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RESEARCH ARTICLE

Regulation of insulin-producing cells in the adult *Drosophila* brain *via* the tachykinin peptide receptor DTKR

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SUMMARY

***Drosophila* insulin-like peptides (DILPs) play important hormonal roles in the regulation of metabolic carbohydrates and lipids, but also in reproduction, growth, stress resistance and aging. In spite of intense studies of insulin signaling in *Drosophila* the regulation of DILP production and release in adult fruit flies is poorly understood. Here we investigated the role of *Drosophila* tachykinin-related peptides (DTKs) and their receptors, DTKR and NKD, in the regulation of brain insulin-producing cells (IPCs) and aspects of DILP signaling. First, we show DTK-immunoreactive axon terminations close to the presumed dendrites of the IPCs, and DTKR immunolabeling in these cells. Second, we utilized targeted RNA interference to knock down expression of the DTK receptor, DTKR, in IPCs and monitored the effects on *Dilp* transcript levels in the brains of fed and starved flies. *Dilp2* and *Dilp3*, but not *Dilp5*, transcripts were significantly affected by DTKR knockdown in IPCs, both in fed and starved flies. Both *Dilp2* and *Dilp3* transcripts increased in fed flies with DTKR diminished in IPCs whereas at starvation the *Dilp3* transcript plummeted and *Dilp2* increased. We also measured trehalose and lipid levels as well as survival in transgene flies at starvation. Knockdown of DTKR in IPCs leads to increased lifespan and a faster decrease of trehalose at starvation but has no significant effect on lipid levels. Finally, we targeted the IPCs with RNAi or ectopic expression of the other DTK receptor, NKD, but found no effect on survival at starvation. Our results suggest that DTK signaling, *via* DTKR, regulates the brain IPCs.**

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Key words: *Drosophila* insulin-like peptides, tachykinin-related peptide, G-protein-coupled receptor, tachykinin-receptor, insulin signaling.

INTRODUCTION

The insulin signaling pathway plays a prominent role in the regulation of metabolism, growth, stress resistance and lifespan both in mammals and in *Drosophila* (Baker and Thummel, 2007; Birse et al., 2010; Broughton et al., 2005; Clancy et al., 2001; Goberdhan and Wilson, 2003; Rulifson et al., 2002; Taguchi and White, 2008; Tatar et al., 2003; Zhang et al., 2009) and is also largely conserved over evolution (Brogiolo et al., 2001; Garofalo, 2002; Giannakou and Partridge, 2007; Grönke et al., 2010). Together, *Drosophila* insulin-like peptides (DILPs) and adipokinetic hormone (AKH) seem to mirror the functions of mammalian insulin and glucagon in *Drosophila* with respect to metabolic homeostasis. DILPs stimulate the uptake of carbohydrate and thus diminishes the circulating blood sugar levels whereas AKH augments the circulating trehalose levels by stimulating glycolysis in the fat body; both hormones also affect lipid homeostasis (Broughton et al., 2005; Géminard et al., 2006; Grönke et al., 2007; Isabel et al., 2005; Kim and Rulifson, 2004; Lee and Park, 2004; Rulifson et al., 2002). Ablation of the insulin-producing cells (IPCs) in the brain results in retarded growth, increased glucose levels in the circulation, increased storage of lipid, reduced fecundity and increased resistance to stress (Broughton et al., 2005; Rulifson et al., 2002). A recent paper demonstrated that DILP signaling to olfactory sensory neurons in the *Drosophila* antennae decreases the sensitivity to odors and thus food search in fed flies (Root et al., 2011). Additionally there is evidence that DILPs, or at least signaling from the IPCs, may participate in the regulation of sleep-wakefulness (Crocker et al., 2010) and ethanol sensitivity (Corl et al., 2005).

In *Drosophila* seven different DILPs have been identified (DILP1–7), some of which resemble mammalian insulin, one that is relaxin-like and one that is similar to insulin-like growth factor (Brogiolo et al., 2001; Grönke et al., 2010; Ikeya et al., 2002; Miguel-Aliaga et al., 2008; Okamoto et al., 2009; Slaidina et al., 2009; Yang et al., 2008). However, only one DILP receptor (dInR) is known in *Drosophila* so far. Extensive work has addressed the mechanisms and roles of signaling downstream of the dInR in *Drosophila,* but the hormonal or neuronal mechanisms that control insulin production and release are not yet understood. The regulation of DILP release in response to nutritional state of the *Drosophila* larva is based on activation of IPCs by nutrient-mediated signals released into the circulation from the fat body where nutrient sensors are located (Geminard et al., 2009). It cannot be excluded that the IPCs also have autonomous nutrient sensors, like the AKH-producing cells (Kim and Rulifson, 2004; Kreneisz et al., 2010), but it is far from clear how DILP production and release is regulated in the fly.

The IPCs of the brain are located in the median neurosecretory cell group of the protocerebrum. These IPCs produce three of the insulin-like peptides, DILP2, DILP3 and DILP5, and are presumed to release these into the circulation from axon terminations in the neurohemal organ corpora cardiaca as well as in the aorta and anterior midgut (Brogiolo et al., 2001; Cao and Brown, 2001; Ikeya et al., 2002; Rulifson et al., 2002). Importantly, the three DILPs are

colocalized in each of the IPCs (Geminard et al., 2009; Ikeya et al., 2002). Some recent studies have suggested neuronal regulation of the brain IPCs in *Drosophila*, and a few neurotransmitters have been suggested as regulators in these neuronal systems: short neuropeptide F (sNPF) (Lee et al., 2008; Lee et al., 2004), octopamine (Crocker et al., 2010), γ-amino butyric acid (GABA) (Enell et al., 2010), and serotonin (Kaplan et al., 2008; Luo et al., 2011). Of these, sNPF and octopamine seem to stimulate insulin signaling, GABA is inhibitory, and serotonin is not yet clear. Thus, the IPCs appear to be under tight neuronal control by multiple neurotransmitters, probably released from distinct neuronal systems. Here we present evidence for yet another type of neuronal regulator of the IPCs, a set of neuropeptides, designated *Drosophila* tachykinins (DTKs), derived from a single precursor and related to mammalian tachykinins of the preprotachykinin A encoding gene (Siviter et al., 2000).

The six *Drosophila* tachykinins, DTK1–6, originate from the gene *Dtk* (Siviter et al., 2000) and can activate two G protein-coupled receptors designated DTKR and NKD (Birse et al., 2006; Poels et al., 2009). The DTKs appear to have pleiotropic functions in the *Drosophila* nervous system, including neuromodulation in olfactory circuits of the antennal lobes and in locomotor circuits of the central complex (Ignell et al., 2009; Kahsai et al., 2010; Nässel and Winther, 2010; Winther et al., 2006). We show here that signaling through DTKR seems to have an inhibitory effect on brain IPCs whereas the other receptor NKD appears not to be expressed in IPCs and thus plays no role in this regulation. Interestingly, mammalian tachykinins, such as substance P, have been shown to increase insulin secretion from the pancreas, indicating a role in regulation of metabolism (Adeghate et al., 2001; Crocker et al., 2010; Schmidt et al., 2000).

MATERIALS AND METHODS

Fly strains

All flies [*Drosophila melanogaster* (Meigen)] were reared at 25°C on a standard yeast, corn meal and agar medium, under 12 h:12 h light:dark conditions. The following fly lines were used: w^{1118} and UAS-cd8GFP were from the Bloomington *Drosophila* Stock Center (BDSC; Indiana University, Bloomington, IN, USA). A *Dilp2*-Gal4 line was donated by Ping Shen [University of Georgia, Athens, GA, USA (Wu et al., 2005)]. The UAS-*Dtkr*-RNAi, UAS-*Dtkr*, UAS-*Dtkr-gfp* and *UAS-Nkd* constructs and fly lines for manipulation of DTKR and NKD receptor levels were described previously (Ignell et al., 2009; Söderberg et al., 2011). Finally, we also used a UAS-*Nkd*-RNAi line from the National Institute of Genetics RNAi Center (NIG-fly) in Kyoto, Japan.

Immunocytochemistry

Rabbit antisera to the amino acids 488–506 of the C-terminus of the *Drosophila* tachykinin receptor DTKR (Birse et al., 2006) and to the full-length cockroach tachykinin LemTRP1, to detect peptides of the DTK precursor (Winther and Nässel, 2001), were used at dilutions of 1:1000–1:2000. These antisera have previously been characterized on *Drosophila* tissues (Birse et al., 2006; Söderberg et al., 2011; Winther et al., 2003). A rabbit antiserum to DILP2 [a gift from M. R. Brown, Athens, GA, USA (Cao and Brown, 2001)] was used at a dilution of 1:4000. To enhance the green fluorescent protein (GFP) signal, we used either a mouse monoclonal antibody to GFP (mAb 3E6; code A-11120; Molecular Probes, Leiden, The Netherlands) or a rabbit anti-GFP (code #A-6455; Invitrogen, Carlsbad, CA, USA).

For immunocytochemistry adult *Drosophila* heads or central nervous systems (CNS) of third instar larvae were dissected in 0.01 mol l^{-1} phosphate buffered saline with 0.25% Triton X-100, pH 7.2 (PBS-TX) and fixed in ice-cold 4% paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer pH 7.4 (PB) for 2–4 h or in Bouin's fixative for 4 h (for the DTKR antiserum applied to adult brains). After rinsing with 0.1 mol l^{-1} PB adult brains or larval CNS were either dissected out for whole-mount immunocytochemistry or whole heads were incubated overnight in 20% sucrose in 0.1 mol l^{-1} PB at 4°C as cryoprotection. Cryostat sections (50 μm thick) of the heads were cut on a cryostat at –23°C. Bouin-fixed tissues were embedded in paraffin and sectioned on a microtome at 12 μm thickness. Incubation with primary antisera for whole-mount tissues was performed for 72 h whereas sections were incubated for 48 h, both at 4°C. For detection of primary antisera Cy3-tagged goat anti-rabbit antiserum (Jackson ImmunoResearch, West Grove, PA, USA) was used at a dilution of 1:1000. Tissues or sections were rinsed thoroughly with PBS-TX, followed by a final wash in PBS and then mounted in 80% glycerol in PBS.

Image analysis and quantification of fluorescence

Microscopic analysis was performed on a Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Jena, Germany) and edited in LSM software and Adobe Photoshop CS3 Extended version 10.0 (Adobe Systems, Mountain View, CA, USA).

Immunocytochemistry with DILP2 antiserum was performed on brain IPCs of different genotypes for quantification of immunofluorescence. Confocal images of the brains from different genotypes were obtained with identical laser intensity and scan settings and monitored for immunofluorescence intensity as described by Broughton et al. and Kaplan et al. (Broughton et al., 2010; Kaplan et al., 2008). Immunofluorescence of both IPC cell bodies and background fluorescence in unlabeled adjacent brain neuropil was quantified in a set of regions of interest, using Image J 1.40 from NIH, Bethesda, MD, USA (http://rsb.info.nih.gov/ij/). The fluorescence of each cell body was measured four times in different regions, and the mean value was obtained. The final immunofluorescence intensity of cell bodies was calculated by subtracting the intensity of tissue background. For each genotype, 40–45 cell bodies in total from eight brains were measured. The data were analyzed in Prism GraphPad 6.0 (San Diego, CA, USA) with Student's *t*-tests.

Assays of lifespan during starvation

Male flies, 4–8 days old, were anesthetized with carbon dioxide and placed individually in 2 ml glass vials with 500 μl of 0.5% aqueous agarose at 25°C in an incubator with 12 h:12 h light:dark conditions and controlled humidity. The vials were checked for dead flies every 12 h. These starvation experiments were run in three replicates with at least 40 flies of each genotype per replicate.

Trehalose assay

Whole-body trehalose was measured according to Isabel et al. (Isabel et al., 2005) with a few minor alterations. Male flies (4–8 days old) were put in groups of 5 in Eppendorf tubes. Flies were weighed (wet mass), then incubated for 1 h in 500 μl of 70% ethanol. Each tube of flies was sonicated (Sonics and Materials Inc., Danbury, CT, USA) for 20 s. 1 ml of 70% ethanol was added, and the tubes were incubated at room temperature for 1 h. A trehalose standard was made with a 2-fold dilution series starting at 200 μg ml^{-1}. The samples were centrifuged for 5 min at 16,000 ***g***, and 1 ml of the samples and 500 μl of the standards were placed in 2 ml Eppendorf tubes and then dried in a vacuum centrifuge (Savant Speed Vac; Speed Vac Plus Sc110A, Farmingdale, NY, USA). 200 μl of 2%

NaOH and 1.5 ml of fresh Anthrone reagent (Sigma Catalogue #A 1631, Sigma Chemical Co., St Louis, MO, USA) were added and the tubes were briefly vortexed. Samples were incubated in a water bath at 90°C for 10 min. 100 μl of each sample was placed in a 96-well ELISA plate and measured in triplicate on an ELISA plate reader (Labsystems Multiscan Plus, Stockholm, Sweden).

Lipid measurements

Lipid content was determined according to the method of Service et al. (Service et al., 1987). Groups of 5 male flies were weighed (wet mass; Mettler MTS, Stockholm, Sweden) and subsequently dried at 65°C for 24 h. Flies were then weighed again to obtain dry mass. Lipids were extracted by placing intact dry flies in glass vials containing 9 ml of diethyl ether for 24 h with gentle agitation at room temperature. The diethyl ether was removed and flies were dried for a further 24 h. Flies were weighed to obtain lean dry mass. The difference between dry mass and lean dry mass was considered the total lipid content of the flies. Lipid content was measured at 0 h and 24 h of starvation.

Quantitative real-time PCR

Total RNA was extracted from whole heads by using TRIzol (GIBCO, Carlsbad, CA, USA), according to the manufacturer's protocol. The RNA was treated with DNase to remove any remaining residual genomic DNA (Turbo DNA-freeTM, Ambion). Treated mRNA was reverse transcribed to cDNA using Quantitect Reverse Transcription Kit (Qiagen, Düsseldorf, Germany), according to the manufacturer's instructions.

The *dilp* primers were as follows: *dilp*2F (forward), TCTGCAGTGAAAAGCTCAACGA; *dilp*2R (reverse), TCGGCA-CCGGGCATG; *dilp*3F, AGAGAACTTTGGACCCCGTGAA; *dilp*3R, TGAACCGAACTATCACTCAACAGTCT; *dilp*5F, GAGGCACCTTGGGCCTATTC; and *dilp*5R, CATGTGGT-GAGATTCGGAGCTA.

PCR was carried out using Taqman Universal PCR Master Mix, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA), with the exception that 25 ml reaction volumes were used, on an ABI Prism 7000 (Applied Biosystems). Endogenous control (*rp49*) primers were as follows: *rp49*F, CACACCAAATCTTACAAAATGTGTGA; and *rp49*R, AATC-CGGCCTTGCACATG. The samples were analyzed in triplicate, and the measured concentration of mRNA was normalized relative to the *rp49* control values. Experiments were made in three replicates, starting from new RNA collection. The normalized data were used to quantify the relative levels of a given mRNA according to the DCt analysis.

We also measured levels of *Dtkr* and *Nkd* transcripts in wild-type flies that were normally fed or starved for 24 h. All flies were 4–8 days old. Approximately 50 flies per tube were placed on 0.5% agarose for 24 h, after which 30 flies per tube were frozen at –80°C. These experiments were made with a different PCR protocol described here. All samples were homogenized in liquid nitrogen and total RNA was extracted using the recommended protocol from the Qiagen RNeasy mini kit (Qiagen catalog no. 74124). RNA was quantified by spectrophotometry and transcribed using the Superscript II First-Strand cDNA synthesis kit (Invitrogen catalog no. 18064). Reverse transcription products were quantified using an ABI PRISM 7700 (Applied Biosystems). PCR reactions were set up as triplicates using SYBER Green Master Mix (Applied Biosystems). The following primers were used: for *Dtkr* 5′GAGTAAGCGAAGGGTGGTGAAG and 3′GAACGGCAGC-CAGCAGAT; for *Nkd* 5′GCATCAATGAGGCCAAAGACTT and 3′TGGCAACTGGTACGGTCTTG; and for *rp49* 5′AGCGCAC-CAAGGACTTCGT and 3′GCCATTTGTGCGACAGCTT.

Experiments were made in four replicates. All calculations were made using software from Applied Biosystems and statistics performed with Prism Graphpad 5.0.

Statistics

Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) was used for collecting and analyzing data. For statistical analysis Prism GraphPad 5.0 was used. Log rank tests (Mantel–Cox) were preformed to analyze trends in lifespan in the starvation assays. One-way and two-way ANOVAs were used for the trehalose and lipid assays to test for any relationships between the different genotypes and starvation times.

RESULTS

Convergence of DTK-expressing neurons and IPCs

Antiserum to cockroach (*Leucophaea maderae*) tachykinin-related peptide 1 (LemTRP-1) is known to recognize DTKs (Siviter et al., 2000; Winther et al., 2003). Immunolabeling with this antiserum revealed neuronal processes with varicose terminations adjacent to the presumed dendrites of IPCs, revealed by *Dilp2*-Gal4-driven GFP (Fig. 1A,B). The DTK-immunolabeled terminations do not make many direct contacts with the IPC branches. However, neuropeptides are known to signal over some distance in the nervous system, so called volume transmission or paracrine signaling (see Maywood et al., 2011; Nässel, 2009; Zupanc, 1996). We could not identify the individual neurons giving rise to these DTK-expressing processes, but an earlier study (Winther et al., 2003) suggests that the candidate neurons have their cell bodies in the tritocerebrum. Immunocytochemistry with an antiserum to a sequence of the DTK receptor, designated DTKR, labels a set of cell bodies in the pars intercerebralis reminiscent of the IPCs, both in adult (Fig. 1C) and larval brains (Fig. 1D). Unfortunately the DTKR antiserum works only on Bouin-fixed tissues where GFP fluorescence is quenched and also the GFP seems denatured because anti-GFP does not work either. Thus, we were unable to perform DTKR immunolabeling of *Dilp2*-Gal4-driven GFP for proper identification. The specificity of this receptor antiserum was tested by running immunocytochemistry on fly brains expressing DTKR in IPCs by means of cross *Dilp2*-Gal4/UAS-*Dtkr*-GFP. A strong DTKR immunolabeling was seen in the IPCs of these flies (supplementary material Fig. S1).

Levels of *Dilp* transcripts in brain after knockdown of DTKR on IPCs

As DTK-expressing neuron processes seem to target the brain IPCs, we tested whether DTK signaling affects levels of *Dilp* transcripts in these cells. Knockdown of DTKR on IPCs was accomplished by crossing the *Dilp2*-Gal4 driver to a UAS-*DTKR*-RNAi line. The efficacy of the UAS-*DTKR*-RNAi in knocking down receptor transcripts has been determined previously by quantitative real-time PCR (qPCR) after pan-neuronal RNAi (using an *Elav*-Gal4 driver); the DTKR transcript was reduced by approximately 40% (Ignell et al., 2009). We sampled RNA from this fly cross at 0 h (fed flies) and 24 h starvation, and performed qPCR to determine levels of *Dilp2*, *Dilp3* and *Dilp5* transcripts after DTKR knockdown in IPCs.

In control flies relative *Dilp2* and *Dilp3* transcripts were not significantly affected by 24 h starvation (Fig. 2A,B) whereas *Dilp5* transcript significantly decreased (Fig. 2C). Knockdown of DTKR in IPCs affected the three *Dilp* transcripts differentially, both in fed

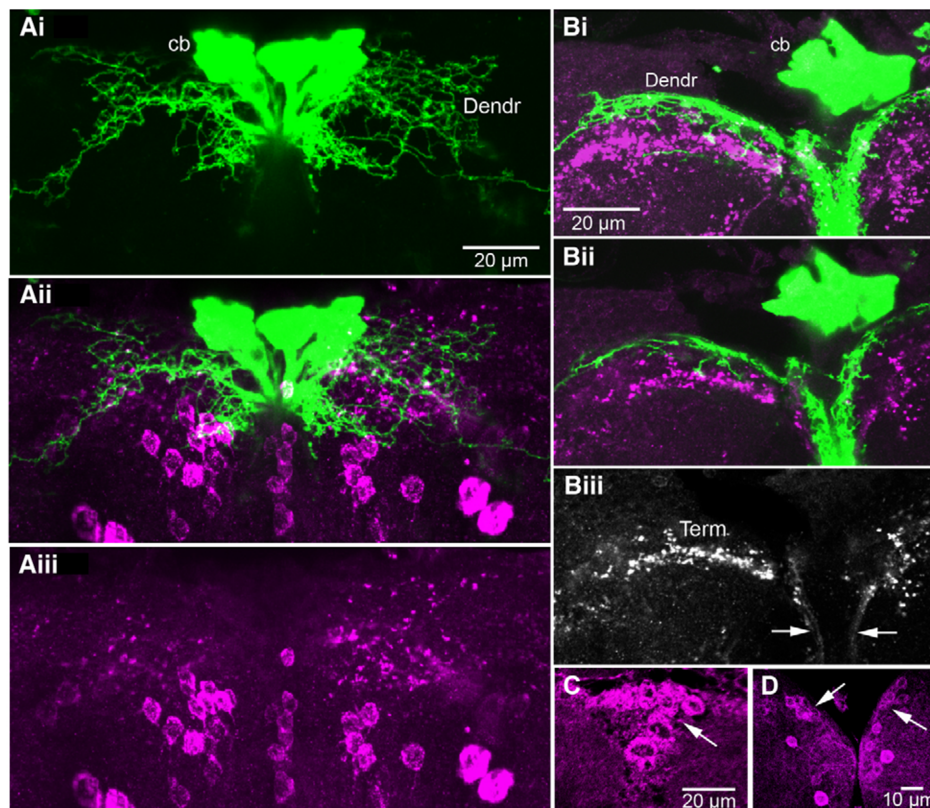

Fig. 1. Neuronal processes expressing *Drosophila* tachykinin, DTK, converge on insulin-producing cells (IPCs) in the pars intercerebralis of the adult brain. (Ai) The brain IPCs are revealed by *Dilp2*-Gal4-driven green fluorescent protein (GFP). The IPC cell bodies (cb) are localized medially in the pars intercerebralis. The presumed dendrites (Dendr) spread laterally in the dorsal median protocerebrum. (Aii) Merged channels showing IPCs (green) and DTK immunolabeling (magenta). In this projection of multiple optical sections, a superposition between IPC dendrites and DTK-expressing varicosities is seen. (Aiii) Single channel displaying DTK-expressing axon terminations with varicosities. The DTK-expressing cell bodies seen in this image are not likely to be the ones impinging on IPCs [the smaller ones supply processes to the central complex (Kahsai et al., 2010; Winther et al., 2003)]. (Bi) Another specimen (a slightly tilted brain compared with A) showing DTK-immunolabeled varicosities (magenta) close to the IPC dendrites (Dendr). This image is a stack of optical sections. (Bii) Single optic section of the same IPCs showing some of the arborizations that appear to be targeted by DTK-expressing axon terminations. (Biii) Single optic section of the DTK-expressing neurons targeting the IPCs. Note the axons (at arrows) ascending from ventral portions of the brain (probably from tritocerebrum) and forming varicose terminations (Term). (C) Antiserum to the DTK receptor DTKR labels a set of median neurosecretory cells in the pars intercerebralis of the adult brain (arrow). As Bouin's fixation and paraffin sections were required for DTKR immunolabeling, it was not possible to simultaneously reveal *Dilp2*-Gal4-driven GFP. (D) DTKR immunolabeled neurons in the larval brain. The cell groups indicated by arrows may correspond to the larval IPCs. The specificity of the DTKR antiserum is verified in supplementary material Fig. S1.

and starved flies (Fig. 2). Both *Dilp2* and *Dilp3* transcripts increased significantly in fed flies after DTKR knockdown in IPCs (Fig. 2A,B), whereas levels of *Dilp5* were not affected compared with controls (Fig. 2C). Furthermore, only the *Dilp2* and *Dilp3* levels were affected by 24 h starvation in the DTKR-knockdown flies. *Dilp2* significantly increases compared with fed flies of the same genotype and compared with controls (Fig. 2A) whereas *Dilp3* drastically decreases after starvation (Fig. 2B). Thus, it appears as if transcription of both *Dilp2* and *Dilp3* increases after reduction of DTK signaling *via* DTKR in normally fed flies and that 24 h starvation drastically decreases *Dilp3* transcription and increases *Dilp2* when DTKR signaling is reduced in IPCs.

We also measured relative DILP2 immunofluorescence in cell bodies of IPCs in control flies and flies with DTKR diminished in the IPCs to obtain an estimate of peptide levels, using a protocol similar to previous studies (Broughton et al., 2010; Kaplan et al., 2008). There is a significantly higher level of DILP2 immunofluorescence after receptor knockdown in the IPCs (Fig. 3).

To determine whether DTK signaling *via* its two receptors is modulated by nutritional status we tested the levels of *NKD* and *DTKR* receptor transcripts by qPCR in wild-type flies that were fed normally or starved for 24 h. We found no change in relative levels of the two receptor transcripts due to starvation (supplementary material Fig. S2). Thus, it is likely that the strength of DTK signaling to the IPCs is mainly determined by the amount of DTK peptide released during starvation and not by changes in receptor expression levels.

DTKR knockdown in IPCs decreases survival of flies exposed to starvation

Insulin signaling from brain IPCs affects the lifespan of flies exposed to starvation and dietary restriction (Broughton et al., 2005; Broughton et al., 2010). To study the influence of DTK signaling to IPCs on survival during starvation, experimental flies were allowed access to water but no food. We determined the effects of starvation on total and median lifespan (when 50% of the flies have succumbed) of different genotypes.

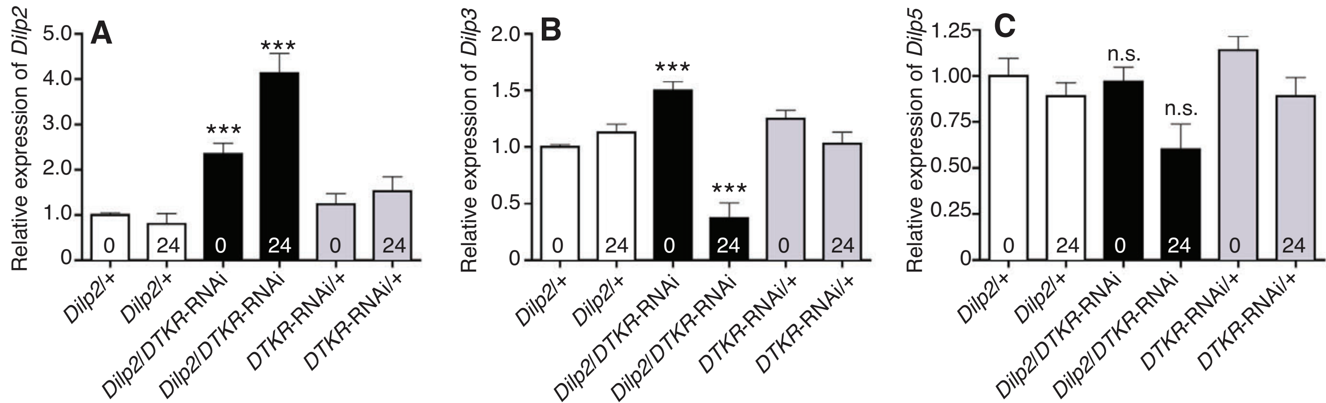

Fig. 2. Levels of *Dilp* transcripts in flies with DTKR knocked down in insulin-producing cells (IPCs). The effect of *DTKR* knockdown in the IPCs (*Dilp2*-Gal4/*DTKR*-RNAi) on *Dilp* expression in the brain of adult flies was measured by quantitative real-time PCR. Fed flies (0 h) and flies starved for 24 h were monitored. Data are shown as mean relative expression ±s.d. (N=10); asterisks denote a significant difference to controls (n.s., not significant, P>0.05; ***P<0.001). (A) The relative levels of *Dilp2* transcript increase in DTKR-knockdown flies compared with parental controls, both in fed flies and after 24 h starvation (P<0.0001, one-way ANOVA). The difference between fed and starved DTKR-knockdown flies is significant (P<0.001, two-way ANOVA, Bonferroni's *post hoc* test). (B) The relative *Dilp3* abundance is higher in flies that express *DTKR*-RNAi in IPCs than in controls in the fed state (P<0.001, one-way ANOVA). Furthermore, the *Dilp3* transcript levels decrease drastically after 24 h starvation in flies with DTKR knocked down. This drop in *Dilp3* is significant (P<0.001, two-way ANOVA). (C) The relative *Dilp5* transcript levels did not differ significantly from those of the controls, neither in fed nor starved flies (P>0.05, two-way ANOVA). However, for each genotype the decrease in *Dilp5* RNA after 24 h starvation is significant (P<0.05; one-way ANOVA). Thus, *Dilp5* seems to be the only transcript affected significantly by starvation in control flies.

Knockdown of DTKR in the IPCs by *Dilp2*-Gal4/*DTKR*-RNAi leads to a shortening of total and median lifespan at starvation by approximately 20% compared with controls (P<0.001; log rank test, Fig. 4A). In contrast, overexpression of DTKR in the IPCs did not result in a significant change of lifespan (Fig. 4A).

To test whether the other DTK receptor NKD plays a role in IPCs at starvation, we monitored the effect of expressing UAS-*NKD*-RNAi or UAS-*NKD* with the *Dilp2*-Gal4 driver. Neither of these crosses led to a change in lifespan at starvation compared with controls (Fig. 4B). These results suggest that NKD is not expressed in the IPCs or at least it plays no role in the regulation of starvation responses.

DTKR knockdown in IPCs affects trehalose levels during starvation

Trehalose is a non-reducing disaccharide and is the major carbohydrate that is used as an energy source by most insects. Insulin signaling from the brain IPCs is known to regulate trehalose levels (Broughton et al., 2005; Rulifson et al., 2002). Specifically, knockdown of *Dilp2* is known to increase the total trehalose content (Broughton et al., 2008). Thus, we tested total trehalose levels in flies with DTKR levels diminished in IPCs. Whole-body trehalose levels in flies of different genotypes were tested at 0 h, 5 h and 12 h of starvation. The wild-type response to starvation is a gradual decrease in trehalose levels over the 12 h starvation period. Here, the 0 h value is a measure of trehalose levels under normal fed conditions.

Knocking down DTKR in the IPCs did not affect trehalose levels in fed flies compared with controls (Fig. 5A). However, after 5 h of starvation the flies with diminished DTKR levels displayed a significantly larger drop in trehalose than controls (P<0.001, one-way ANOVA), but after 12 h levels were the same in all genotypes (Fig. 5A). Thus, the trehalose levels in DTKR-knockdown flies fell more rapidly at onset of starvation than in control flies. At starvation the trehalose is likely to be metabolized, partly due to hunger-induced hyperlocomotion, and with increased DILP signaling carbohydrates are depleted more rapidly.

DTKR knockdown in IPCs does not affect lipid content during starvation

Insects, including *Drosophila*, utilize lipids as fuel for prolonged exercise or in other situations of high-energy demands, as well as at times of restricted availability of nutrients (Baker and Thummel, 2007; Gäde et al., 1997; Van der Horst et al., 2001). Lipid levels in *Drosophila* are regulated in part by insulin signaling from brain IPCs (Broughton et al., 2005; Grönke et al., 2010), but also by AKH (Baker and Thummel, 2007; Grönke et al., 2007). Thus, we investigated whole-body lipid levels in flies with DTKR knockdown targeted to IPCs in fed conditions (0 h starvation) and after 24 h of

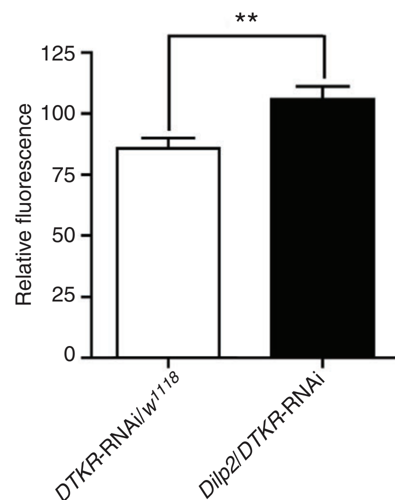

Fig. 3. DILP2 peptide levels in insulin-producing cells (IPCs) after DTKR knockdown in IPCs. Relative immunofluorescence was measured after DILP2 immunolabeling of IPCs in normally fed experimental flies. After DTKR knockdown (*Dilp2*/*DTKR*-RNAi) the relative fluorescence increased significantly compared with the control (P=0.004, Student's t-test; N=8 brains of each genotype).

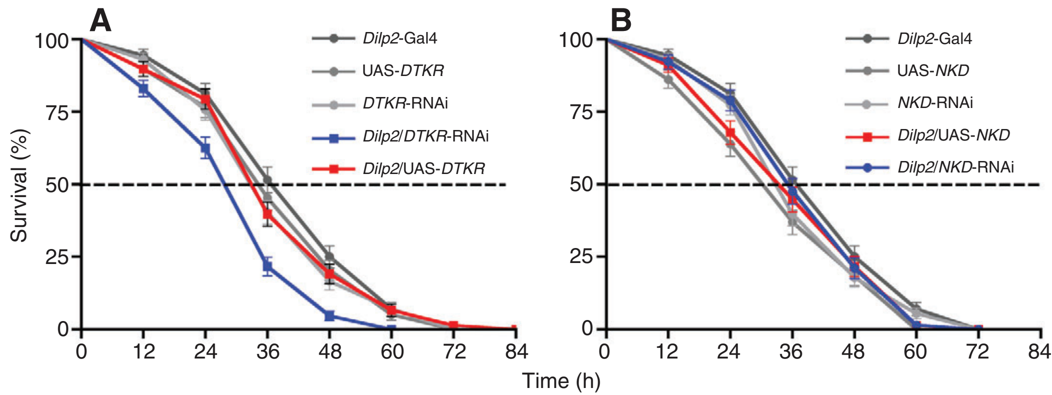

Fig. 4. DTKR knockdown in insulin-producing cells (IPCs) increases sensitivity to starvation. We tested the survival of flies with DTKR knocked down in IPCs (*Dilp2*/*DTKR*-RNAi) or with DTKR overexpressed in IPCs (*Dilp2*/UAS-*DTKR*), compared with parental controls, in a starvation assay where flies were kept on aqueous agarose. All experiments were run in triplicate. Dashed lines indicate 50% survival. (A) A significant decrease in survival was seen after knockdown of DTKR in IPCs. The median survival decreased by 20% ($P<0.0001$, log rank test, N=128–171 for each genotype). However, no significant difference in survival was observed in flies with DTKR ectopically expressed IPCs ($P>0.05$, N=128–137). (B) Neither knockdown (*Dilp2*/*NKD*-RNAi) nor ectopic expression (*Dilp2*/UAS-*NKD*) of the other tachykinin receptor, NKD, in IPCs had any effect on survival at starvation ($P>0.05$, log rank test, N=128–146).

starvation. In fed animals there is no significant difference in lipid levels between controls and DTKR-knockdown flies (Fig. 5B). After 24 h starvation there is a significant drop in lipids both in experimental and control flies (Fig. 5B). This suggests that DTK signaling to IPCs does not affect lipid levels in fed or starved flies or that such an action is masked by compensatory DILP or AKH signaling (see Grönke et al., 2010; Grönke et al., 2007).

DISCUSSION

We have investigated the effects of DTK signaling to IPCs in the *Drosophila* brain by monitoring *Dilp* transcript levels and survival at starvation, as well as trehalose and lipid levels in fed and starved flies. The brain IPCs are presumed to release DILP2, DILP3 and DILP5, orthologs of mammalian insulins (Cao and Brown, 2001; Ikeya et al., 2002). Since these insulin-like peptides have been shown to play a significant role in lifespan, in nutritional stress responses and in metabolic regulation (Baker and Thummel, 2007; Broughton et al., 2005; Giannakou and Partridge, 2007; Grönke et al., 2010; Rulifson et al., 2002; Tatar et al., 2001), the DTK signaling onto these cells may be of significance for the regulation of vital physiological functions. However, the IPCs are also known to regulate feeding behavior, locomotor activity, sleep-wakefulness and ethanol sensitivity, and they may do so independent of the insulin signaling pathway (Cognigni et al., 2011; Corl et al., 2005; Crocker et al., 2010; Mattaliano et al., 2007; Wu et al., 2005). Thus, activation or inhibition of signaling in the IPCs may result in actions that are

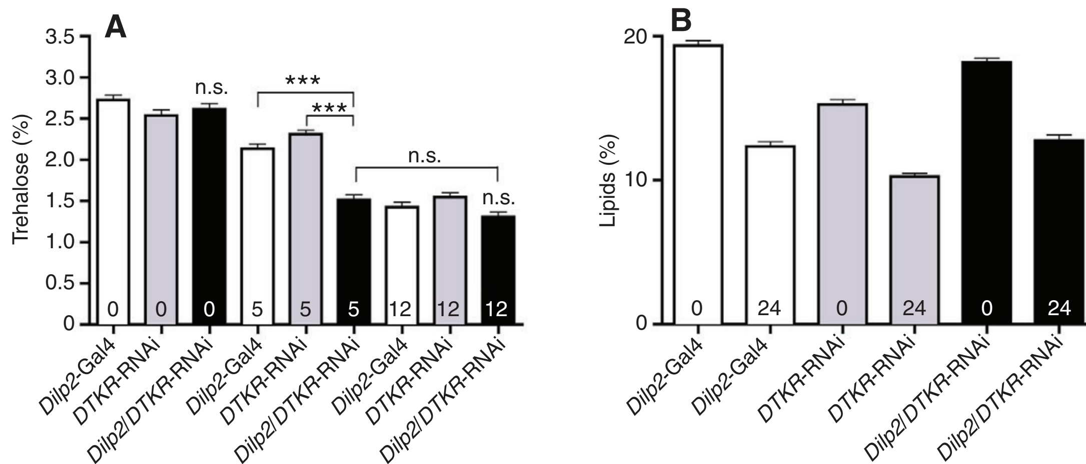

Fig. 5. Diminished DTKR signaling in insulin-producing cells (IPCs) affects total trehalose but not lipid content in starved flies. (A) Trehalose levels were determined in flies with DTKR receptor knockdown in IPCs (*Dilp2*/*DTKR*-RNAi) and parental controls. The trehalose levels were measured in fed flies (0 h), and flies starved for 5 h and 12 h (run in triplicate, N=120 for each genotype and condition). In control flies the trehalose levels gradually diminish after 5 h and 12 h starvation ($P<0.001$, two-way ANOVA). However, *Dilp2*/*DTKR*-RNAi flies (black bars) display a much more pronounced decrease between 0 h and 5 h starvation than controls ($P<0.001$, one-way ANOVA, Tukey's *post hoc* test) and display similar trehalose levels as controls at 12 h starvation ($P>0.05$, one-way ANOVA, Tukey's *post hoc* test). Asterisks denote a significant difference to controls (n.s., not significant, $P>0.05$; ***$P<0.001$). (B) Whole-body lipid levels were determined in fed flies (0 h) and flies starved for 24 h. In control flies, the total lipid content significantly decreases after 24 h starvation ($P<0.001$, one-way ANOVA). Flies expressing *DTKR*-RNAi in IPCs (black bars) displayed the same decrease in lipid as the control flies. We detected no significant change in lipid content over time in the test flies compared with controls. (n.s., two-way ANOVA, Bonferroni's *post hoc* test; N=120, run in triplicate).

non-insulin mediated, either *via* other messengers released by the same cells or indirectly by the action of DILPs on specific neurons (Root et al., 2011; Wu et al., 2005).

The most direct evidence that DTK signaling affects the brain IPCs is that knock down of the receptor DTKR in IPCs leads to altered expression levels of *Dilp2* and *Dilp3* transcripts in fed flies and that *Dilp3* RNA drops drastically in knockdown flies after 24 h starvation whereas *Dilp2* levels increase. Interestingly, the *Dilp5* transcript is not affected by *DTKR*-RNAi, but is the only one that seems affected by starvation both in controls and knockdown flies. It is known that restricted diet conditions in adult (control) flies alter the transcript level of *Dilp5*, but not *Dilp2 and Dilp3* (Broughton et al., 2010), corroborating our findings. To our knowledge our data here are the first to quantify *Dilp* transcripts at complete starvation in adults, but an earlier report monitored *Dilp* transcripts by *in situ* hybridization of fed and starved third instar larvae (Ikeya et al., 2002). These authors noted decreased *Dilp3* and *Dilp5* transcripts but unaffected levels of *Dilp2*. This difference could be either dependent on a difference in larval and adult functions of the IPCs or could be due to the difference in techniques used for monitoring transcript levels. Certainly the feeding behavior and metabolism differs greatly between larvae and adults in *Drosophila* (Baker and Thummel, 2007). It should be noted here that insulin expression/signaling also involves autocrine or paracrine feedbacks so that DILP3 may act in stimulatory regulation of expression of DILP2 and DILP5 in the IPCs (Broughton et al., 2008; Grönke et al., 2010) whereas DILP6 released from the fat body may negatively regulate the IPCs (Grönke et al., 2010).

Unfortunately there are no reports that unequivocally demonstrate the release of DILPs into the circulation of *Drosophila* in a quantitative fashion (but see Geminard et al., 2009). Thus, indirect measurements, such as *Dilp* transcript or DILP peptide-immunofluorescence levels in cell bodies of IPCs, have to be matched against the physiological effects seen after manipulations of IPCs. A few indicators of altered insulin signaling have been used here: levels of carbohydrate and lipids as well as effects on lifespan at starvation. As one of the functions of DILPs is to stimulate uptake of circulating blood sugar and thereby decreasing trehalose levels in the circulation (Giannakou and Partridge, 2007; Rulifson et al., 2002; Tatar et al., 2003), we monitored whole-body trehalose levels in fed and starved flies after DTKR knockdown in IPCs. Knockdown of DTKR in IPCs had no effect on trehalose in fed flies, but induced an acute drop in trehalose after 5 h starvation, compared with controls, suggesting an increase in insulin signaling. Analysis of *Dilp* mutants or knockdown indicated that trehalose levels are regulated by DILP2 (Broughton et al., 2008; Grönke et al., 2010); one of the peptides whose transcripts was indeed altered by DTKR knockdown at starvation. In our experiments lipid levels were not affected by manipulations of DTKR on IPCs in fed or starved flies. Lipid metabolism may be regulated by multiple DILPs, including *Dilp6* (Grönke et al., 2010), or by compensatory AKH signaling (Baker and Thummel, 2007; Grönke et al., 2007), and this may explain our lack of effect after manipulating only IPC activity.

Diminishment of DTKR expression on IPCs results in flies that display a shortened lifespan at starvation. This would also indicate increased insulin signaling, as deletion of IPCs or knocking down combinations of DILPs produce the opposite phenotype (Broughton et al., 2005; Buch et al., 2008; Grönke et al., 2010), and over-expression of *Dilp2* in IPCs resulted in decreased resistance to starvation (Enell et al., 2010). A similar reduction of lifespan at starvation was seen after knock down of the inhibitory $GABA_B$ receptor on IPCs (Enell et al., 2010). It is not clear which of the DILPs regulates the lifespan at dietary restriction or starvation, but DILP2 has been suggested as a candidate (Bauer et al., 2007; Grönke et al., 2010; Hwangbo et al., 2004; Wang et al., 2005).

The second known DTK receptor, designated NKD (Li et al., 1991; Poels et al., 2009), does not seem to play a role in the regulation of IPCs. NKD can be activated only by one of the DTKs, the N-terminally extended DTK-6 (Poels et al., 2009), which has not been detected in the *Drosophila* brain, in contrast to the DTK-1-5 known to activate DTKR (Winther et al., 2003; Yew et al., 2009).

It can be mentioned that an earlier report shows that DTKR is expressed in renal (Malpighian) tubules where it regulates DILP5 signaling (Söderberg et al., 2011). It is proposed that this regulation is mediated by DTKs released hormonally from endocrine cells of the midgut. DTKs circulating locally act on DTKR expressed in principal cells of the renal tubules, resulting in a local activation of DILP5 signaling. The DTKR-regulated DILP5 signaling in renal tubules does not affect trehalose levels in fed or starved flies, but seems to be part of the defense against oxidative stress (Söderberg et al., 2011). Thus, this DTK-controlled DILP5 signaling in the tubules is probably independent of the paracrine DTK-mediated IPC regulation in the brain, but further studies of gut-derived DTK action are required to confirm this.

In summary our results indicate that in wild-type flies the activated DTKR inhibits insulin signaling in the brain IPCs, and knockdown of the receptor therefore leads to increased insulin signaling. This can be seen in the decreased lifespan and a considerable decrease in trehalose levels during short-term starvation compared with controls, similar to what is expected at increased DILP signaling. The most direct evidence that DTKR is involved in IPC regulation is the effect on *Dilp2* and *Dilp3* transcript levels seen after receptor knockdown in the IPCs in fed and starved flies. However, it is important for the future to develop a sensitive assay for quantifying hemolymph levels of individual DILPs to monitor how their release is affected by the DTKR signaling to IPCs.

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