

Tachykinin-related peptides modulate odor perception and locomotor activity in *Drosophila*

Åsa M.E. Winther,^a Angel Acebes,^b and Alberto Ferrús^{b,*}

^aDepartment of Zoology, Stockholm University, S-106 91 Stockholm, Sweden

^bDepartment of Developmental Neurobiology, Instituto Cajal, Consejo Superior de Investigaciones Científicas, Ave. Dr. Arce 37, E-28002 Madrid, Spain

Received 30 June 2005; revised 9 October 2005; accepted 14 October 2005

Available online 11 November 2005

The invertebrate tachykinin-related peptides (TKRPs) constitute a conserved family, structurally related to the mammalian tachykinins, including members such as substance P and neurokinins A and B. Although their expression has been documented in the brains of insects and mammals, their neural functions remain largely unknown, particularly in behavior. Here, we have studied the role of TKRPs in *Drosophila*. We have analyzed the olfactory perception and the locomotor activity of individuals in which TKRPs are eliminated in the nervous system specifically, by using RNAi constructs to silence gene expression. The perception of specific odorants and concentrations is modified towards a loss of sensitivity, thus resulting in a significant change of the behavioral response towards indifference. In locomotion assays, the TKRP-deficient flies show hyperactivity. We conclude that these peptides are modulators of olfactory perception and locomotion activity in agreement with their abundant expression in the olfactory lobes and central complex. In these brain centers, TKRPs seem to enhance the regulatory inhibition of the neurons in which they are expressed.

© 2005 Elsevier Inc. All rights reserved.

Keywords: *Drosophila*; Neuropeptide; RNA interference; Antennal lobe; Central body complex; Olfaction; Locomotion

Introduction

Neuropeptides are the most chemically diverse and numerous (>100) type of neurotransmitters. Their role in the central nervous system (CNS) of insects remains largely unknown because antagonists and pharmacological data are scant. Tachykinin-like peptides (TKLPs) constitute a large and diverse family, found in both vertebrates and invertebrates. TKLPs can be divided into two distinct groups based on their C-terminal sequence motif. All known vertebrate tachykinins share the C-terminal pentapeptide FXGLM, whereas the tachykinin-related

peptides (TKRPs), found exclusively in invertebrates, contain the somewhat different pentapeptide FX₁GX₂R (Nachman et al., 1999; Nässel, 1999; Vanden Broeck et al., 1999). Both groups, however, are assumed to be ancestrally related, hence the name tachykinin-related peptides (Schoofs et al., 1990; Nässel, 1999; Vanden Broeck et al., 1999; Nässel, 2002). Based on immunocytochemical data, insect TKRPs are predominantly located in interneurons within the CNS and in endocrine cells of the intestine (Nässel, 1999, 2002; Kwok et al., 2005), suggesting a role in neuromodulation and in gut function. In the *Drosophila* TKRP prohormone gene *dtk* (CG 14734), five different peptide isoforms (DTK-1 to 5) have been identified (Siviter et al., 2000; Winther et al., 2003). Two TKRP receptors, NKD and DTKR, have been cloned from *Drosophila* based on sequence similarities to mammalian tachykinin receptors (Li et al., 1991; Monnier et al., 1992). DTKR was shown to be activated by substance P, indicating that the corresponding functions may be conserved as well (Li et al., 1991). More recently, this receptor has been demonstrated to bind various DTK peptides (Birise et al., 2005). The TKRPs show effects in a number of different in vitro assays (Nässel, 1999; Vanden Broeck et al., 1999; Nässel, 2002). Predominantly, these actions are on different types of muscle, but there are few studies of the modulatory actions of the TKRPs on insect and crustacean neurons or in neuronal networks. For instance, TKRPs enhance the hyperpolarizing response to GABA in photoreceptors of the crayfish *Pacifastacus leniusculus* (Glantz et al., 2000) and trigger bursts of action potentials in the dorsal unpaired median neurons of the metathoracic ganglia of the locust *Locusta migratoria* (Lundquist and Nässel, 1997). TKRPs also regulate the modulation of rhythmic motor pattern of the stomatogastric ganglion of the crab *Cancer borealis* (Nusbaum et al., 2001; Swensen and Marder, 2001). However, no in vivo data on TKRPs physiological role in the CNS are available. Here, we have taken advantage of the genetic tools that *Drosophila* offers to perform an in situ study of TKRPs. We have analyzed the olfactory perception and the locomotor activity of individuals in which expression of these peptides is eliminated in the nervous system specifically. This study is the first to demonstrate behavioral effects of TKRPs.

* Corresponding author. Fax: +34 91 5854754.

E-mail address: aferrus@cajal.csic.es (A. Ferrús).

Available online on ScienceDirect (www.sciencedirect.com).

Results

Design of the RNA interference to inactivate the *dtk* gene

We used genomic–cDNA RNA interference (RNAi) to target the *dtk* gene where the resulting transcript is predicted to form loopless double-stranded RNA after splicing (Kalidas and Smith, 2002). Including functional introns in the RNAi construct increases the stability of the inverted-repeat sequences in bacteria and thereby facilitates the cloning process and, in addition, enhances the silencing effect of the RNAi (Kalidas and Smith, 2002; Lee and Carthew, 2003). We fused a genomic DNA fragment encoding the progenitor sequences of DTK-1–5 (Siviter et al., 2000) to the corresponding inverted cDNA fragment (Fig. 1). The *dtk*-RNAi construct was cloned into the pUAST vector (Brand and Perrimon, 1993) where it is under the control of the Gal4 UAS regulatory sequences. By expressing the Gal4 transcription factor under the control of a *Drosophila* promoter, we can direct the expression of the construct to tissues of interest. The construct was injected into *yellow-white* (*y w*) embryos, and two separate lines were obtained (UAS-*dtk*-RNAi37A and UAS-*dtk*-RNAi37D, on chromosome II and III respectively).

Interestingly, driving the expression of the *dtk*-RNAi construct to all cell types (*tub*-Gal4-LL7) resulted in lethality, mostly at the embryonic stage, demonstrating that the construct produces interference. This result suggests a vital, and yet undescribed, function for DTK in embryonic development. Less likely, the lethality could result from cross-interference of the *dtk*-RNAi construct with another putative vital gene. 97% of the UAS-*dtk*-RNAi37A; *tub*-Gal4-LL7 individuals died at the embryo stage, and the remaining 3% died as first instar larvae. Among animals expressing the construct UAS-*dtk*-RNAi37D, 77% died at the embryo stage and 23% as first larval instar. The usage of a more restricted Gal4 (Gal4-T80) (Hrdlicka et al., 2002) that allowed viability until adulthood enabled us to quantify the effect of the interference. To monitor the effect of the *dtk*-RNAi construct, we employed quantitative real-time PCR (QRT-PCR) and immunocytochemistry. QRT-PCR was performed on flies expressing the UAS-*dtk*-RNAi construct in one copy driven by either Gal4-T80 or the neural *elav*-Gal4-C155. The levels of *dtk* transcripts in these flies were compared to the levels in the different parental lines and *y w*. An average transcript reduction of 91% (± 0.8 SEM) was measured (Fig. 2). Since these two drivers allow viability, in contrast to the general *tub*-Gal4-LL7, the remaining 10% of *dtk* transcription must be responsible for this survival.

The interference of *dtk* expression was confirmed by immunocytochemistry using an antiserum to the C-terminus of one of the cockroach TKRPs. This antiserum is known to recognize the C-

terminal residues FX₁GX₂R-amide of insect TKRPs, including *Drosophila* DTKs (Winther et al., 2003). Immunocytochemistry was carried out on whole third instar CNS of control larvae and of larvae expressing one copy of the RNAi construct driven by *elav*-Gal4-C155 (Figs. 3A–D). As expected, TKRP immunoreactivity could be detected in control flies (Figs. 3A and C), showing neuronal cell bodies with varicose processes in brain, subesophageal and ventral ganglia in agreement with previous findings (Winther et al., 2003). Immunoreactivity was strongly reduced in the *dtk*-RNAi expressing nervous systems (Figs. 3B and D), faint staining was detectable in cell bodies in the protocerebrum and in the subesophageal ganglion. As an example, a pair of strongly TKRP-immunoreactive neurons in the anterior part of the protocerebrum that give rise to axonal processes descending throughout the length of the ventral nerve cord (designated descending neurons) (Winther et al., 2003) was observed in control flies (Figs. 3A and C) but could not be detected in *dtk*-RNAi expressing flies (Figs. 3B and D).

The same test performed on adult flies bearing two copies of the transgene showed a stronger reduction of *dtk* expression (Figs. 3E–H). TKRP immunoreactivity in sections of adult brain was not detectable in *elav*-Gal4-C155; UAS-*dtk*-RNAi37DAS-*dtk*-RNAi37D flies (Figs. 3F and H). TKRP immunoreactivity in control parental line (*y w*; UAS-*dtk*-RNAi37D) was detected in several cell bodies and neuropils similar to the immunoreactivity detected in wild-type (Winther et al., 2003). For example, a prominent signal was detected in the antennal lobes, which are innervated by varicose processes arising from two clusters of cell bodies in the anterior deutocerebrum (Fig. 3E). Strong immunoreactivity was also detected in the fan-shaped body of the central body complex (Fig. 3G). We conclude that the *dtk*-RNAi drastically reduces the expression of the *dtk* gene in vivo. In the CNS, in particular, the gene expression seems abolished by *elav*-Gal4-C155 driving two copies of the construct. This genotype, however, is viable probably thanks to the non-abolished *dtk* expression in cells outside the C155 domain allowing behavioral studies.

Olfactory behavioral effect of TKRPs in the adult

The antennal lobe neuropil in the brain of *Drosophila* is densely innervated by TKRP expressing interneurons (Fig. 3E). This distribution suggests a role in olfactory processing. Thus, we tested the neural *dtk* knock-down flies for olfactory behavioral response to a set of odorants over a range of concentrations in a T-maze setup. We tested four odorants (acetone, benzaldehyde, butanol and isoamyl acetate) belonging to different chemical classes. Flies were allowed to choose between the odorant and the solvent (control) compartment. The results are expressed as an olfactory index, RI

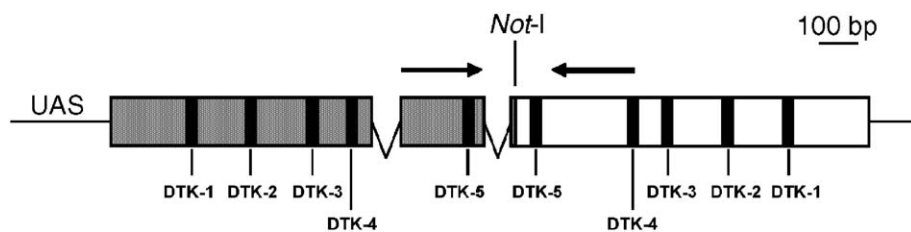


Fig. 1. Structure of the UAS-*dtk*-RNAi construct. Genomic DNA (including part of exon 2, exon 3, part of exon 4 and introns 2 and 3) was cloned using a *NotI* restriction site to the corresponding inverted cDNA fragment. Gray boxes represent genomic exons, white boxes represent inverted cDNA. DTK precursor sequences are labeled and indicated by black bars. UAS: upstream activating sequence. Arrows indicate sense of coding sequence.

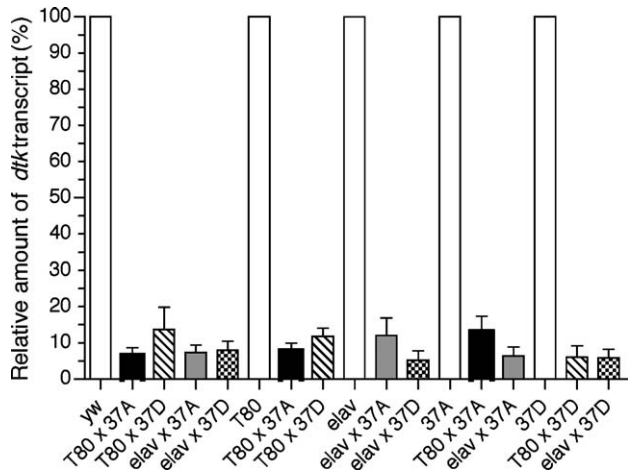


Fig. 2. QRT-PCR analysis of *dtk* expression and effects of *dtk*-RNAi. Transcription levels in flies expressing a *dtk*-RNAi construct, 37A or 37D, driven either by Gal4-T80 or *elav*-Gal4-C155 are compared to *y w* and to parental lines (set as 100%). Transcript levels are normalized to *RNApolIII* as internal standard. Each bar represents the average of 3–5 independent RNA extractions. Vertical bars indicate SEM.

(see Experimental methods), and are based on the behavioral response of approximately 500 flies in 50 independent choice tests.

Experimental and control flies displayed similar responses to acetone on the range of concentrations tested (Fig. 4). With benzaldehyde and butanol, however, there was a significant difference in the responses to high concentration (10^{-1} vol/vol) (Student's *t* test $P < 0.01$ and $P < 0.05$, respectively). The TKRP-deficient flies were less repelled than the control flies (Fig. 4). Dose-response curves to isoamyl acetate revealed significant differences over a wider range of concentrations. A common feature of the three odorant perceptions is that the behavioral responses changed in the direction of a loss of perception sensitivity in the deficient flies (Fig. 4). Since the behavioral effect elicited could be considered as subtle, further experiments to confirm this feature were conducted.

Olfactory behavioral effect of TKRPs in larvae

Like the adult, larvae also respond specifically to a large number of odorants, albeit based on a neuronal circuit very different from that of the adult, due to substantial reorganization during metamorphosis (Heimbeck et al., 1999). We assayed larvae of the same genotypes as in the adult tests under a similar olfactory choice test and scored by the same index (RI). Larvae (total $n = 400$ per genotype) were allowed to choose between the odorant (undiluted) and the solvent (see Experimental methods). The data for the two odorants tested, benzaldehyde and isoamyl acetate, show that control larvae become attracted with RI values consistent with previous reports (Heimbeck et al., 1999). By contrast, larvae expressing two copies of the *dtk*-RNAi construct appeared as indifferent, particularly in the case of benzaldehyde (Fig. 5). These results are coincident with those from the adult and confirm the behavioral change caused by the neural deficiency of TKRPs.

Behavioral effects of TKRPs in other sensory modalities

Since the TKRP depletion was elicited throughout the central nervous system, we reasoned that the observed effects on olfactory

perception, although subtle, should be detected in other sensory modalities as well. The TKRP expression data on the wild type indicated the central complex as one of the major brain centers where immunoreactive cells are present (Winther et al., 2003) (Fig.

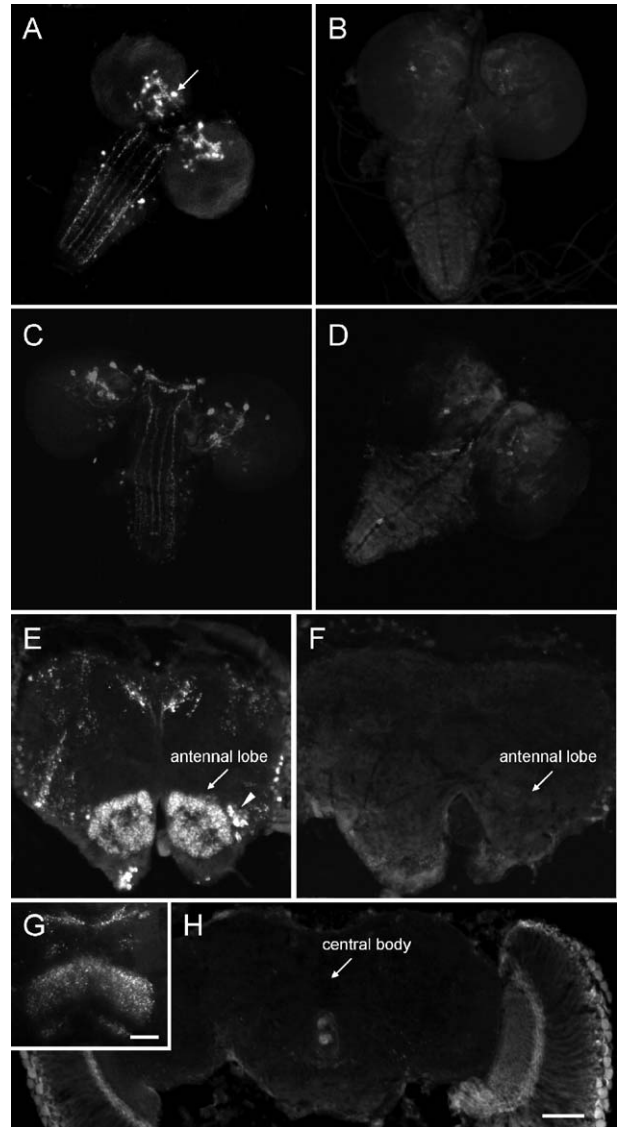


Fig. 3. Effect of *dtk*-RNAi upon TKRP expression. Immunoreactivity in whole mounts of larval third instar CNS (A–D) and sections of adult brain (E–H) using a general insect TKRP primary antiserum with Cy3-tagged secondary antiserum. Control parental lines (A and C) show immunoreactive cell bodies with processes in brain, subesophageal and ventral ganglia. Arrow indicates one of the descending neurons with axons running throughout the ventral nerve cord. The signal is strongly reduced in *dtk*-RNAi expressing larvae (B and D). (E) Control parental line shows immunoreactive cell bodies (arrow head) in the anterior deutocerebrum with varicose processes in the glomeruli of the antennal lobe (arrow). Immunoreactivity is also seen in other parts of the brain. (G) Detail from control parental line showing the fan-shaped body (of the central complex) with immunoreactive varicosities. Immunoreactivity is not detectable in flies expressing two copies of the *dtk*-RNAi construct (F and H). A: *elav*-Gal4-C155. B: *elav*-Gal4-C155; UAS-*dtk*-RNAi37D/+. C: *y w*; UAS-*dtk*-RNAi37A. D: *elav*-Gal4-C155; UAS-*dtk*-RNAi37A/+. E and G: *y w*; UAS-*dtk*-RNAi37D. F and H: *elav*-Gal4-C155; UAS-*dtk*-RNAi37D. Scale bars = 25 μm in G; 100 μm in H.

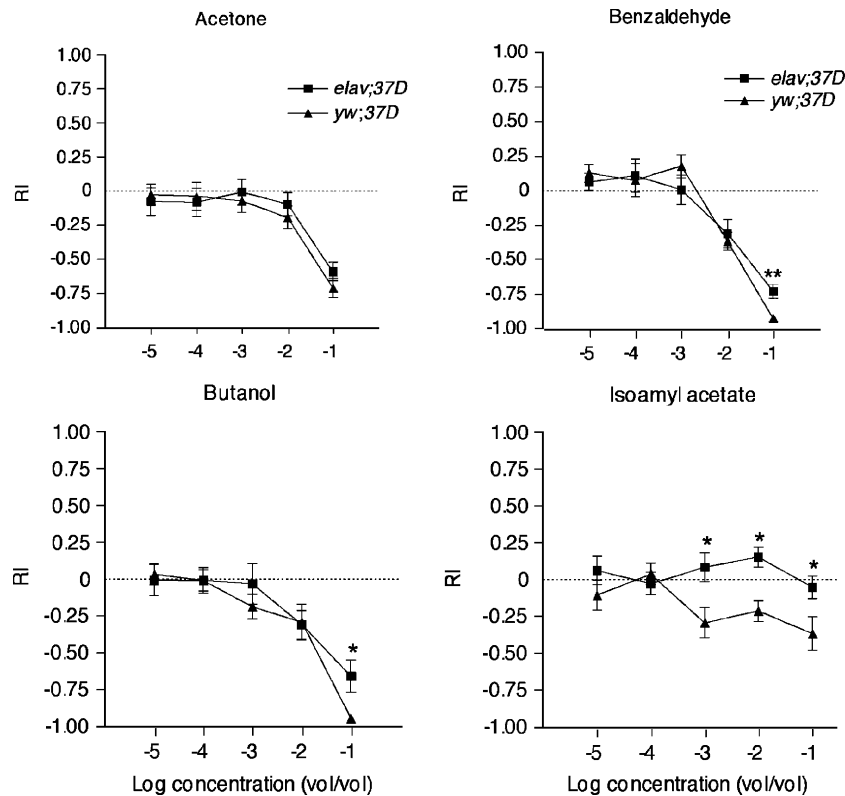


Fig. 4. Olfactory behavior of neural DTK-deficient flies. Dose–response curves for *dtk*-RNAi expressing flies (*elav*-Gal4-C155; UAS-*dtk*-RNAi37D) (squares) and control flies (*yw*; UAS-*dtk*-RNAi37D) (triangles) on an olfactory choice assay. Positive index values (RI) represent attraction, while negative RIs indicate repulsion. Average RI values are based on 10 replicates of ~25 flies each. Vertical bars indicate SEM. Asterisks denote statistical significance (Student's *t* test, **P* < 0.05, ***P* < 0.01).

3G). The central body complex has been implicated in the control of locomotor activity (Strauss and Heisenberg, 1993; Strauss, 2002). In that context, we tested the *dtk*-abolished flies in the Buridan's arena, a locomotion test based on the walking activity between two opposing visual stripes (see Experimental methods).

The same genotypes as in previous assays were used in single fly tests (total *n* = 12 per genotype). The two experimental groups showed the same type of effect, higher locomotion activity than the controls (Fig. 6). The effect was reproduced in one RNAi construct as well as in flies carrying one copy of each RNAi construct (*elav*-Gal4-C155; UAS-*dtk*-RNAi37A/+; UAS-*dtk*-RNAi37D/+). Of the three parameters measured, percentage of active time and distance walked were significantly increased. As expected, the speed of movement was not changed, as it is controlled by thoracic motoneurons and the leg muscles, while the central complex in the brain is thought to control the initiation and maintenance of locomotion (Strauss, 2002). We concluded that *Drosophila* TKRPs, in addition to modulation of olfactory perception, are also involved in the modulation of locomotor activity, and these functional roles are in agreement with the brain centers where these peptides are expressed.

Discussion

The *Drosophila dtk* gene, which encodes the pre-protein of the DTK1–5 peptides, is expressed in late stage embryo, larvae and adult (Siviter et al., 2000; Winther et al., 2003). Beyond the endocrine cells of the gut, these peptides are predominately

expressed in neuropils of the antennal lobe and central body complex of the adult CNS suggesting neural functions, perhaps related to olfaction and locomotion. These functions, however, were unknown. Here, we addressed possible roles of DTKs using RNAi to produce neural *dtk* knock-down flies in conjunction with the corresponding behavioral tests. We found that DTK-deficient larvae and adult flies display subtle odorant-specific defects consistent between adults and larvae. These flies also exhibited increased locomotor activity. These experiments reveal the first possible physiological roles for *Drosophila* TKRPs in fine-tuning of olfactory processing and locomotor activity. The distribution of TKRP-immunoreactive material has been studied in a number of different insect species. In all of them, TKRPs have been found in the antennal lobe and in the central body complex (Nässel, 1999, 2002). Hence, we suggest that the involvement of the DTKs in olfaction and locomotion shown here may be a general characteristic for TKRPs in insects.

Interference with *dtk* expression

The effectiveness of the RNAi constructs to virtually abolish *dtk* expression was proven by QRT-PCR and immunocytochemical analysis. We determined the expression level in whole flies in which the *dtk*-RNAi construct was driven by a general, but not ubiquitous, Gal4 (T80), as well as in heads from flies with the construct driven with a neural Gal4 (*elav*-C155). Using only the heads from these flies was justified since, in the adult head, TKRPs are expressed exclusively in the brain. We determined a consistent ~90% decrease of the gene expression when measuring the

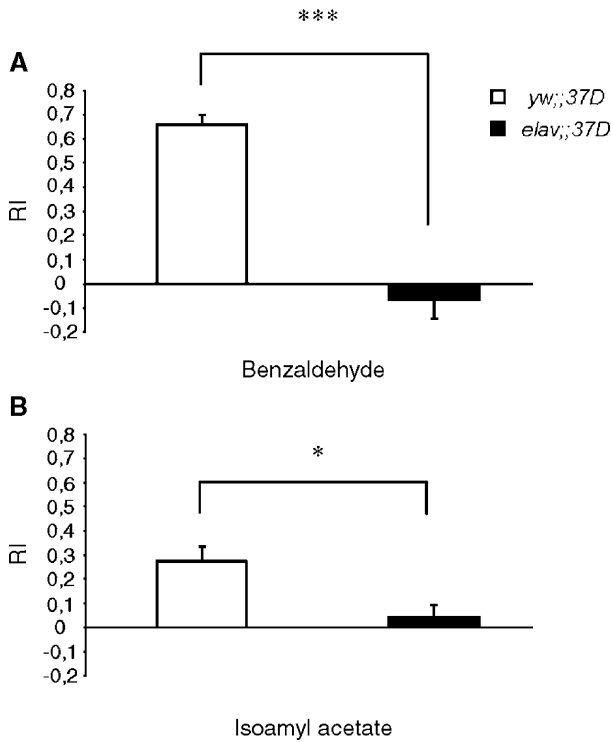


Fig. 5. Larval olfactory behavior. Response from control and experimental larvae (same genotypes as in Fig. 4). Histograms represent mean olfactory index (RI) obtained from 400 larvae per odorant and experimental group. Note the shift from attraction towards indifference in mutant larvae in both odorants. Vertical bars indicate SEM. Asterisks indicate statistical significance (Student's *t* test, ****P* < 0.01 and **P* < 0.05).

transcript levels in either of the two independent transgenic *dtk*-RNAi lines (37A and 37D) and when using two different drivers (Gal4-T80 and *elav*-Gal4-C155). The suppression of *dtk* expression was confirmed by immunocytochemistry. In sections of adult heads from flies homozygous for both the Gal4 and the *dtk*-RNAi, no expression could be detected, suggesting that two copies of RNAi construct further increase the efficiency of interference, reducing it to a virtually null condition.

Driving the construct with the ubiquitous *tub*-Gal4LL7 resulted in lethality, mostly at the embryo stage. This observation is consistent with the expression of the two identified *Drosophila* TKRP receptors, DTKR and NKD. Based on transcript expression, DTKR displays the strongest signal in late embryogenesis and is detected only in the embryonic CNS (Li et al., 1991). By contrast, NKD expression commences at 3 h and remains throughout embryonic development, with a peak around 12–16 h (Monnier et al., 1992). The regulation of NKD receptor gene is carried out by two enhancer systems, the distal sequence of the promoter controls the late embryonic expression in neurosecretory cells of the CNS, whereas the proximal region regulates the early expression in the peripheral nervous system (Rosay et al., 1995). Concerning the peptides, in situ hybridization data show expression of the precursor in a group of midgut cells in stage 17 embryos (Siviter et al., 2000). TKRP-immunoreactive material was also detected in the primordium of the stomatogastric nervous system in stage 16 embryos (Ryan Birse, personal communication). Taken together, with these earlier studies, our findings demonstrate that TKRPs in *Drosophila* regulate embryonic development, and this expression appears required for viability. Most likely, the *dtk* expression

outside of the *elav*-Gal4-C155 and Gal4-T80 domains, which amounts roughly to the 10% of whole body gene expression, is enough to allow survival, albeit with impaired behavior.

TKRPs in olfactory perception

We detected subtle changes in behavioral responses to olfactory stimuli in flies deficient in DTK in neurons by use of *elav*-Gal4-C155 driver. The responses to isoamyl acetate were shifted towards indifference of choice between the odorant and the solvent at concentrations that normally evoke repulsion. Responses to benzaldehyde and butanol were affected in the same direction but only at high concentrations. Responses to acetone remained normal. As suggested in earlier studies, odor specificity decreases at high concentrations (de Bruyne et al., 2001; Stensmyr et al., 2003; Wang et al., 2003). Hence, the data could be interpreted as a

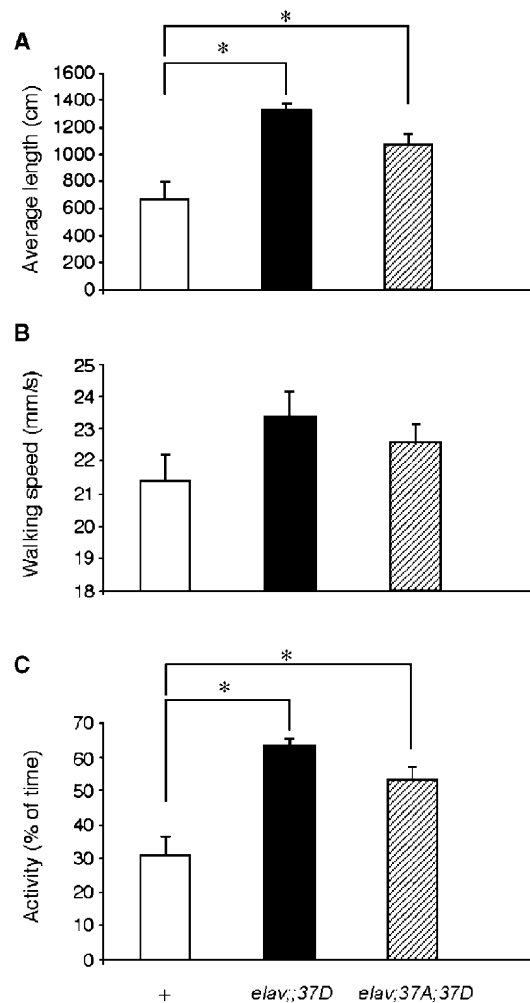


Fig. 6. Locomotor activity. Histograms of walking performances from control *yw*; UAS-*dtk*-RNAi37D (white) and two experimental genotypes: *elav*-Gal4-C155; UAS-*dtk*-RNAi37D (black) and *elav*-Gal4-C155; UAS-*dtk*-RNAi37A/+; UAS-*dtk*-RNAi37D/+ (stripped) tested in the Buridan's arena. Mean values obtained from 12 flies per group. Note major differences in walked distance (average length, A) and active time (activity time, C) in experimental flies compared to controls. Walking speed (B) remains normal. No significant differences were observed between the two experimental groups. Vertical bars indicate SEM. Asterisks indicate statistical significance (ANOVA, *P* < 0.05).

loss of specificity at high concentrations. It is important to notice, however, that the effect seems to be odorant-specific as well as concentration-dependent since isoamyl acetate perception was affected over a wider range of concentrations when compared to benzaldehyde or butanol. It is unlikely that this apparent specificity is the result of the remaining ~10% of *dtk* expression. Since many other peptides are present in the fly brain, we speculate that the receptors may be cross activated by other peptides given the absence of DTKs. This is often the case with receptors when deprived of their natural ligand as shown in multiple in vitro assays for neurotrophins as well as G-coupled peptide receptors (Bibel et al., 1999; Meeusen et al., 2003). In these cases, however, the functional substitution is not complete, and physiological defects become evident (De Meyts, 2004). The observed changes in behavioral responses to isoamyl acetate and benzaldehyde in the adult fly were also observed in the larval responses to high concentrations of these odors, confirming that the observed effects are consistent in both olfactory systems. Adult and larval tests were designed according to the actual living conditions of each type of organism, that is, variable odorant concentrations for the flying adults while only high concentration to the feeding larvae. The involvement of TKLPs in the olfactory system is probably not a peculiarity of *Drosophila*. In the rat, substance P has been detected in neural networks within the olfactory bulb, and its receptors have been localized to the external layer of the same organ, suggesting that this peptide has a role in olfactory processing in mammals (Quirion et al., 1983; Ribeiro-da-Silva and Hökfelt, 2000). In *Drosophila*, TKRPs have been found in local interneurons with processes in the antennal lobe neuropil (Siviter et al., 2000; Winther et al., 2003). These peptidergic interneurons have been suggested to actively elaborate odor responses through synaptic interactions with three other converging neurons: sensory, local and projection neurons in a connectivity network (Wilson et al., 2004). In fact, large dense core vesicle terminals are found in all antennal lobes very close (<5 µm) to tripartite synapses (our unpublished data), suggesting peptidergic modulation of synapse activity. The data reported here are consistent with the active elaboration of sensory perception in the antennal lobe and involve TKRPs directly in that process. The molecular mechanism for this process is likely to be based in the collaborative roles of TKRP and GABA. Both are expressed in the antennal lobes in *Drosophila* and colocalize in cells of the cockroach antennal lobe (Nässel, 2002). In crayfish photoreceptors, TKRPs enhance the inhibitory activity of GABA (Glantz et al., 2000). If this functional role is conserved in antennal lobe neurons, a TKRP deficiency would lead to a diminished inhibitory activity. A similar loss of inhibition in the central complex would lead to an excess of locomotion.

TRKPs in locomotor activity

Like the vertebrate tachykinins, the DTKs seem to be involved in several different physiological processes. The abundant expression of DTKs in the central complex of the adult brain justified the locomotion tests. Our data clearly show hyperactivity in the TKRP-depleted animals. The central complex is an integrative center where pathways of many sensory modalities converge and from where many output pathways emerge (Strausfeld, 1976; Renn et al., 1999). The abnormal locomotor behavior observed in *Drosophila* might be related to the recently reported correlation between reduced levels of human tachykinins in amygdala neurons and mood disorders (Carletti et al., 2005). Furthermore, tachyki-

nins and their receptors have been reported in the rat pineal gland (Mukda et al., 2005), an organ involved in the control of circadian locomotor activity. Finally, substance P injected in the brain stem of lampreys induced bouts of locomotor activity (Brocard et al., 2005). These facts point to a novel role of TKRPs in at least the maintenance and perhaps in the transition between states of locomotion activity. Thus, further behavioral analysis of TKRPs in a suitable experimental organism is justified.

Experimental methods

Fly strains and cultures

The tachykinin RNAi construct was injected into *y w* embryos. Transformants carrying the construct were designated: *y w*; UAS-*dtk*-RNAi37A and *y w*; UAS-*dtk*-RNAi37D. As Gal4 driver lines, we used: *tub*-Gal4-LL7, Gal4-T80 and *elav*-Gal4-C155. For determining the stage of death in flies expressing the RNAi construct when driven by the Gal4-LL7, crosses of Gal4-LL7/TM6,Tb,GFP with either *y w*; UAS-*dtk*-RNAi37A or *y w*; UAS-*dtk*-RNAi37D were used. Fly strains were obtained from the Bloomington *Drosophila* Stock Center, (Fly Base <http://flybase.bio.indiana.edu>).

For QRT-PCR, crosses of *elav*-Gal4-C155 with either *y w*; UAS-*dtk*-RNAi37A or *y w*; UAS-*dtk*-RNAi37D and crosses of Gal4-T80 with *y w*; UAS-*dtk*-RNAi37A or *y w*; UAS-*dtk*-RNAi37D were used. Behavioral experiments were performed on: *elav*-Gal4-C155; UAS-*dtk*-RNAi37A, *elav*-Gal4-C155; UAS-*dtk*-RNAi37D and *elav*-Gal4-C155; UAS-*dtk*-RNAi37A/+; UAS-*dtk*-RNAi37D/+ flies. Corresponding parental strains were used as controls. All flies were raised on standard commel–yeast–agar medium at 22–25°C.

RNA interference

The RNAi construct was targeted to the last 654 bp of exon 2, exon 3 and the first 18 bp of exon 4. Genomic and cDNA fragments were amplified using PCR with primers containing unique restriction sites. The genomic fragment was isolated by PCR as an *Eco*RI to *Not*I fragment. This was ligated to the corresponding inverted cDNA fragment. The inverted cDNA fragment was isolated using PCR primers containing a *Not*I site and an *Xho*I site. PCR products were subcloned into pBluescript II KS (+) and sequenced. The entire construct was digested with *Eco*RI and *Xho*I and cloned into pUAS (Brand and Perrimon, 1993) digested with *Eco*RI and *Xho*I. Ligated RNAi constructs were transformed into *SURE2* cells (Stratagene, La Jolla, CA, USA). Primer sequences for RNAi constructs were: genomic 5'-CCG GAA TTC TCC AAT GCG CCC TCT GAG; genomic 3'-TAA AAT ATG CGG CCG CCC TAC TCG AAA AGT GCT GT; cDNA 5'-TAA AAA ATG CGG CCG CCC TAC TCG AAA AGT GCT GG; cDNA 3'-CCC CTC GAG TCC AAT GCG CCC TCT GAG. The RNAi construct was injected into *y w* embryos via standard germline transformation techniques (Spradling and Rubin, 1982).

Staging time of death

tub-Gal4-LL7/TM6,Tb,GFP flies were crossed with flies homozygous for the RNAi construct. Eggs were briefly sorted under UV light. Non-fluorescent eggs were incubated on apple–agar plates with yeast paste in humid chambers at 25°C. Number of living individuals was counted and collected every 24 h. About 100 individuals were screened for each of the *tub*-Gal4-LL7/uAS-*dtk*-RNAi37D and *tub*-Gal4-LL7/uAS-*dtk*-RNAi37A F₁.

Quantitative real-time PCR (QRT-PCR)

To examine the efficiency of the UAS-*dtk*-RNAi construct in the down-regulation of the native gene, the levels of *dtk* transcripts were quantified in

flies expressing one copy of the construct driven by either Gal4-T80 or *elav*-Gal4-C155. Total RNA was extracted from whole 1- to 3-day-old flies (Gal4-T80AS-*dtk*-RNAi and corresponding controls) or liquid nitrogen frozen heads (*elav*-Gal4-C155AS-*dtk*-RNAi and corresponding controls) using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. RNAs were quantified by spectrophotometry and transcribed using the first-strand cDNA synthesis kit from Amersham Biosciences (Piscataway, NJ, USA). Reverse transcription products were quantified using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR reactions were set up as triplicates using SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA). Whole experiments were repeated 3 to 5 times starting from new and independent tissue collections. PCR conditions were step 1: 50°C for 2 min, step 2: 95°C for 10 min, step 3: 95°C for 45 s, step 4: 54°C for 30 s, step 5: 68°C for 30 s, cycle back 40 times to step 3. After PCR, the absence of unwanted by-products was confirmed by melting curve analysis and agarose gel electrophoresis of the products. The housekeeping gene of the 140-kDa subunit of RNA polymerase II (*RNAPolIII*) was chosen as an internal transcriptional standard (Falkenburg et al., 1987; Radonic et al., 2004). The level of *dtk* transcripts was normalized with respect to those of *RNAPolIII* in the same sample. Standard curves were obtained using serial dilutions of y *w* template yielding correlation coefficients of at least 0.98 in all experiments. Separate curves for *dtk* and *RNAPolIII* were included in each PCR run. The relative amount of *dtk* transcripts was calculated based on the efficiency calibrated mathematical model (Soong et al., 2000; see also Pfaffl, 2001). The relative expression ratio was calculated only from the real-time PCR efficiencies and the crossing point deviation of the sample versus the control. Real-time PCR efficiencies (*E*) were calculated from standard curves according to the equation: $E = 10^{(-1/\text{slope})}$ (Rasmussen, 2001). Primers were designed using the Primer Select software (Lasergene, Madison, WI). Amplicons were confirmed by sequencing. Primers for *dtk* were: 5'-GCG AGG CAT ACG GCC AGC ACT TT and 3'-CCG GTC CCC TGT GTC CAT CTA CTT. Primers for *RNAPolIII* were 5'-GAG TCC GCG TAA CAC CTA TCA AA and 3'-ACA AGT GGC TTC ATC GGA TAG TAA AG.

Immunocytochemistry

Whole CNS from third instar larvae and adult heads was used for fixation. Tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 2–4 h. Immunocytochemistry was performed either on whole tissues or cryostat sections (15 μ m). A general insect TKRP antiserum (code K 9836, see Winther and Nässel, 2001) was used at a dilution of 1:4000. Tissues were incubated in antiserum for 16 h at 4°C and detected with cyanamide-tagged secondary antibody (Cy3; Jackson ImmunoResearch, West Grove, PA). The specificity of the TKRP antiserum has been established previously (Winther and Nässel, 2001; Winther et al., 2003). Microscopic analysis was performed on a Zeiss Axioplan 2 microscope coupled to a CCD camera (Hamamatsu, Hamamatsu City, Japan) and edited with Adobe Photoshop 8.0. Confocal stacks (on whole-mounts of larval CNS) were acquired with a Zeiss LSM 510 confocal microscope based on an Axiovert S100 M microscope (Zeiss, Germany) and processed with LSM software or on a Leica TLS4D confocal microscope equipped with a Leitz DMIRB microscope and processed with Q500 MC software (Leica, Germany).

Olfactory choice assay

To test the olfactory behavioral effect of the DTK deficiency, we used a T-maze test adapted from the protocol previously described (Tully and Quinn, 1985; Devaud et al., 2001). Tested odorants were diluted in paraffin oil in concentrations ranging from 1×10^{-5} to 1×10^{-1} vol/vol. A piece of filter paper was impregnated with either 100 μ l of the test odorant or, as a control, 100 μ l of the solvent paraffin oil. Groups of ~25 females, 3–5 days old were placed in the T-maze apparatus and allowed 30 s to choose between the odorant and the control side. Flies were only tested once, and all experiments were conducted in the dark at room temperature. The

response index, RI, (Ayyub et al., 1990) was calculated as the number of flies trapped in the odor compartment subtracted by the number of flies trapped in the control compartment and divided by the total number of flies. RI values range from 1 (total attraction) to –1 (total repulsion). Flies not making any choice were excluded. Tests where more than one-third of the flies did not make any choice were discarded. Average RI values are based on 10 replicates of 25 flies each.

Larval olfactory test

We followed a previously described protocol (Heimbeck et al., 1999). Briefly, groups of 20 third instar wandering larvae were collected from the food and washed in a 15% sucrose solution. Odor (1 μ l undiluted) and control (1 μ l paraffin oil) substances were disposed on two small filter paper disks, placed on plastic cups (lids of 1.5 ml Eppendorf vials) and situated on opposite sides of Petri dishes (diameter 85 mm) covered with a layer of 4% agar. Twenty larvae were deposited in the center of the plate before adding the test substances and immediately covered with a lid. Test duration was 5 min in the dark. The RI was calculated as the number of larvae situated in the proximity of the odorant cup (30 mm radius) minus that of larvae located in an identical surface on the control side and divided by the total. Positive responses indicate attraction, whereas negative RIs indicate avoidance. A value of 0 indicates indifference to the odorant tested. Isoamyl acetate and benzaldehyde were employed as odorants. A total of $n = 400$ larvae were tested in each experimental and control group.

Locomotor assay

We used the “Buridan’s paradigm” (Götz, 1980). Briefly, flies aged 3 to 5 days with their wings clipped were allowed to walk freely between two dark landmarks (vertical bar stripes) in a uniformly brighter (white light) landscape. The flies were confined to an elevated circular disk (8.5 cm diameter) surrounded by a water-filled tank. Tracks were recorded at a 10 Hz sampling rate using a video scanning device and a software (Tracker v5 program) developed by R. Wolf (University of Würzburg, Germany). Path lengths were calculated for five successive 3-min intervals of undisturbed walking and averaged between individuals of the same genotype. Walking speeds were calculated for every transition between the black stripes. A total of $n = 12$ flies were analyzed per genotype.

Acknowledgments

Research was funded by grants BMC2003-05051, GR/SAL/0837/2004 and 01/1185. The department of Developmental Neurobiology, Instituto Cajal, belongs to the CIEN network. Å.W. was a recipient of a Marie Curie fellowship HPMF-CT-2001-01326 and A.A. of a Marie Curie contract MERG-CT-2004-502902. Å.W. received support from Carl Tryggers Foundation and the Royal Swedish Academy of Sciences. The critical reading of the manuscript by D. Nässel, V. Sahota and B. Grau is appreciated as well as the assistance of S. Jordan on the behavioral tests.

References

- Ayyub, C., Paranjape, J., Rodrigues, V., Siddiqi, O., 1990. Genetics of olfactory behavior in *Drosophila melanogaster*. J. Neurogenet. 6, 243–262.
- Bibel, M., Hoppe, E., Barde, Y.A., 1999. Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J. 18, 616–622.
- Birse, R.T., Johnson, E.C., Taghert, P.H., Nössel, D.R., 2005. Widely distributed G-protein-coupled receptor (CG7887) is activated by endogenous tachykinin-related peptides. J. Neurobiol. 65 (electronic publication ahead of print).

- Brand, A., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401–415.
- Brocard, F., Bardy, C., Dubuc, R., 2005. Modulatory effect of substance P to the brain stem locomotor command in lampreys. *J. Neurophysiol.* 93, 2127–2141.
- Carletti, R., Corsi, M., Melotto, S., Caberlotto, L., 2005. Down-regulation of amygdala preprotachykinin A mRNA but not 3H-SP receptor binding sites in subjects affected by mood disorders and schizophrenia. *Eur. J. Neurosci.* 21, 1712–1718.
- de Bruyne, M., Foster, K., Carlson, J.R., 2001. Odor coding in the *Drosophila* antenna. *Neuron* 30, 537–552.
- De Meyts, P., 2004. Insulin and its receptor: structure, function and evolution. *BioEssays* 26, 1351–1362.
- Devaud, J.-M., Acebes, A., Ferrús, A., 2001. Odor exposure causes central adaptation and morphological changes in selected olfactory glomeruli in *Drosophila*. *J. Neurosci.* 21, 6274–6282.
- Falkenburg, D., Dwornickzak, B., Faust, D.M., Bautz, E.K.F., 1987. RNA polymerase II of *Drosophila*. Relation of its 140,000 Mr subunit to the β subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.* 195, 929–937.
- Glantz, R.M., Miller, C.S., Nässel, D.R., 2000. Tachykinin-related peptide and GABA-mediated presynaptic inhibition of crayfish photoreceptors. *J. Neurosci.* 20, 1780–1790.
- Götz, K.G., 1980. Visual guidance in *Drosophila*. In: Siddiqi, O., Babu, P., Hall, L.M., Hall, C. (Eds.), *Development and Neurobiology of Drosophila*. Plenum, New York, pp. 391–407.
- Heimbeck, G., Bugnon, V., Gendre, N., Haberin, C., Stocker, R.F., 1999. Smell and taste perception in *Drosophila melanogaster* larva: toxin expression studies in chemosensory neurons. *J. Neurosci.* 19, 6599–6609.
- Hrdlicka, L., Gibson, M., Kiger, A., Micchelli, C., Schober, M., Schöck, F., Perrimon, N., 2002. Analysis of twenty-four Gal4 lines in *Drosophila melanogaster*. *Genesis* 34, 51–57.
- Kalidas, S., Smith, D.P., 2002. Novel genomic cDNA hybrids produce effective RNA interference in adult *Drosophila*. *Neuron* 33, 177–184.
- Kwok, R., Chung, D., Te Brugge, V., Orchard, I., 2005. The distribution and activity of tachykinin-related peptides in the blood-feeding bug, *Rhodnius prolixus*. *Peptides* 26, 43–51.
- Lee, Y.S., Carthew, R.W., 2003. Making a better RNAi vector for *Drosophila*: use of intron spacers. *Methods* 30, 322–329.
- Li, X.J., Wolfgang, W., Wu, Y.N., North, R.A., Forte, M., 1991. Cloning, heterologous expression and developmental regulation of a *Drosophila* receptor for tachykinin-like peptides. *EMBO J.* 10, 3221–3229.
- Lundquist, C.T., Nässel, D.R., 1997. Peptidergic activation of locust dorsal paired median (DUM) neurons: depolarization induced by locust tachykinins may be mediated by cAMP. *J. Neurobiol.* 33, 297–315.
- Meeusen, T., Mertens, I., De Loof, A., Schoofs, L., 2003. G-protein coupled receptors in invertebrates: a state of the art. *Int. Rev. Cytol.* 230, 189–261.
- Monnier, D., Colas, J.-F., Rosay, P., Hen, R., Borrelli, E., Maroteaux, L., 1992. NKD, a developmentally regulated tachykinin receptor in *Drosophila*. *J. Biol. Chem.* 267, 1298–1302.
- Mukda, S., Chetsawang, B., Govitrapong, P., Schmidt, P.T., Hay-Schmidt, A., Moller, M., 2005. Tachykinins and tachykinin-receptors in the rat pineal gland. *Eur. J. Neurosci.* 21, 2743–2751.
- Nachman, R.J., Moyna, G., Williams, H.J., Zabrocki, J., Zadina, J.E., Coast, G.M., Vanden Broeck, J., 1999. Comparison of active conformations of the insect tachykinin/tachykinin and insect kinin/Tyr-W-MIF-1 neuropeptide family pairs. *Ann. N. Y. Acad. Sci.* 897, 388–400.
- Nässel, D.R., 1999. Tachykinin-related peptides in invertebrates: a review. *Peptides* 20, 141–158.
- Nässel, D.R., 2002. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog. Neurobiol.* 68, 1–84.
- Nusbaum, M.P., Blitz, D.M., Swensen, A.M., Wood, D., Marder, E., 2001. The roles of co-transmission in neural network modulation. *Trends Neurosci.* 24, 146–154.
- Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, 2002–2007.
- Quirion, R., Shults, C.W., Moody, T.W., Pert, C.B., Chase, T.N., O'Donohue, T.L., 1983. Autoradiographic distribution of substance P receptors in the central nervous system. *Nature* 303, 714–716.
- Radonic, A., Thulke, S., Mackay, I.M., Landt, O., Siebert, W., Nitsche, A., 2004. Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* 313, 856–862.
- Rasmussen, R., 2001. Quantification on the LightCycler. In: Meuer, S., Wittwer, C., Nakagawara, K. (Eds.), *Cycle Real Time PCR, Methods and Applications*. Springer Press, Heidelberg, pp. 21–34.
- Renn, S.C., Armstrong, J.D., Yang, M., Wang, Z., An, X., Kaiser, K., Taghert, P.H., 1999. Genetic analysis of the *Drosophila* ellipsoid body neuropil: organization and development of the central complex. *J. Neurobiol.* 41, 189–207.
- Ribeiro-da-Silva, A., Hökfelt, T., 2000. Neuroanatomical localization of substance P in the CNS and sensory neurons. *Neuropeptides* 34, 256–271.
- Rosay, P., Colas, J.-F., Maroteaux, L., 1995. Dual organization of the *Drosophila* neuropeptide receptor NKD gene promoter. *Mech. Dev.* 51, 329–339.
- Schoofs, L., Holman, G.M., Hayes, T.K., Nachman, R.J., De Loof, A., 1990. Locusta tachykinin I and II, two novel insect neuropeptides with homology to peptides of the vertebrate tachykinin family. *FEBS Lett.* 261, 397–401.
- Siviter, R.J., Coast, G.M., Winther, Å.M.E., Nachman, R.J., Taylor, C.A.M., Shirras, A.D., Coates, D., Isaac, R.E., Nässel, D.R., 2000. Expression and functional characterization of a *Drosophila* neuropeptide precursor with homology to mammalian preprotachykinin A. *J. Biol. Chem.* 275, 23273–23280.
- Soong, R., Ruschhoff, J., Tabati, K., 2000. Detection of colorectal micro-metastasis by quantitative RT-PCR of cytokeratin 20 mRNA. Roche Diagnostics internal publication.
- Spradling, A.C., Rubin, G.M., 1982. Transposition of cloned P elements into *Drosophila* germline chromosomes. *Science* 218, 341–347.
- Stensmyr, M.C., Giordano, E., Balloi, A., Angioy, A.-M., Hansson, B.S., 2003. Novel ligands for *Drosophila* olfactory receptor neurons. *J. Exp. Biol.* 206, 715–724.
- Strausfeld, N.J., 1976. *Atlas of an Insect Brain*. Springer, Heidelberg.
- Strauss, R., 2002. The central complex and the genetic dissection of locomotor behaviour. *Curr. Opin. Neurobiol.* 12, 633–638.
- Strauss, R., Heisenberg, M., 1993. Higher control center of locomotor behavior in the *Drosophila* brain. *J. Neurosci.* 13, 1852–1861.
- Swensen, A.M., Marder, E., 2001. Modulators with convergent cellular actions elicit distinct circuit outputs. *J. Neurosci.* 21, 4050–4058.
- Tully, T., Quinn, W.G., 1985. Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J. Comp. Physiol., A* 157, 263–277.
- Vanden Broeck, J., Torfs, H., Poels, J., Van Poyer, W., Swinnen, E., Ferket, K., De Loof, A., 1999. Tachykinin-like peptides and their receptors. *Ann. N. Y. Acad. Sci.* 897, 374–387.
- Wang, J.W., Wong, A.M., Flores, J., Vosshall, L.B., Axel, R., 2003. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell* 112, 271–282.
- Wilson, R.I., Turner, G.C., Laurent, G., 2004. Transformation of olfactory representations in the *Drosophila* antennal lobe. *Science* 303, 366–370.
- Winther, Å.M.E., Nässel, D.R., 2001. Intestinal peptides as circulating hormones: release of tachykinin-related peptide from locust and cockroach midgut. *J. Exp. Biol.* 204, 1269–1280.
- Winther, Å.M.E., Siviter, R.J., Isaac, R.E., Predel, R., Nässel, D.R., 2003. Neuronal expression of tachykinin-related peptides and gene transcript during postembryonic development of *Drosophila*. *J. Comp. Neurol.* 464, 180–196.