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Neuropeptides in the *Drosophila* central complex in modulation of locomotor behavior

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SUMMARY

The central complex is one of the most prominent neuropils in the insect brain. It has been implicated in the control of locomotor activity and is considered as a pre-motor center. Several neuropeptides are expressed in circuits of the central complex, and thus may be modulators of locomotor behavior. Here we have investigated the roles of two different neuropeptides, *Drosophila* tachykinin (DTK) and short neuropeptide F (sNPF), in aspects of locomotor behavior. In the *Drosophila* brain, DTK and sNPF are expressed in interneurons innervating the central complex. We have directed RNA interference (RNAi) towards DTK and sNPF specifically in different central complex neurons. We also expressed a temperature-sensitive dominant negative allele of the fly ortholog of dynamin called *shibire*^{ts1}, essential in membrane vesicle recycling and endocytosis, to disrupt synaptic transmission in central complex neurons. The spontaneous walking activity of the RNAi- or *shibire*^{ts1}-expressing flies was quantified by video tracking. DTK-deficient flies displayed drastically increased center zone avoidance, suggesting that DTK is involved in the regulation of spatial orientation. In addition, DTK deficiency in other central complex neurons resulted in flies with an increased number of activity–rest bouts. Perturbations in the sNPF circuit indicated that this peptide is involved in the fine regulation of locomotor activity levels. Our findings suggest that the contribution of DTK and sNPF to locomotor behavior is circuit dependent and associated with particular neuronal substrates. Thus, peptidergic pathways in the central complex have specific roles in the fine tuning of locomotor activity of adult *Drosophila*.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/13/2256/DC1

Key words: *Drosophila* tachykinin, short neuropeptide F, fan-shaped body, centrophobism, locomotor activity levels, *shibire*.

INTRODUCTION

The central complex is the most central and the only unpaired neuropil in the insect brain. It receives multimodal inputs from most parts of the brain, mainly through tangential neurons in both hemispheres (Hanesch et al., 1989; Homberg, 2004). The central complex consists of four interconnected substructures: the protocerebral bridge, the fan-shaped body (fb), the ellipsoid body and the noduli (Hanesch et al., 1989; Williams, 1975). It has been proposed as a higher center for locomotor control that regulates several aspects of walking and flying behavior. Flies with structural mutations in the central complex or those in which central complex neurons have been inactivated walk slower and are less motivated for walking (Poeck et al., 2008; Strauss et al., 1992; Strauss and Heisenberg, 1993). Additionally, they show altered temporal structure of walking and disturbed orientation ability (Ilius et al., 1994; Martin et al., 2002; Martin et al., 1999b).

The central complex has also been suggested to act as a higher center for integration of visual input (Homberg, 2004). In locusts and crickets, neurons found in the central complex have a function in sky-compass navigation (Heinze and Homberg, 2007; Sakura et al., 2008; Vitzthum et al., 2002). Recently it has been shown that in *Drosophila* the central complex is associated with spatial orientation memory (Neuser et al., 2008) and with visual learning (Liu et al., 2006; Wang et al., 2008).

Immunocytochemical analysis has revealed that a large number of different neuropeptides are expressed in the central complex of different insects (Nässel and Homberg, 2006). Thus, neuropeptides seem to be significant mediators of neuronal activity in the central complex. However, the functional roles of the peptidergic neurons innervating the central complex are still poorly understood. One previous study explored the behavioral effects of peptide deficiency: global down-regulation of the neuropeptide *Drosophila* tachykinin (DTK) resulted in impaired olfactory and locomotor behavior (Winther et al., 2006). These findings were in agreement with the prominent distribution of DTK in the antennal lobes and in the fb. In the *Drosophila* brain, DTK is expressed in about 150 interneurons with processes in several neuropils where they are likely to act as neuromodulators (Winther et al., 2003). Two DTK receptors, DTKR and NKD, have been identified in *Drosophila* and both of them are expressed in the central complex (Birse et al., 2006; Poels et al., 2009). Short neuropeptide F (sNPF) is another example of a peptide expressed in many different neuropils; it is expressed in thousands of interneurons, including a small subpopulation that innervates the fb (Nässel et al., 2008). As DTK and sNPF display the most prominent immunostaining in the central complex compared with the labeling of other peptides (L.K. and Å.M.E.W., unpublished observation), it seemed reasonable to target these two peptides to gain insight into peptidergic neuromodulation of locomotor behavior.

Here we initiated an analysis of peptide function in the central complex and explored the contribution of DTK and sNPF to spontaneous locomotor behavior, employing RNA interference (RNAi). As these peptides are broadly expressed in multiple neuronal systems we directed RNAi exclusively to distinct sub-

populations of fb neurons to study the *bona fide* contribution of the peptides to locomotor behavior. To further explore the role of central complex circuits in the regulation of locomotor behavior and to corroborate our RNAi findings, we set out to interfere with vesicular traffic specifically in the same subset of neurons using the *Drosophila* ortholog of dynamin, *shibire*. The thermosensitive dominant negative allele *shi*^{ts1} perturbs vesicle traffic and inhibits synaptic transmission at the restrictive temperature (30°C) (Kitamoto, 2001).

MATERIALS AND METHODS

Fly strains

Adult white-eyed *Drosophila melanogaster* (*w*^{1118}) flies as well as several lines of transgenic flies were used for immunocytochemistry and experiments. To analyze peptide expression pattern in neurons of the fb, different fb-GAL4 lines driving green fluorescent protein (GFP) were used in combination with peptide immunocytochemistry. Tangential fb neurons were visualized by GAL4 lines: 121y, 154y, 210y, c5, c205, c584 and NP6510 (Liu et al., 2006; Martin et al., 1999b). Columnar neurons were identified by c739-GAL4 (Armstrong et al., 1998) and pontine neurons by NP2320-GAL4 (Liu et al., 2006). The GAL4 lines 121y, 154y, c584, c739 and 210y were a gift of J. Douglas Armstrong, University of Edinburgh, UK; NP6510 and NP2320 were provided by Martin Heisenberg, University of Würzburg, Germany. Neurons were visualized by crossing the GAL4 lines to UAS-mcd8-GFP flies (Bloomington *Drosophila* Stock Center, Indiana University, USA).

For analysis of locomotor behavior, fb-GAL4 lines selected for coexpression of GAL4 and peptide were crossed with UAS-*dtk*-RNAi37A;UAS-*dtk*-RNAi37D and with UAS-*sNPF*-RNAi;UAS-*sNPF*-RNAi flies to knock down levels of DTK and sNPF, respectively. Generation and characterization of UAS-*dtk*-RNAi and UAS-*sNPF*-RNAi have been described previously (Winther et al., 2006; Lee et al., 2004). The UAS-*sNPF*-RNAi line was a gift of Kweon Yu, Daejeon, Korea. Silencing of selected fb neurons was accomplished by driving UAS-*shi*^{ts1} with the GAL4 lines c739, NP2320 and c584. For behavioral experiments the corresponding heterozygous parental flies (crossed with Canton-S *w*^{1118}) were used as controls.

Immunocytochemistry

Adult *Drosophila* heads were fixed in ice-cold 4% paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer pH 7.4 for 4 h. Brains were dissected for whole-mount immunocytochemistry or whole heads were used for cryostat sectioning. Incubation with primary antisera for whole-mount tissues was performed for 72 h while sections were incubated overnight at 4°C. The following primary antisera were used: antiserum to a generic sequence of insect tachykinin-related peptides likely to recognize the different DTK isoforms (anti-LemTRP-1) (Winther and Nässel, 2001; Winther et al., 2003) at a 1:4000 dilution, sNPF antiserum (Johard et al., 2008) at a 1:4000 dilution, and mouse monoclonal GFP antibody (mAb3E6; code A-11120; Molecular Probes, Leiden, The Netherlands) at a 1:1500 dilution. For detection of primary antisera, tissues were incubated in Cy3-tagged goat anti-rabbit antiserum or Cy2-tagged goat anti-mouse antiserum (Jackson ImmunoResearch, West Grove, PA, USA) at 1:1500 dilution for 2 h at room temperature. Finally, tissues were mounted in 80% glycerol. For each experiment at least 10 adult brains were used.

Image analysis

Specimens were imaged with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany). Images were obtained at an optical section thickness of 1–2 μm and assembled using Zeiss LSM software. The images were processed with Zeiss LSM software and edited for contrast and brightness in Adobe Photoshop CS3 Extended version 10.0 (www.adobe.com).

Quantification of immunofluorescence

Immunocytochemistry and imaging were performed as described above. Dissected 4 day old female brains were used for immunocytochemistry. Specimens were imaged under identical conditions. The immunofluorescence emitted from the specimens was quantified in a set region of interest (ROI) using ImageJ v1.42 (http://rsb.info.nih.gov/ij). Fluorescence was quantified in several adjacent ROI so that approximately the entire fb was covered. As the expression patterns of *w*^{1118} and the GAL4 lines used to knock down peptide levels appeared to be identical, *w*^{1118} was used as a control. Quantification of immunofluorescence was performed blind. The data were analyzed using Prism v4.0 (GraphPad, CA, USA). Three brains per genotype and experiment were measured.

Locomotor assay

We used a video-tracking paradigm described elsewhere (Martin, 2004). In brief, single flies aged 3–4 days were allowed to walk freely in a square chamber of 4 cm×4 cm and 3.5 mm high. Flies were recorded at 5 Hz sampling rate using EthoVision software (Noldus, Wageningen, The Netherlands). Video recordings of controls and peptide-deficient flies were performed at 25°C for 7 h in 60% relative humidity. For the neuronal silencing experiments, control flies and flies expressing *shi*^{ts1} were video recorded at 30°C, for 5 h. A total of 18–26 flies were analyzed for each genotype and sex.

Analysis of data

Analysis of data was carried out as detailed previously (Martin, 2004). Four parameters were extracted from EthoVision software: frequency of passages through a virtual zone located at the centre of the arena (2 cm in diameter), total distance moved (m), mean walking speed (mm s^{-1}) and number of episodes of activity and inactivity (start/stop). In order to account for differences in overall activity, the frequency of passages in the central zone was normalized to the duration of movement, i.e. the number of passages in the central zone was divided by the total time spent in activity (s). Statistical analysis was done using analysis of variance (ANOVA) and Tukey's *post hoc* test (Statistica v4; www.statsoft.com).

RESULTS

DTK-expressing fb neurons modulate central zone avoidance

Using a generic insect tachykinin (TK) antiserum, known to identify DTKs, we found that different populations of DTK-expressing neurons innervate the fb. The fb is composed of a network of horizontal layers and vertical columnar elements (Hanesch et al., 1989). The horizontal layers of the fb are composed of tangential neurons providing input from several areas of the brain (Hanesch et al., 1989; Strauss, 2002). We found two GAL4 lines that displayed expression patterns partly overlapping with TK immunoreactivity: c739-GAL4 and 121y-GAL4. The GAL4 line c739 (Armstrong et al., 1998) drives expression in a group of approximately 16 columnar neurons in the superior median protocerebrum (SMP) that innervate the fb (Fig. 1Ai). In the SMP, we detected approximately 24 immunoreactive neurons likely to innervate the fb with varicose axonal processes (Fig. 1Bi); of these 10–12 neurons were included in the c739-GAL4 (Fig. 1Ci). In the

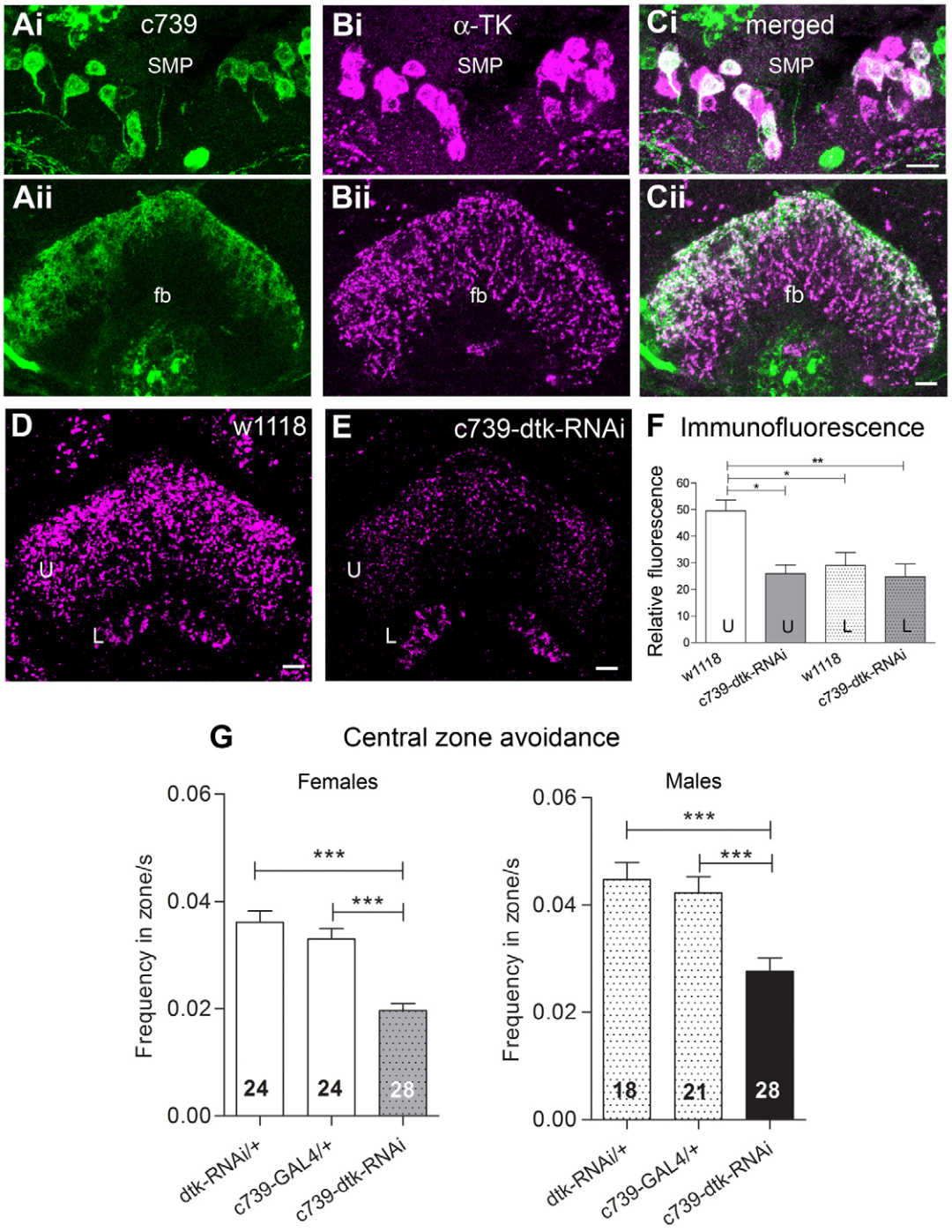

Fig. 1. *Drosophila* tachykinin (DTK) depletion in columnar neurons affects spatial orientation. Distribution of tachykinin (TK) immunoreactivity in relation to c739-GAL4-driven green fluorescent protein (GFP) in neuronal cell bodies (Ai–Ci) that innervate the fan-shaped body (fb) (Aii–Cii). (Ai) GFP expression driven by c739-GAL4 shows columnar neurons in the superior median protocerebrum (SMP) innervating the fb. (Bi) TK-immunolabeled neurons innervating the fb. (Ci) Coexpression of TK-immunolabeling and c739-GAL4 is detected in 10–12 neurons in the SMP likely to innervate the upper part of the fb. (D–F) When c739-GAL4 was used to drive UAS-*dtk*-RNAi reduced immunofluorescence was observed in the upper part (U) of the fb but not in the non-targeted area of the fb (lower part, L) (E). TK-immunolabeling of control brain (w^{1118}) is shown in D. (F) Effect of *dtk*-RNAi on the relative immunofluorescence across three specimens per genotype comparing both upper and lower parts of the fb. (G) Flies bearing the c739-GAL4 and UAS-*dtk*-RNAi transgenes were monitored for locomotor behavior. Female and male flies with DTK depletion in c739 neurons (c739-*dtk*-RNAi) passed into the central zone of the arena less frequently than controls (*dtk*-RNAi/+ and c739-GAL4/+). Values are corrected for the activity levels of the flies. Numbers in boxes indicate number of flies recorded. Vertical bars indicate s.e.m. ANOVA: **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars 10 μm.

fb, we detected coexpression of immunoreactivity and c739-GAL4 in the upper part (Fig. 1Aii–Cii). c739-GAL4 also drives expression in the ellipsoid body where DTK is not expressed. For clarity, we chose to show a projection not including the ellipsoid body, which would if included obscure DTK expression; hence, immunoreactivity in the lower part of the fb is not shown.

The 121y-GAL4 drives expression in tangential neurons innervating the fb (Liu et al., 2006). We detected colocalization of

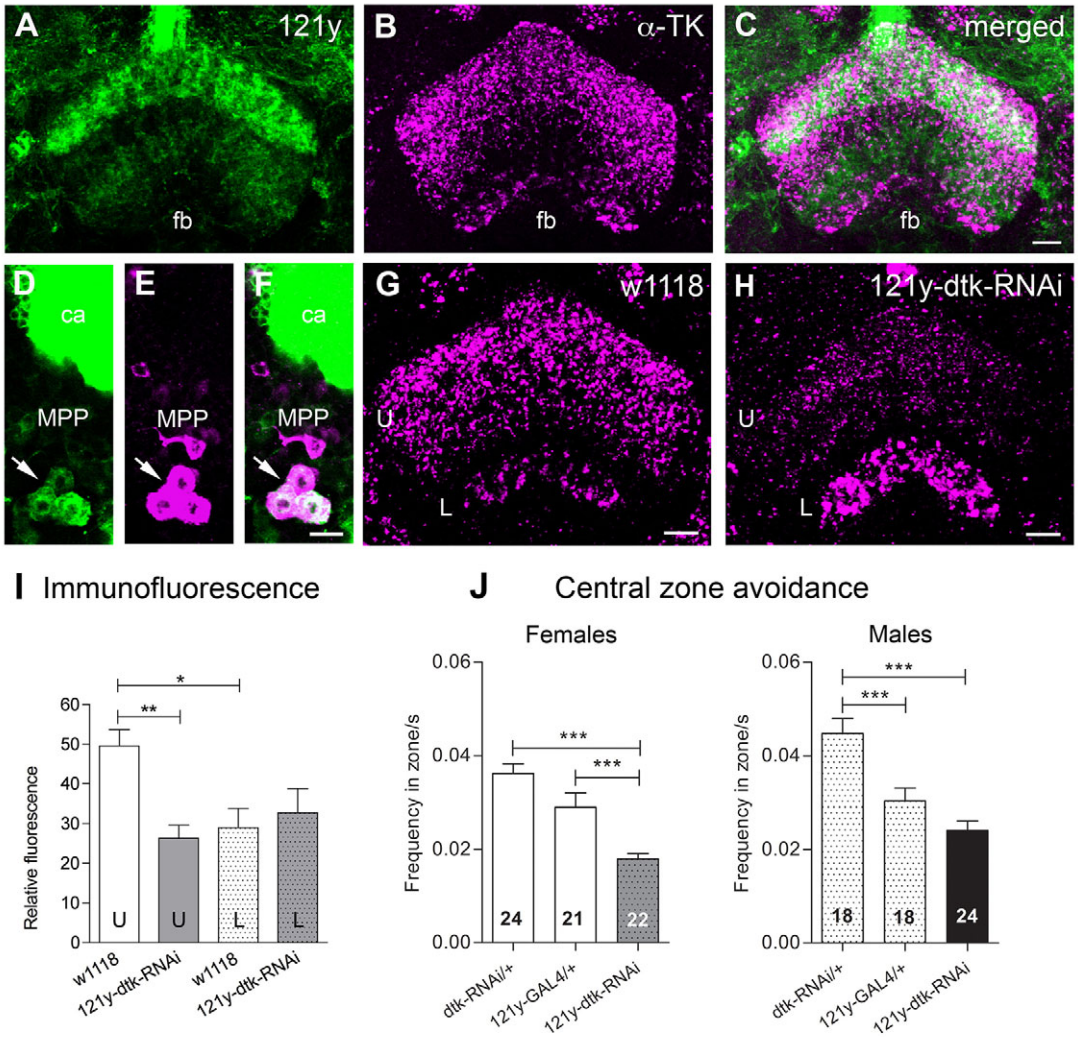

Fig. 2. DTK depletion in tangential neurons increases centrophobism. (A–F) Immunolabeling with antiserum that identifies insect TKs in adult brain expressing 121y-GAL4-driven GFP. 121y-GAL4 drives GFP expression in the upper and lower part of the fb (A) and in tangential neurons ventral to the calyxes (ca) of the mushroom bodies in the median posterior protocerebrum (MPP) (D). Immunolabeling is detected in the fb (B) and in neurons in the MPP (E). Colocalization of immunolabeling and 121y-GAL4 was detected in the upper part of the fb (C). Colocalization was also detected in three neurons in each brain hemisphere in the MPP that are likely to innervate the fb; one brain hemisphere is shown and arrows indicate coexpressing neurons (D–F). DTK levels were knocked down in the upper part (U) of the fb when driving *dtk*-RNAi with the 121y-GAL4 as indicated by reduced TK immunofluorescence (G,H); note that TK immunofluorescence in the non-targeted area of the fb (lower part, L) did not decrease. Immunolabeling of control brain (w^{1118}) is shown in G. (I) Effect of *dtk*-RNAi on the relative immunofluorescence across three specimens per genotype comparing upper and lower parts of the fb. (J) Flies with down-regulated levels of DTK in the fb (121y-*dtk*-RNAi) were monitored for locomotor behavior. This resulted in female flies that moved into the central zone of the arena less frequently compared with controls (*dtk*-RNAi/+ and 121y-GAL4/+). Also male knock-down flies displayed an increase in central zone avoidance; however, since 121y-GAL4/+ control flies displayed an increase in central zone avoidance as well, the mean values of the two were not significantly different. Values are corrected for the activity levels of the flies. Numbers in boxes indicate number of flies recorded. Vertical bars indicate s.e.m. ANOVA: **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars 10 μm.

121y and immunoreactivity in the upper part of the fb (Fig. 2A–C), and in three to four neurons in each brain hemisphere in the median posterior protocerebrum (MPP) (Fig. 2D–F). Both c739-GAL4 and 121y-GAL4 drive expression outside the central complex; however, we could not detect any colocalization of GAL4 and immunoreactivity other than that described above, either in the brain or in the ventral ganglia (not shown).

Since the central complex is suggested to regulate locomotor activity, we studied the effect of DTKs on locomotion. Therefore we down regulated DTK levels in the c739- and 121y-expressing neurons by RNAi and monitored the effect on locomotion. First, we wanted to establish that c739-GAL4 causes a reduction of DTK when driving RNAi. We measured a significant decrease in immunofluorescence in targeted areas of the fb (Fig. 1D–F). Individual flies bearing the c739-GAL4 and *dtk*-RNAi transgenes were video tracked and four parameters of the fly's walking behavior were extracted: total distance moved, number of start/stop actions (activity/inactivity period), mean walking speed and frequency of passages in the central zone. Like other organisms, flies have a tendency to avoid the center of the arena and prefer to walk in the periphery, a phenomenon known as centrophobism (Martin, 2004). To quantify centrophobism, we measured the frequency of passages across a virtual central zone. We found that female flies with DTK depletion in c739-expressing neurons (c739-*dtk*-RNAi) crossed the center of the arena less often

than controls (by 54% compared with *dtk*-RNAi/+ and by 50% compared with c739-GAL4/+) (Fig. 1G). Male c739-*dtk*-RNAi flies also displayed a decrease in frequency of passages into the central zone by 37% and 34% compared with controls (Fig. 1G). We could not detect any significant differences in the other parameters studied (supplementary material Fig. S1). To further confirm the role of DTK in fb neurons, we drove *dtk*-RNAi with 121y-GAL4. Initially, we confirmed that this GAL4 line was able to reduce DTK levels by measuring the immunofluorescence (Fig. 2G–I). We found that female flies with DTK depletion in 121y-expressing neurons (121y-*dtk*-RNAi) also showed a reduction in the number of passages across the central zone (by 62% compared with *dtk*-RNAi/+ and by 50% compared with 121y-GAL4/+) (Fig. 2J). Male flies displayed a decreased number of passages across the central zone by 46% (compared with *dtk*-RNAi/+) (Fig. 2J). However, the GAL4 parental control (121y-GAL4/+) also showed increased central zone avoidance (Fig. 2J), therefore making it somewhat difficult to interpret the contribution of DTK in 121y-expressing neurons in male flies. Additionally, male GAL4 control flies (121y-GAL4/+) showed a significantly different mean walking speed compared with the RNAi control flies (*dtk*-RNAi/+) (supplementary material Fig. S2D). Together these data indicate that 121y-GAL4 males seem to have deficits in some aspects of locomotor behavior, likely due to the insertion of the GAL4 itself. We also detected a decrease in mean walking speed (supplementary material Fig. S2C) and an increased number of start/stop actions (supplementary material Fig. S2E) compared with one of the controls (*dtk*-RNAi/+) in female 121y-*dtk*-RNAi flies, and male 121y-*dtk*-RNAi flies displayed an increase in the total distance moved compared with one of the controls (121y-GAL4/+) (supplementary material Fig. S2B). Differences from only one of the parental controls were not considered pertinent.

In summary, based on the use of two different and independent GAL4 lines, c739 and 121y (except for 121y males), our data suggest that DTK is expressed in two specific populations of neurons innervating the fb that modulate spatial orientation, as measured here by increasing central zone avoidance or centrophobism in DTK-deficient flies.

DTK depletion in pontine neurons increases number of activity–rest phases

Since only a subpopulation of the DTK-expressing neurons innervating the fb were part of the c739-GAL4 and 121y-GAL4, we employed other GAL4 lines to attempt to identify appropriate drivers to knock down peptide levels in the remaining DTK-expressing neurons. We employed the following GAL4 lines: 154y, 210y, c5, c205, c584, NP2320 and NP6510 (supplementary material Table S1). Of these lines, NP2320-GAL4 revealed colocalization of GAL4-driven GFP expression and TK-immunoreactivity. We detected colocalization in the upper part of the fb (Fig. 3Aii–Cii), and in 8–10 immunoreactive neurons in the SMP (Fig. 3Ai–Ci). NP2320-GAL4 has been characterized as driving expression in pontine neurons of the fb (Liu et al., 2006). Pontine neurons are believed to be intrinsic neurons that connect different regions of one central complex substructure (Hanesch et al., 1989; Heinze and Homberg, 2008). Also, 210y-GAL4 displayed colocalization with DTK-expressing neurons (not shown); however, this GAL4 line appeared to have deficits in several aspects of locomotor behavior (not shown); thus we could not use 210y-GAL4 for locomotor behavior analysis.

To explore the walking pattern of flies with diminished DTK levels in pontine neurons we drove *dtk*-RNAi with NP2320-GAL4. First we verified that DTK levels were reduced in the targeted area by measuring immunofluorescence (Fig. 3D–F). Interestingly, female but not male flies with DTK depletion in pontine neurons (NP2320-*dtk*-RNAi) displayed an increase in the number of start/stop actions compared with controls (by 15% compared with *dtk*-RNAi/+ and by 24% compared with NP2320-GAL4/+) (Fig. 3G). No significant difference was observed between NP2320-*dtk*-RNAi and controls for the other parameters studied (supplementary material Fig. S3).

Additionally, we tested flies bearing the c205-GAL4 and the UAS-*dtk*-RNAi constructs for locomotor behavior. The expression of c205-GAL4 does not overlap with immunolabeling and was used as a negative control. As expected we did not detect any significant differences in the parameters measured (supplementary material Fig. S4).

sNPF knock-down in tangential neurons increases distance moved and mean walking speed

sNPF has been reported to be expressed in the central complex (Nässel et al., 2008). In order to locate GAL4 drivers to knock down sNPF levels in the fb we employed a sNPF antiserum in combination with the different GAL4 lines mentioned previously (supplementary material Table S1). Of these GAL4 lines we detected coexpression of sNPF- and GAL4-driven GFP expression in c584-GAL4 and 210y-GAL4. We detected colocalization of sNPF immunoreactivity and c584 expression in the lower part of the fb (Fig. 4Aii–Cii) and in a cluster of five dorso-lateral neurons posterior to the fb in each hemisphere (Fig. 4Ai–Ci). We did not detect coexpression of immunoreactivity and c584-GAL4 in any other cells or neuropils, either in the brain or in the ventral ganglia (not shown).

To examine the effect of sNPF on locomotion, we drove *sNPF*-RNAi with c584-GAL4 and monitored walking behavior. As mentioned above, the 210y-GAL4 flies appeared to have deficits in locomotor behavior and thus were not used in the locomotor assay. First, we verified that the *sNPF*-RNAi produced a reduction of peptide levels with c584-GAL4 by measuring immunofluorescence (Fig. 4D–F). Female flies with sNPF depletion in c584 neurons (c584-*sNPF*-RNAi) traveled longer distances than their controls (by 29% compared with *sNPF*-RNAi/+ and by 27% compared with c584-GAL4/+) (Fig. 4G). Additionally, these flies exhibited a higher mean walking speed by 10% and 13% compared with controls (Fig. 4H). Male sNPF-depleted flies also displayed an increase in distance moved by 17% and 26% compared with controls (Fig. 4G). In contrast to female sNPF knock-downs, male c584-*sNPF*-RNAi did not display a significant increase in mean walking speed (Fig. 4H). We did not detect any significant differences between test and control flies in the number of start/stop actions and in central zone avoidance (supplementary material Fig. S5).

In summary, our data suggest that sNPF expressed in c584 neurons inhibits the overall activity level of the fly, as reduced sNPF levels in c584 neurons increased distance traveled and mean walking speed. Additionally, the modulation of walking speed by sNPF may be sexually dimorphic.

As a negative control, we tested one GAL4 line (c205) that does not coexpress sNPF; as predicted we could not detect any significant differences in the parameters measured (supplementary material Fig. S4).

shi^{ts1} affects locomotor behavior

To further investigate how the fb regulates locomotor behavior we employed a UAS-*shibire*^{ts1} (*shi*^{ts1}) construct under the control of three different fb-GAL4 lines (c739, NP2320 and c584, each of them producing different phenotypes when driving peptide RNAi). *shibire* encodes a temperature-sensitive dominant negative allele of

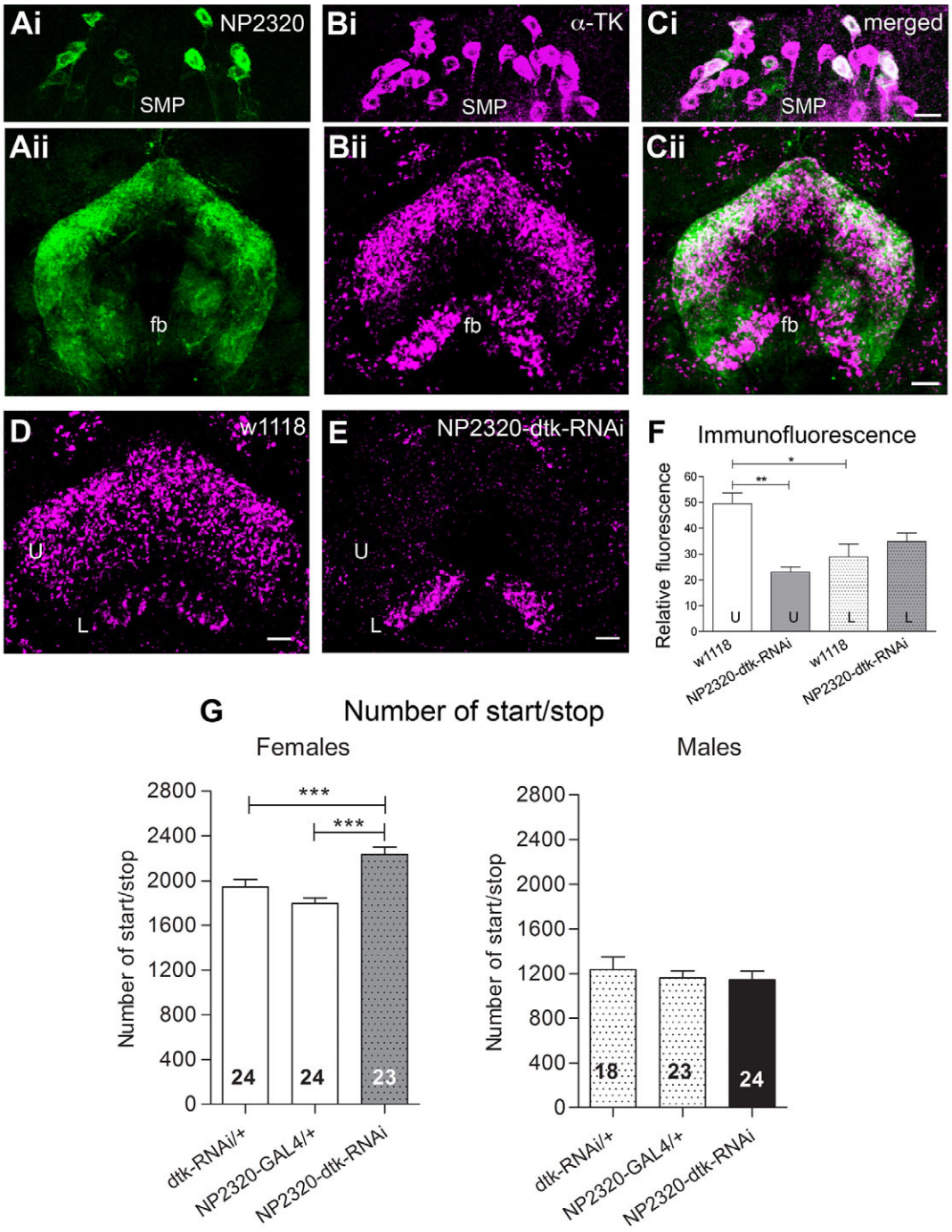

Fig. 3. DTK deficiency in pontine neurons increases the number of activity phases. Distribution of immunolabeling of insect TKs in relation to NP2320-GAL4-driven expression of GFP in the fb (Aii–Cii). (A) NP2320-GAL4-driven GFP expression in pontine neurons in the SMP (Ai) and neuropil (Aii). (B) TK-immunoreactive neurons in the SMP (Bi) innervating the fb (Bii). (C) Coexpression of TK-immunoreactivity and NP2320 in 8–10 neurons in the SMP (Ci) and in the upper part of the fb (Cii). Flies bearing the UAS-*dtk*-RNAi and the NP2320-GAL4 transgenes displayed reduced TK immunofluorescence in the upper part (U) of the fb but not in the non-targeted lower part (L) of the fb (E). Immunolabeling of control brain (w^{1118}) is shown in D. (F) Effect of *dtk*-RNAi on the relative immunofluorescence across three specimens per genotype comparing upper and lower parts of the fb. (G) Driving *dtk*-RNAi in NP2320 neurons altered the number of start/stop actions in female but not in male flies. Numbers in boxes indicate number of flies recorded. Vertical bars indicate s.e.m. ANOVA: **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars 10 μm.

dynamin, a protein associated with endocytosis and release of vesicles from the trans-Golgi network (Kitamoto, 2001; van der Bliek, 1999). The *shi*^{ts1} allele has been used for blocking synaptic transmission at the restrictive temperature (30°C) (Kitamoto, 2001); however, *shi* may also affect other endocytic pathways (Guha et al., 2003). Thus perturbing *shi* function may interfere with receptor-mediated endocytosis, recycling of membrane proteins as well as vesicular trafficking.

Blocking *shi* function in neurons included in the c739-GAL4 line resulted in increased central zone avoidance in both females (32%) and males (25%) (Fig. 5A), thus corroborating a role for c739 positive neurons in centrophobism. Comparing the levels of increased centrophobism in flies with blocked endocytosis with peptide knock-downs may be problematical since they were recorded at different temperatures. Diminishing DTK levels in NP2320-expressing neurons resulted in an increase in start/stop actions in female flies. Using NP2320-GAL4 to drive UAS-*shi*^{ts1} produced female and male flies that started and stopped more frequently than controls (52% and 153%, respectively) (Fig. 5B). Finally, female flies bearing the UAS-*shi*^{ts1} and c584-GAL4 transgenes did not display any significant changes in total distance walked and mean walking speed, whereas male flies walked shorter distances (23%) and were slower (14%) (Fig. 5C,D), in contrast to when knocking down sNPF levels with this GAL4. However, when blocking *shi* function we cannot exclude a prolongation of sNPF signaling in target neurons because it may interfere with receptor-mediated endocytosis and receptor internalization in postsynaptic neurons. For all GAL4 lines tested

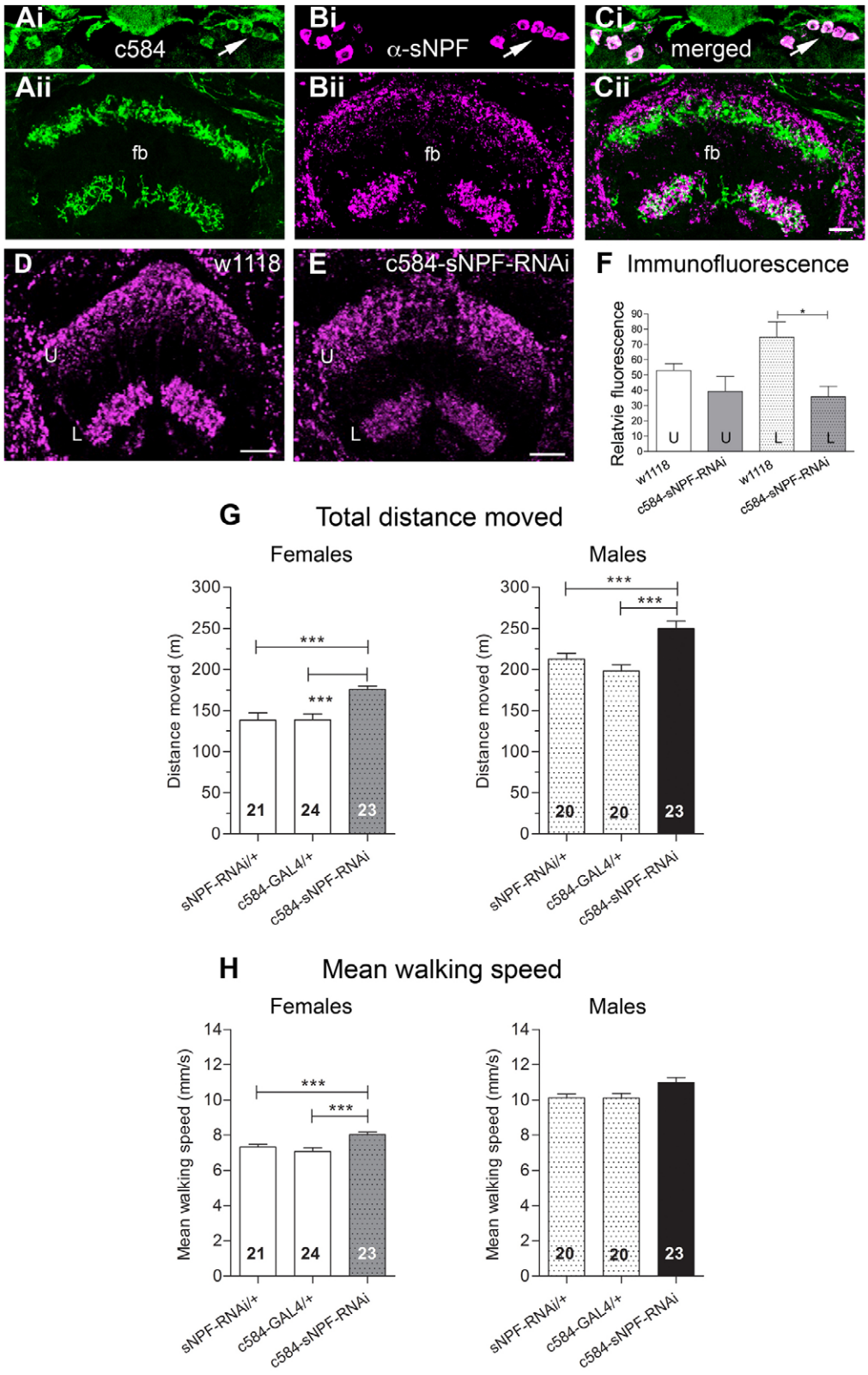

Fig. 4. sNPF depletion in c584 neurons modifies the total distance walked and walking speed. Immunostaining was performed on brain expressing GFP in c584-GAL4 neurons, using an antiserum towards sNPF (A–F). (A) GFP expression of c584-GAL4 in the upper and lower part of the fb (Aii) and in neuronal cell bodies (Ai, arrow indicating cell bodies in one brain hemisphere). (B) sNPF-immunoreactivity in the fb (Bii) and in five sNPF-immunoreactive neurons in each brain lobe (Bi). (C) Colocalization of immunoreactivity and c584-GAL4 expression was detected in the lower part of the fb (Cii) and in five neurons in the dorso-lateral posterior protocerebrum of each brain lobe (Ci). Reduced sNPF immunofluorescence is observed in the lower part (L) of the fb in sNPF knock-down flies (c584-*sNPF*-RNAi) (E), compared with the non-targeted area of the fb (upper part, U) and control brain (w^{1118}) (D). (F) Effect of *sNPF*-RNAi on the relative immunofluorescence across three specimens per genotype. (G,H) *sNPF*-RNAi was expressed in c584-GAL4 neurons and peptide knock-down flies were monitored for locomotor behavior. Female and male flies with sNPF depletion in c584 neurons (c584-*sNPF*-RNAi) walked longer distances than controls (*sNPF*-RNAi/+ and GAL4-c584/+) (G). Female but not male sNPF knock-down flies displayed significantly higher mean walking speed (H). Numbers in boxes indicate number of flies recorded. Vertical bars indicate s.e.m. ANOVA: **P*<0.05, ****P*<0.001. Scale bars 10 μm.

additional parameters were also affected (supplementary material Fig. S6), which may be accounted for by GAL4 expression outside the fb.

Taken together these data suggest the possibility that neurotransmission in specific fb neurons, of which some are expressing DTK, is essential for centrophobism. The role of pontine fb neurons in controlling the temporal organization of walking activities is also supported.

DISCUSSION

Here we report that specific DTK-expressing neurons may be involved primarily in the regulation of spatial distribution of flies

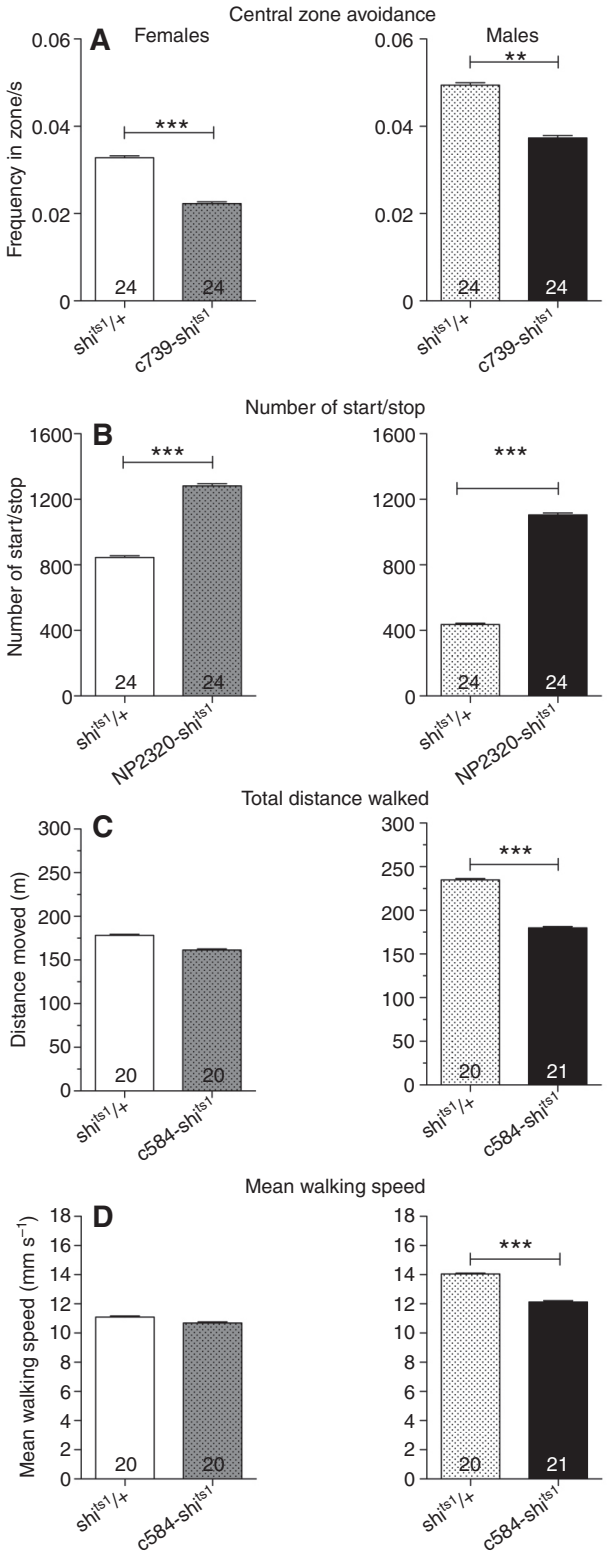

Fig. 5. Bolcking endocytosis with *shi*^{ts1} alters several aspects of locomotor behavior. Analysis of locomotor patterns in flies with selective neuronal inhibition. Blocking endocytosis by expressing *shi*^{ts1} in c739 neurons increased central zone avoidance in both male and female flies (A). Both sexes of NP2320-GAL4-UAS-*shi*^{ts1} displayed an increase in the number of start/stop actions (B). Total distance walked and mean walking speed were decreased in male c584-GAL4-UAS-*shi*^{ts1} (C,D). Numbers in boxes indicate number of flies recorded. Vertical bars indicate s.e.m. ANOVA: $^{**}P<0.01$, $^{***}P<0.001$.

and in the modification of the number of activity–rest bouts, whereas sNPF appears to play a role in the fine tuning of walking speed and distance walked. Altogether, our results suggest that different peptidergic neurons of the *Drosophila* central complex modulate distinct aspects of locomotor behavior.

Interference with DTK expression in the fb

The effectiveness of the RNAi in down regulating peptide expression was determined by measuring immunofluorescence. As this method does not give an absolute measure of peptide levels it is difficult to address the actual amount of peptide still remaining. However, it is worth noting that there was no reduction in immunofluorescence in other parts of the fb where no overlap of peptide and GAL4 was detected. Additionally, the GAL lines used to drive RNAi produced distinct patterns of down regulation, confirming that the different GAL4/peptide-positive neurons supply the fb in specific patterns.

DTKs: distribution and contribution to locomotor behavior

We identified three GAL4 lines that drive expression in TK immunoreactive cells innervating the fb. Two of these (c739 and NP2320) are expressed in a group of immunolabeled cells located in the SMP, a cell group that previously have been described as fb-invading neurons (Winther et al., 2003). We also located a second group of neurons, situated in the MPP, which appears to give rise to fine immunoreactive fibers projecting to the fb. The axons of these neurons could not be resolved in detail as they enter the fb. However, by comparing the expression pattern of 121y-GAL4 and the pattern of immunoreactivity we can narrow down the neuropil where they are co-expressed to a stratum in the upper part of the fb and to a group of cells in the MPP. Thus, the expression patterns indicate that the immunolabeled cells in the MPP project to the fb.

Using these markers for different neuronal types (c739, 121y and NP2320), we noted that the central complex of the *Drosophila* brain is likely to be innervated by TK-expressing tangential, columnar and pontine neurons. TK immunostaining of the central complex in various other insect species also shows obvious diversity in neuron types. For instance, in the locust four different populations of columnar fb neurons and two different populations of tangential neurons have been identified (Vitzthum and Homberg, 1998). Based on morphology it has been suggested that the main inputs to the central complex are through large field tangential neurons and that main output channels are through columnar elements, while pontine neurons are believed to be intrinsic elements connecting columns and layers within the central complex (Hanesch et al., 1989; Heinze and Homberg, 2008). From these assumptions, one could speculate that immunolabeled tangential neurons provide input and are presynaptic in the fb, and that immunolabeled columnar neurons are postsynaptic in the fb.

After knock-down of DTK levels in a subpopulation of columnar neurons (c739-GAL4) and in DTK-expressing tangential neurons (121y-GAL4), flies spent an increased amount of time in the periphery of the open field arena. The behavior of avoiding the potentially dangerous central zone of an open field has previously been described in both vertebrates (Kallai et al., 2007; Lamprea et al., 2008) and flies (Besson and Martin, 2005; Lebreton and Martin, 2009; Martin, 2004). In *Drosophila*, this phenomenon has been referred to as centrophobism and it has been shown to be regulated, at least partially, by the mushroom bodies (Besson and Martin, 2005; Lebreton and Martin, 2009). The mushroom bodies are major brain structures primarily studied for their role in olfactory memory formation (Keene and Waddell, 2007) and also, but to a lesser extent, for their contribution to locomotor activity (Besson and Martin,

2005; Martin et al., 1998). Disruption of mushroom body functions decreases central zone avoidance (Besson and Martin, 2005) whereas DTK deprivation in specific central complex neurons increased central zone avoidance. Therefore it seems that the role of the central complex is opposite to that of the mushroom bodies. Our results thus appear to be congruent with previous findings of antagonistic roles of the mushroom bodies and the central complex in the control of locomotion, where the mushroom bodies suppress locomotor activity (Martin et al., 1998) and the central complex increases walking activity (Martin et al., 1999b). Moreover, when disrupting signaling from two other substructures of the central complex, the protocerebral bridge and the ellipsoid body, no significant changes in centrophobic behavior could be detected (Besson and Martin, 2005). In concert with our findings it is possible to suggest that the fb, but neither the protocerebral bridge nor the ellipsoid body (or at least the partial neuronal components of these complex structures that have been investigated in these former studies) contributes to the central zone avoidance behavior.

Centrophobism relies on visual features (Besson and Martin, 2005), and fb neurons have been shown to be part of a circuitry implicated in visual memory traces (Liu et al., 2006). Interestingly, we show here that the same neurons (121y-GAL4) that were shown to participate in visual memory traces are also suggested to be involved in the modulation of centrophobism. However, up to the present the neurons supplying the fb with visual input have not been identified and they need to be resolved for a more complete understanding of visually oriented behaviors. DTK deficiency in both a set of columnar neurons and selected large field tangential neurons affected central zone avoidance, suggesting that these identified neurons are part of a circuitry required for the proper maintenance of centrophobism.

As suggested previously, the function of DTKs is dependent on circuitry (Ignell et al., 2009; Winther et al., 2003). Here, we have shown that relatively small subpopulations of neurons (24–30 of the total of ~150 DTK-expressing neurons in the brain) innervating the central complex are modifying different parameters of walking behavior. In addition to the contribution to central zone avoidance, our results indicate that DTKs also modify the number of activity–rest bouts in female flies as demonstrated when down regulating DTK levels in a set of pontine neurons (NP2320-GAL4). The organization of locomotor activity in active and inactive periods is strictly regulated, where the change between an active and an inactive period is suggested to involve the switching on and off of a central pattern generator (CPG) (Martin, 2004; Martin et al., 1999a). In mammals and in lampreys, the TK Substance P initiates locomotion when injected into the brain stem (Dubuc et al., 2008; Garcia-Rill et al., 1990). Furthermore, endogenously released TK has been found to contribute to baseline frequency and initiation of activity in networks generating locomotor activity in the lamprey (Perez et al., 2007). Thus, these findings point to a role of DTK in the modulation of neuronal networks contributing to CPGs.

Modulation of locomotor behavior by sNPF

We show here that sNPF may be involved in the fine tuning of locomotor activity levels, since flies with sNPF deficiency in the fb increased the distance that they covered and female flies showed an increased mean walking speed. We have down regulated sNPF levels in approximately 10 out of thousands of sNPF-expressing neurons in the brain. These neurons are located in pairs of five on either side of the brain and this cell cluster has previously been characterized as fb-invading neurons (Martin et al., 1999b). Recent studies have suggested that sNPF probably has multiple functions and possibly acts as a cotransmitter (Nässel et al., 2008) or as a local neuromodulator (Park et al., 2008). Previously, sNPF has been shown to regulate feeding behavior (Lee et al., 2004) and has also been suggested to regulate production/secretion of *Drosophila* insulin-like peptides (Lee et al., 2008). Our findings suggest that besides a role in feeding and metabolism sNPF is also involved in the inhibition of locomotor activity levels.

Interfering with endocytosis in the fb

When blocking endocytosis in c739-GAL4- and NP2320-GAL4-expressing neurons we confirmed that these neurons are involved in the regulation of centrophobism and of activity phases, respectively. Blocking *shibire* function resulted in alterations of locomotor behavior in addition to those found when down regulating peptide levels. It is possible that these parameters are controlled by neurons included in the GAL4 lines not overlapping with peptide expression, e.g. neurons in the thoracic ganglia. CPGs are localized to the thoracic nervous system and are modulated by thoracic interneurons (Ritzmann and Büschges, 2007); hence, neurons in the thoracic ganglia may contribute to the supplementary locomotor phenotypes. Neuropeptides frequently colocalize with fast neurotransmitters and are likely to act as cotransmitters (Burnstock, 2004; Nässel and Homberg, 2006). Thus, blocking *shibire* function in peptidergic neurons will also target possible cotransmitters. In fact, it is not known how shi^{ts1} interferes with peptide signaling as mechanisms of peptide vesicle release and termination of peptide signals are different from those of neurotransmitter signaling (Merighi, 2002). Recently, shi^{ts1} was shown to interfere with receptor internalization of the PDF neuropeptide rather than with PDF peptide vesicle trafficking (Wülbeck et al., 2009). Flies with shi^{ts1}-blocked endocytosis in c584 neurons displayed different changes in activity levels to flies bearing the *sNPF*-RNAi and c584-GAL4 transgenes. It is possible that c584-GAL4 also includes neurons expressing the sNPF receptor. Hence, shi^{ts1}-induced perturbation of endocytosis may increase sNPF signaling by blocking receptor internalization, a key step in peptide signal termination. However, the distribution of the sNPF receptor in the brain has not yet been determined. Alternatively, sNPF may have a developmental role in c584 neurons and interfering with sNPF signaling with RNAi may produce different effects from those with temporally restricted shi^{ts1} silencing.

Sexual dimorphism of locomotor behavior

Some aspects of locomotor behavior are known to be sexually dimorphic in wild-type flies (Martin, 2004; Martin et al., 1999a). In this study we observed that interfering with peptide signaling in NP2320-GAL4- and c584-GAL-expressing neurons only perturbed the behavior of female flies. Additionally, disrupting vesicular traffic by expressing shi^{ts1} affected activity levels in male flies only. Whether this dimorphism depends on subtle sex-specific differences in the expression of the different GAL4s and effectors or on an inherent sex-specific regulation of locomotor behavior is unknown. We have not detected any dimorphic peptide expression pattern. Alternatively, the receptor distribution may be expressed in sexually distinct patterns, which remain to be determined.

CONCLUSIONS

We have analyzed the contribution to locomotion of two out of several neuropeptides expressed in the *Drosophila* central complex. We have thus gained examples of the diverse roles that neuropeptides may have in the fine tuning of locomotor behavior. Though the

picture is far from complete, it is tempting to speculate that peptidergic signaling circuits, by acting on discrete targets within the central complex, play important roles in increasing flexibility and dynamics in the networks modulating locomotor activity.

LIST OF ABBREVIATIONS

CPG	central pattern generator
DTK	*Drosophila* tachykinin
fb	fan-shaped body
GFP	green fluorescent protein
MPP	median posterior protocerebrum
SMP	superior median protocerebrum
sNPF	short neuropeptide F
TK	tachykinin

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Table S1. Coexpression of peptide immunoreactivity and GAL4 driver line

GAL4	Neuron type[a]	Coexpression of TK-IR (number of neurons)	Coexpression of sNPF-IR (number of neurons)	Ref.[b]
121y	Tangential	2×3–4[c]	n.d.	1
154y	Tangential	n.d.	n.d.	1
210y[d]	Tangential	2×3–4[c], 2×4[c,e]	2×2[c]	1
c5	Tangential	n.d.	n.d.	1,2
c205	Tangential	n.d.	n.d.	2
c584	Tangential	n.d.	2×5[c]	2
c739	Columnar	10–12	n.d.	3
NP2320	Pontine	8–10	n.d.	1
NP6510	Tangential	n.d.	n.d.	1

TK-IR, tachykinin immunoreactivity; sNPF-IR, short neuropeptide F immunoreactivity; n.d., not detected.

[a]Type of fan-shaped body neuron marked by GAL4.

[b]References: 1, Liu et al., 2006; 2, Martin et al., 1999b; 3, Armstrong et al., 1998.

[c]Pairs of neurons in each brain hemisphere.

[d]The 210y-GAL4 displayed a locomotor phenotype and thus could not be used for the locomotor behavioral assay.

[e]Two different populations of 210y-tangential neurons were coexpressing TK-IR.