thereby triggering apoptosis. A substantial difference was not observed with the addition of *α-synuclein* overexpression, and therefore, no conclusion can be made regarding possible links between the PI3K signaling pathway and α-synuclein cytotoxicity.

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Copulation duration in *Drosophila melanogaster* reared on different diets: a multiple choice test.

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Duration of copulation in *Drosophila* is a species-specific trait, which considerably varies among different species (Spieth, 1952; Grant, 1983; Kraaijeveld *et al*., 2008). Variation in this trait was also recorded among geographical or inbred strains (for review see Hirai *et al*., 1999). Mating duration in *Drosophila* is related to many traits, like courtship vigor (Gromko *et al*., 1991), fertility (Gromko *et al*., 1991; Singh and Singh, 1999), female fitness (Friberg, 2006), and paternity (Gilchrist