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Expression of human uncoupling protein-3 in *Drosophila* insulin-producing cells increases insulin-like peptide (DILP) levels and shortens lifespan

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ABSTRACT

Uncoupling proteins (UCPs) can dissipate mitochondrial protonmotive force by increasing the proton conductance of the inner membrane and through this effect could decrease ROS production, ameliorate oxidative stress and extend lifespan. We investigated whether ubiquitous, pan-neuronal or neurosecretory cell-specific expression of human UCP3 (hUCP3) in adult *Drosophila melanogaster* affected lifespan. Low, ubiquitous expression of hUCP3 at levels found in rodent skeletal muscle mitochondria did not affect proton conductance in mitochondria isolated from whole flies, but high pan-neuronal expression of hUCP3 increased the proton conductance of mitochondria isolated from fly heads. Expression of hUCP3 at moderate levels in adult neurons led to a marginal lifespan-extension in males. However, high expression of hUCP3 in neuronal tissue shortened lifespan. The life-shortening effect was replicated when hUCP3 was expressed specifically in median neurosecretory cells (mNSC), which express three of the *Drosophila* insulin-like peptides (DILPs). Expression of hUCP3 in the mNSC did not alter expression of *dilp2*, *dilp3* or *dilp5* mRNA, but led to increased amounts of DILP2 in fly heads. These data suggest that lowering mitochondrial coupling by high expression of hUCP3 alters mNSC function in a way that appears to increase DILP-levels in fly heads and lead to a concomitant decrease in lifespan.

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1. Introduction

The oxidative damage theory of ageing postulates that accumulated damage to macromolecules caused by reactive oxygen species (ROS) ultimately leads to tissue failure and death (Beckman and Ames, 1998). Most ROS produced in animal cells originate from the mitochondria, as a by-product of respiration. Electrons can escape from the respiratory chain and reduce molecular oxygen to form superoxide (Boveris and Chance, 1973; Boveris, 1977). Correlative evidence has come, for instance, from the finding that ROS-production from isolated mitochondria is associated with lifespan across five species of flies (Sohal et al., 1995; Sohal and Weindruch, 1996). Attenuation of ROS production could therefore be one way to ameliorate ageing-related damage. However, other studies have produced contrary findings (Guo et al., 2001; Orr et al., 2003; Bayne et al., 2005). Precise modulations of ROS-production may be required to ameliorate oxidative damage in a way that extends lifespan because ROS are also involved in signalling pathways (D'Autreaux and Toledano, 2007) and immune function (Bogdan et al., 2000). ROS-production by isolated mitochondria is highly sensitive to mitochondrial protonmotive force in both mammals (Liu, 1997; Korshunov et al., 1997; Lambert and Brand, 2004), and *Drosophila* (Miwa et al., 2003). Decreasing protonmotive force by mild uncoupling of mitochondria could attenuate oxidative damage and decrease the rate of ageing (Skulachev, 1996; Brand et al., 2004).

The uncoupling proteins (UCPs) are a family of mitochondrial proteins whose best-characterised function (in the presence of suitable activators) is to cause partial uncoupling of oxidative phosphorylation by dissipating the protonmotive force generated by the electron transport chain. The capacity of UCPs to circumvent endogenous oxidative stress was demonstrated in mitochondria from *Ucp3* knockout mice, which have significantly higher levels of oxidative damage than wild-type controls (Vidal-Puig et al., 2000; Brand et al., 2002). The presence of UCP3 was also found to protect the mitochondrial tricarboxylic acid cycle enzyme aconitase against inactivation by oxidative damage in vitro (Talbot and Brand, 2005). An increase in macrophage ROS production in $Ucp2^{-/-}$ mice (Arsenijevic et al., 2000) and the finding that pancreatic islets from $Ucp2^{-/-}$ mice show significantly raised mitochondrial membrane potential and superoxide production (Krauss et al.,

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2003) provide further evidence for in vivo ability of uncoupling proteins to lower levels of mitochondrial ROS. Furthermore, a recent study of over-expression of UCP1 in mouse skeletal muscle showed increased median (but not maximal) lifespan and alteration of several ageing-related pathologies (Gates et al., 2007).

Mitochondrial oxidative stress in neuronal tissue has been implicated in age-associated neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases (Jenner, 1993; Finkel and Holbrook, 2000; Wang et al., 2005). Based on observations in *Ucp2*$^{-/-}$ mice, it has been suggested that UCP2 has a role in helping to maintain normal dopaminergic neuronal function (Andrews et al., 2006). UCP2 over-expression significantly decreases infarct size after ischemia reperfusion brain injury in mice (Mattiasson et al., 2003), although, as with most UCP overexpression studies, it is unclear whether this represents a native or an artifactual activity of the UCP2 (Brand and Esteves, 2005). Over-expression of human Cu, Zn-superoxide dismutase in motor neurons of *Drosophila* has been reported to extend lifespan up to 40% in wild-type flies, and also to rescue lifespan by up to 80% in *Sod*$^{-/-}$ flies (Parkes et al., 1998), suggesting that neuronal superoxide production can limit lifespan (but see Ford et al., 2007). More specifically, it was found that inducible, adult-specific expression of hUCP2 in neuronal tissue of *Drosophila* can lead to extension of lifespan, supporting the hypothesis that attenuating production of ROS by mild uncoupling catalysed by UCPs can increase lifespan (Fridell et al., 2005). However, recent studies using cultured mammalian neurons suggest that mild uncoupling is not protective against ROS-production (Johnson-Cadwell et al., 2007; Tretter and Adam-Vizi, 2007).

In mammals, UCP2 also plays a central role as a negative modulator of glucose-stimulated insulin-secretion in pancreatic β-cells (Zhang et al., 2001). Deficiency of UCP2 increases coupling efficiency in clonal β-cells (Affourtit and Brand, 2008) and protects against hyperglycemic (Krauss et al., 2003) and lipid-induced β-cell dysfunction (Joseph et al., 2004). Insulin/insulin-like growth factor (IGF)-like signalling (IIS), which is conserved in the nematode worm, *Caenorhabditis elegans*, the fruit fly, *Drosophila*, and the mouse has recently gained attention for its involvement in the determination of lifespan (reviews: Gems and Partridge, 2001; Garofalo, 2002; Tatar et al., 2003; Giannakou and Partridge, 2007; Piper et al., 2008). Three of the *Drosophila* insulin-like peptides (DILPs) are secreted from the median neurosecretory cells (mNSCs) in the brain, and play a fundamental role in the IIS pathway in *Drosophila* (Ikeya et al., 2002; Rulifson et al., 2002; Giannakou and Partridge, 2007). Partial ablation of this small group of cells leads to a decreased expression of *dilp2*, *dilp3* and *dilp5* mRNA and up to 33% extension of lifespan (Broughton et al., 2005). The *Drosophila* genome does not contain orthologues of UCP2 or UCP3. Recently, however, a *Drosophila* homologue of the brain mitochondrial carrier protein 1 (dBMCP1) was characterised in the heterologous yeast system and was found to exhibit some properties associated with mitochondrial uncoupling (Fridell et al., 2005). There is still some debate as to whether the primary function of BMCP1 and the related UCP4 is mitochondrial uncoupling; especially since BMCP1 and UCP4 are more divergent and are located on the phylogenic tree at a greater distance from other uncoupling proteins, closer to oxoglutarate carriers. Problems commonly associated with overexpression of membrane spanning proteins giving false-positive mitochondrial uncoupling (Brand and Esteves, 2005) led Sánchez-Blanco et al. (2006) to examine the function of dBMCP1 in the fly. Upon knocking out dBMCP1 it was found that the mitochondria displayed the same respiration kinetics to control fly mitochondria and dBMCP1 appeared to play a more major role in metabolic homeostasis and control of metabolism during starvation stress (Sánchez-Blanco et al., 2006). Thus, the importance of mitochondrial mild uncoupling and ROS in insulin-secreting tissues of invertebrates awaits investigation.

In the present work, building on the work of Fridell et al. (2005) using human UCP2, we focused on the expression of human UCP3 (hUCP3) in *Drosophila*, which has not been studied previously in this model, and which has been shown to have a strong phenotype when overexpressed in mammals (Clapham et al., 2000). We used high ectopic expression of hUCP3 as a tool that is known to artificially uncouple mitochondria (Harper et al., 2002; Brand and Esteves, 2005) in order to investigate whether mild uncoupling caused by expression of human UCP3 in *Drosophila* (ubiquitously, pan-neuronally or specifically in the mNSCs) affects lifespan or DILP expression. We find that high, ubiquitous expression of hUCP3 does not increase lifespan, while adult-specific neuronal expression causes a marginal lifespan-extension only in males. In contrast, increasing the amount of neuronal hUCP3 expression to an extent that produces measurable increases in proton conductance results in increased DILP protein levels in head samples and dramatic shortening of lifespan, suggesting that mitochondrial coupling in the mNSCs affects DILP secretion.

2. Materials and methods

2.1. Fly maintenance and lifespan experiments

Drosophila melanogaster were reared and lifespan experiments were conducted on SY food: 50 g sucrose (Tate & Lyle Sugars, London, UK), 100 g brewer's yeast (MP Biomedicals, London, UK), 15 g agar (Sigma), 3 g Nipagin® M (methyl 4-hydroxybenzoate, Clariant UK Ltd., Pontypridd, UK) and 3 ml propionic acid (Sigma) per litre. Nipagin® M was added in a solution containing 100 g/L Nipagin® M in 95% ethanol. Ubiquitous drivers *actin-GAL4* (stock # 4414) and *da-GAL4* (stock # 8641) were obtained from Bloomington *Drosophila* Stock Center (Indiana, USA). *dilp2-GAL4* was obtained from Dr. Tomoatsu Ikeya (Ikeya et al., 2002; Broughton et al., 2005) and elav-GS was kind gift from Dr. Ronald Davis (Osterwalder et al., 2001). All transgenic lines (including *UAS-hUCP3* lines, below) were backcrossed to existing w^{1118} stock for 10–12 generations and tested negative for *Wolbachia* endosymbiont using PCR-based detection, as described in Toivonen et al. (2007). To generate experimental flies, age-matched parents were mated in 2–4 cages containing grape juice agar plates with live yeast paste. After 2 days of acclimatization, eggs laid during a 6 h window were collected, and the same volume of embryos (approximately 300 flies) was transferred to each rearing bottle. Newly eclosed flies were transferred to new bottles without anaesthesia and left for 48 h to mate. Sexes were separated by brief CO_2 exposure and the flies were transferred into experimental vials (with and without RU486), 10 flies per vial. Fresh food vials were provided three times per week and deaths were counted. Raw data from the lifespan studies is presented in Supplementary Table 2.

2.2. Generation of UAS-hUCP3 transgenic flies

hUCP3 cDNA was amplified and cloned in p{UAST} vector under the control of a yeast *GAL4* upstream activation sequence (*UAS*, Phelps and Brand, 1998). Four independent UAS-hUCP3 lines (2E-yw, 2E-or, 2H and 2A) were obtained by microinjection into w^{1118} embryos using standard methods (Spradling, 1986). To produce the *UAS-hUCP3-high* strain, females from line 2A were crossed with males from line 2H (both in chromosome 3). Transheterozygous (*2A/2H*) female progeny were crossed with existing balancer stock w^{1118}; *CyO/Sp*; *TM6B/MKRS* and resultant w^{1118}; *+/CyO*; *2A,2H/TM6B* progeny were collected based on intense eye colour indicating the presence of recombinant (*2A,2H*) chromosome. These females were then crossed with w^{1118}; *2E-yw; +/MKRS* males and w^{1118}; *2E-yw/CyO*; *2A,2H/MKRS* progeny were collected. The

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