Genes, Brain and Behavior (2006) **5:** 602–613

© 2006 The Authors
Journal compilation © 2006 Blackwell Munksgaard

Thermal nociception in adult *Drosophila*: behavioral characterization and the role of the *painless* gene

S. Y. Xu[†,‡], C. L. Cang[§], X. F. Liu[¶], Y. Q. Peng[¶], Y. Z. Ye[¶], Z. Q. Zhao[§] and A. K. Guo[*,†,¶]

[†]State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, CAS, [‡]Graduate School of CAS, Beijing, [§]Institute of Neurobiology, Fudan University, Shanghai, and [¶]Institute of Neuroscience, Key Laboratory of Neurobiology, SIBS, CAS, Shanghai, China

**Corresponding author: A. Guo, State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, CAS, Beijing 100101, China. E-mail: akguo@ion.ac.cn*

Nociception, warning of injury that should be avoided, serves an important protective function in animals. In this study, we show that adult *Drosophila* avoids noxious heat by a jump response. To quantitatively analyze this nociceptive behavior, we developed two assays. In the CO_2 laser beam assay, flies exhibit this behavior when a laser beam heats their abdomens. The consistency of the jump latency in this assay meets an important criterion for a good nociceptive assay. In the hot plate assay, flies jump quickly to escape from a hot copper plate (>45 °C). Our results demonstrate that, as in mammals, the latency of the jump response is inversely related to stimulus intensity, and innoxious thermosensation does not elicit this nociceptive behavior. To explore the genetic mechanisms of nociception, we examined several mutants in both assays. Abnormal nociceptive behavior of a mutant, *painless*^{1}, indicates that *painless*, a gene essential for nociception in *Drosophila* larvae, is also required for thermal nociception in adult flies. *painless* is expressed in certain neurons of the peripheral nervous system and thoracic ganglia, as well as in the definite brain structures, the mushroom bodies. However, chemical or genetic insults to the mushroom bodies do not influence the nociceptive behavior, suggesting that different *painless*-expressing neurons play diverse roles in thermal nociception. Additionally, *no-bridge*^{KS49}, a mutant that has a structural defect in the protocerebral bridge, shows defective response to noxious heat. Thus, our results validate adult *Drosophila* as a useful model to study the genetic mechanisms of thermal nociception.

Keywords: CO_2 laser, *Drosophila*, hot plate, jump response, mushroom body, nociception, *painless*

Received 9 June 2005, revised 12 January 2006, accepted for publication 16 January 2006

Pain is quite important for human survival. Painful sensations, such as pricking, burning, aching, stinging and soreness, warn of injury that should be avoided or treated and, consequently, have a protective function in humans. Nevertheless, persistent or chronic pain, which is usually induced by inflammation, nerve damage or cancer, is harmful to human health (Smith 2000).

In response to nociceptive sensations, animals exhibit behavioral and physiological reactions that resemble those of humans, although they lack the ability to verbally report their sensations. Vertebrates, such as mice and rats, are usually used as models to study nociception. These studies have a long history (Ben-bassat *et al*. 1959; Woolfe & MacDonald 1944) and have mainly been concerned with the physiological or pathophysiological mechanisms of nociception (Dickenson & Sullivan 1987; Gao *et al*. 2004; Guo *et al*. 2005). However, the genetic mechanisms of nociception are poorly understood, although they are being uncovered gradually (Caterina *et al*. 1997, 1999).

Caenorhabditis elegans and *Drosophila melanogaster* have been established as models to study the genetic mechanisms of nociception. These studies benefited from the powerful genetic tools available in the two invertebrates and their short life cycles. Wild-type *C. elegans* withdraws from a heated metal tip, which serves as a noxious thermal stimulus (Wittenburg & Baumeister 1999). Three mutants, *eat-4(n2474)*, *flp-1(yn2)* and *flp-1(yn4)*, showed a defect during the withdraw reaction. *eat-4(n2474)* is a mutant allele of the gene *eat-4*, which encodes a protein necessary for glutamatergic neurotransmission in *C. elegans* (Lee *et al*. 1999). *flp-1(yn2)* and *flp-1(yn4)* are two mutant alleles of the gene *flp-1*, which encodes seven distinct neuropeptides of the FMRFamide-related family (Nelson *et al*. 1998). Therefore, the behavioral results above suggest that glutamate and FMRFamide-related neuropeptides have an important modulatory function for thermal nociception in *C. elegans* (Wittenburg & Baumeister 1999). Recently, Tracey and colleagues developed a simple behavioral assay for *Drosophila* larvae and used it to screen for mutants defective in nociception. They found that when touched with a probe heated to 46 °C, *painless*^{1} mutant larvae responded significantly slower than wild-type larvae. Moreover, *painless*^{1} larvae responded as quickly as wild-type larvae when touched with a 52 °C probe and also exhibited a defective response to noxious mechanical stimuli. They further found that *painless*^{1} is a mutant of the *painless* gene, which encodes a protein of the transient receptor potential ion channel family (Tracey *et al*. 2003). Their work suggests that *Drosophila* larva is a good model to explore the genetic mechanisms of nociception.

doi: 10.1111/j.1601-183X.2006.00213.x

Adult flies exhibited escape behavior from heat punishment in visual learning at flight simulators (Wolf & Heisenberg 1991), suggesting adult flies can sense noxious thermal stimulus. Using adult *Drosophila* as a model to study nociception has two important benefits. First, adult flies exhibit abundant behavioral patterns, which provide a plentiful behavioral repertoire for developing precise assays. Second, the expression patterns of many genes change during *Drosophila* development (Gilbert 2000). Comparing the results from adults with those from larvae may manifest a dynamic map of gene expression associated with nociception. However, neither a method to quantitatively analyze nociceptive behavior nor the behavioral characteristics of nociception in adult *Drosophila* has been reported.

In this study, we reported a robust jump response to noxious thermal stimulus in adult *Drosophila*. Two assays were developed to quantitatively analyze this behavior. We then tested *painless*^{1} flies in the two assays and found that they were defective in response to noxious heat. We observed that *painless* is expressed in the mushroom bodies (MBs) and the other neural structures in adult flies. However, the normal nociceptive behavior of *mushroom body miniature*[1] (*mbm*^{1}) females and hydroxyurea-treated flies, whose MBs were very incomplete, suggested that MBs are unimportant for thermal nociception.

Materials and methods

Fly strains

Adult male *D. melanogaster* (2–3 days old) were used for all experiments except where otherwise indicated. These flies were grown on standard cornmeal/molasses medium (Guo *et al*. 1996), at 25 °C and 60% relative humidity in a 12:12 h light/dark cycle.

painless^{1} was generated in a *w*^{1118} background (Tracey *et al*. 2003). *pain-rescue;painless*^{1} is a transgenic strain in which a genomic DNA rescue-fragment containing the *painless* transcript unit has been transformed into the *painless*^{1} strain (Tracey *et al*. 2003). Thus, the Canton Special (CS) wild-type strain was used as the control for *painless*^{1} and *pain-rescue; painless*^{1}. The three strains and *pain*-Gal4 were a generous gift from Dr Tracey (in the Duke University, Durham, NC, USA).

no-bridge^{KS49} (*nob*^{KS49}) and *mbm*^{1} were generated in a wild-type Berlin (WTB) background (de Belle & Heisenberg 1996; Heisenberg & Bohl 1979). The WTB strain was used as the control for *nob*^{KS49} and *mbm*^{1}. The three strains were kindly supplied by Reinhard Wolf and Dr Heisenberg (in the University of Wuerzburg, Wuerzburg, Germany).

bizarre (*biz*), heterozygous with the balancer strain FM7A, was generated in a CS background (Benzer 1967) and was a generous gift from Dr Zars (in the University of Missouri-Columbia, Columbia, MO, USA). To keep the same genetic background with *biz*, the CS strain serving as the control for *biz* was back-crossed with FM7A at least five generations.

The back-crossed CS flies were also used in the experiments represented in Figs 2(a,b,c) and 3(b).

CO_2 laser beam assay

A CO_2 laser generator (Chengyong Photoelectric Technology Co., Shanghai, China) was used to produce the laser beams ($\lambda = 10.6$ μm, diameter 1.5 mm) of different powers (calibrated by the National Center of Measurement and Testing for East China, Shanghai, China) (Fig. 1a). A red laser beam ($\lambda = 810$ nm, <2 mW), with the same optical pathway as the CO_2 laser beam, was used for aiming. In this assay, all the flies were tested only once with the 0.02-W laser unless specifically indicated otherwise.

A metallic hook (3×10^{-5} g, approximately one-third the weight of a male fly) was firmly glued to the thorax and head of each fly between 0900 and 1200 h (Wolf & Heisenberg 1991). Subsequently, they were allowed to rest in our culture conditions for 3–4 h. At the beginning of the assay, a glued fly with a metallic clamp clamping its hook was fixed on a 3-D micromanipulator and was adjusted in the center of the red laser's spot (diameter 1.5 mm) with its ventral side upward. The experimenter then gave the fly a cotton cord (length 3–5 mm) as a marker to illustrate its jump response. The fly held the cord by its legs and was allowed to acclimate to the environment for 1 min before the CO_2 laser beam hit its abdomen. After the CO_2 laser generator was switched on, the fly threw the cotton cord upward, which was regarded as an indication of a 'jump' (see *Supplemental material* Video S1). A similar method has been described previously (Engel & Wu 1996).

A digital video camera (30 f/s; Logitech Co., Romanel-sur-Morges, Switzerland) was used to record the entire process. The video recordings were analyzed frame by frame (MainActor v5.1, Mainconcept Co., Aachen, Germany). The latency was defined accurately from the disappearance of the red laser to the moment when the cotton cord was launched (see *Supplemental material* Video S1). Only the latencies of the flies that exhibited successful jumps (our criterion was that the cotton cord was thrown upward completely from its previous position in the video recording) were included in our analysis.

The room temperature was controlled at 24–25 °C, and the relative humidity was 40–50%. The experimenters were blind to mutants and wild-type controls (or treated and untreated groups). The hot plate (HP) assay and the jump test (see below) were also carried out in the same conditions.

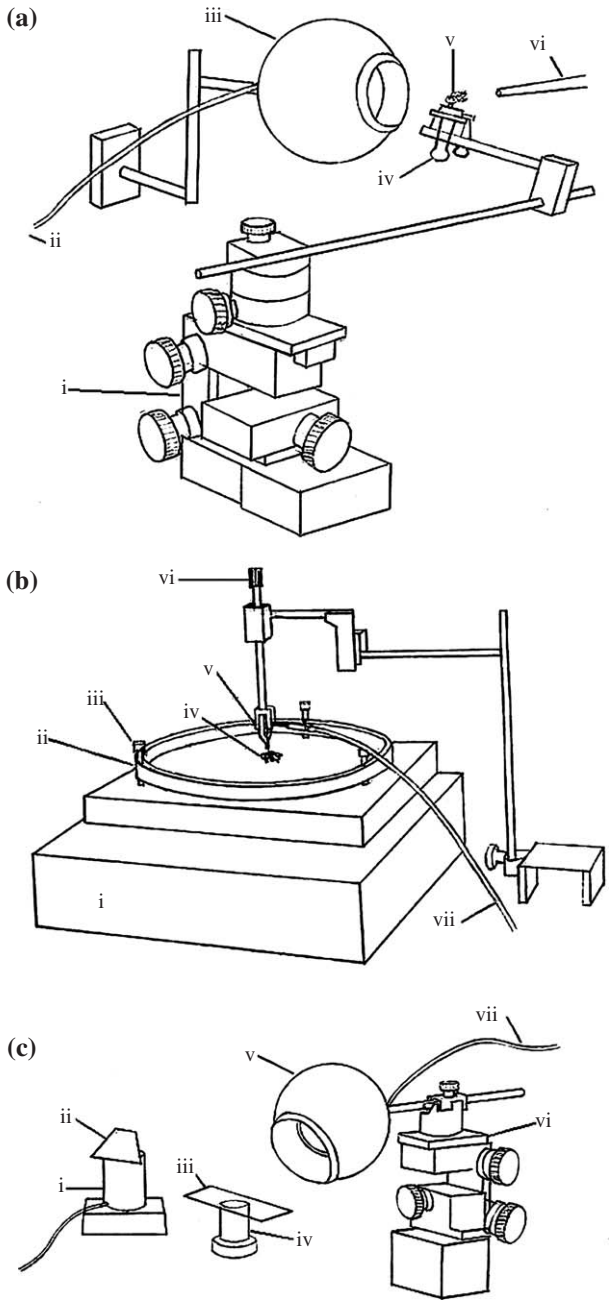
HP assay

A nylon thread was firmly glued to the thorax and head of each fly (thread length 4–5 mm; weight 2×10^{-5} g, approximately one-tenth the weight of a male fly; diameter 50 μm; donated by Dupont Co., Hayward, CA, USA). When a glued fly reached the rim of the copper plate (see below), a Plexiglas ring placed at the rim would bar further movement of its nylon thread. Consequently, the fly would be prevented from leaving the plate (Fig. 1b).

Flies were glued between 0900 and 1200 h and subsequently were allowed to rest in our culture condition for 3–4 h. At the beginning of this assay, a glued fly with a

metallic clamp clamping its nylon thread was suspended at 3–4 mm above a heated copper plate (Longxing R&D Center of Medicinal Technique, Tianjing, China). When the fly was released onto the plate, a stopwatch began to record the latency until the fly jumped. Jump latencies <1 second were regarded as 1 second. In this assay, all the flies were tested on a 47 °C plate except where otherwise indicated.

Because adult flies tend to jump spontaneously under natural light, the HP assay was carried out in a room illuminated only with a red lamp. We observed that all the flies showed little spontaneous jumping under the red light ($\lambda = 625$ nm; data not shown), perhaps because the photoreceptors of adult *Drosophila* have little response to red light with wavelength above 600 nm (Heisenberg & Wolf 1984).



Jump test

Each fly was kept in its own small covered chamber in advance (to inhibit interaction between flies). A 40-W lamp was placed at 20–25 cm above the chamber. After the chamber was uncovered, the fly in it usually climbed up to the rim of the chamber. Then the experimenters switched on an electromotor that drove a horizontal fan. With a quickly moving shadow, the fan ran above the chamber. The fly exhibited a jump response at this moment (see *Supplemental material* Video S2). The same digital video camera was used to record this response, and the method used in the CO_2 laser beem (CLB) assay was used to measure the jump latency in this test (Fig. 1c).

In adult *Drosophila*, the known output pathway of the jump response is the giant fibers, and their function is defective in some mutants (Engel & Wu 1998; Thoms & Wyman 1984). Thus, a mutant that exhibits behavioral defect in the two assays may not be indeed defective in thermal nociception, because the mutation may influence the function of the giant fibers. However, if the mutant exhibits a normal jump response to innocuous stimuli, its abnormal behavior in the two assays is indeed caused by abnormal thermal nociception. Considering that adult flies can jump to escape from innocuous stimuli, such as visual, olfactory, or mechanical stimuli (Campbell & Nash 1998; Kaplan & Trout 1974; Tully & Koss 1992), we designed this test as the control for the two assays.

Figure 1: CO_2 laser beam (CLB) assay, hot plate (HP) assay and jump test. (a) Experimental apparatus of the CLB assay. (i) 3-D manipulator; (ii) wire, connected to a computer; (iii) camera (30 f/s); (iv) metallic clamp; (v) a glued fly; (vi) the output tube of a CLB. A glued fly was fixed on a 3-D micromanipulator with its ventral side facing upward. The position of the fly was adjusted into the center of the red laser's spot. After the CO_2 laser was switched on to stimulate the fly, it threw the cotton cord upward, which was regarded as an indication of 'jump' (see *Supplemental material* Video S1) (b) Experimental apparatus of the hot-plate assay. (i) hot plate; (ii) plastic bar (which could be turned up or down by rotating the knob); (iii) metallic knob; (iv) a glued fly; (v) metallic clamp; vi) the knob to control the elevator; (vii) shutter release. A glued fly with a metallic clamp clamping its nylon thread was suspended 3–4 mm above the hot plate. When the fly was released onto the plate, a stopwatch was used to record the latency from the releasing until the fly jumped. (c) Experimental apparatus of the jump test. (i) electrometer; (ii) black fan (sector 45′, half diameter 70 mm) parallel to the table; (iii) glass cover; (iv) a fly in a small chamber; (v) video camera; (vi) 3-D manipulator; and (vii) wire, connected to a computer. When a fly climbed up to the rim of the chamber, the electric fan was turned on and run above the fly to elicit a jump response, which were recorded with the video camera for further analysis.

Histology and microscopy

According to a procedure described previously (Lee & Luo 1999), the central nervous systems of young adult flies were dissected in 100 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2) [phosphate-buffered saline, (PBS)] and fixed in PBS containing 4% formaldehyde for 20 min at room temperature. For GFP visualization, samples were washed in PBT (PBS + 0.3% Triton) several times for a total time of 30 min. For immunofluorescence, samples were blocked in 5% normal goat serum for 30 min in PBT, incubated with primary antibody (mAb1D4, 1:10) at 4 °C overnight, washed 6 × 10 min in PBT, incubated with secondary antibody (fluorescein isothiocyanate, 1:200) for 2 h at room temperature and further washed 6 × 10 min in PBT. Samples were viewed and images were taken under a 1024 laser-scanning confocal microscope (LSM510 Axiovert 200 M, Zeiss). Images were processed using Adobe Photoshop.

Hydroxyurea treatment

Groups of 500 WTB larvae that had hatched less than 1 h before were fed a heat-killed yeast suspension containing 50 mg/ml hydroxyurea (HU; Sigma) for 4 h. Control larvae were fed yeast only. Larvae were then washed in distilled water and allowed to develop in fresh cornmeal/molasses medium at 25 °C, 60% relative humidity with a 12:12 h light/dark cycle (de Belle & Heisenberg 1994).

Ablation technique

Each fly was held under a soft plastic piece after cold anesthesia, with the head protruding, providing access to the antennae. Fine forceps were used to remove the third antennal segments. At least 4 h intervened between surgery and testing (Sayeed & Benzer 1996).

Control flies were also held down under the soft plastic piece, but their third antennal segments were not removed.

Statistical analysis

The Student's *t*-test was used to analyze all the data from the CLB assay unless specifically indicated otherwise. In the HP assay, data usually did not fit normal distribution (perhaps because latencies shorter than 1 second were recorded as 1 second). Thus, the Wilcoxon rank-sum test was used to analyze all the data from the HP assay. A *P*-value <0.05 was considered statistically significant.

Results

Noxious heat elicits the jump response in the two assays

Flies jumped quickly after the CO_2 laser beam heated their abdomens in the CLB assay. The higher the laser power, the shorter the jump latency (Fig. 2a). Approximately 6% of the flies did not exhibit an obvious jump response. In the HP assay, flies jumped within a short time after they were released on the copper plate. The jump latency was shortened with the increase of the plate temperature (Fig. 2b). Approximately 5% of the flies did not show a jump response before they were paralyzed on the 45 or 47 °C plate.

A definite characteristic of a good assay for nociception is the consistency of latencies despite repeated tests (Vyklicky 1984). We stimulated a group of wild-type flies repeatedly at 30-min intervals in the CLB assay. The mean latencies in the first, second and third tests did not significantly differ from each other (Fig. 2c, ANOVA test was used), suggesting that the CLB assay is a good method to assess thermal nociception in adult *Drosophila*.

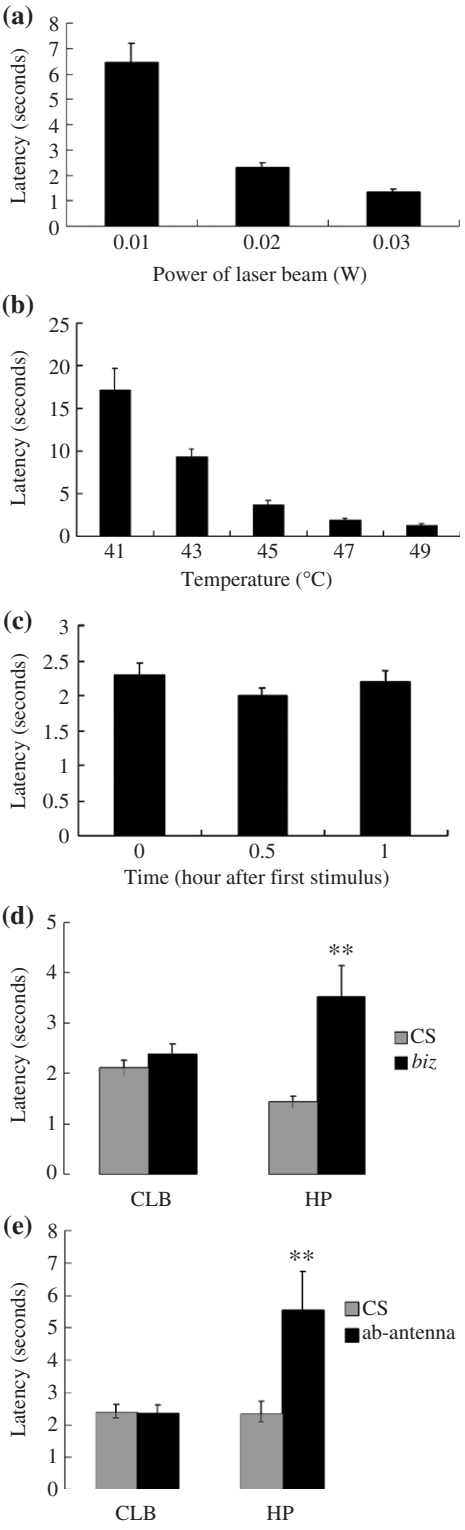
Previous studies reported two types of thermosensors in adult flies. One type, located in the third antenna segment, responds to approximately 27 °C (Sayeed & Benzer 1996; Zars 2001). Ablation of the third antenna segments eliminates this type of thermosensation (Sayeed & Benzer 1996). The other type responds to approximately 33 °C. Its location, however, is still unknown (Zars 2001). Exposure to mild temperatures, 27 or 33 °C, does not induce acute injury in adult *Drosophila*. Thus, we proposed that the two types of thermosensation are innoxious thermosensation, although flies are averse to them. *biz* flies distributed randomly across the temperature gradient from 23 to 36.5 °C, and even to 45 °C, indicating that they are completely thermoblind (Sayeed & Benzer 1996). The result above demonstrates that *biz* flies have a defect in both types of thermosensors and therefore are defective in innoxious thermosensation.

Comparing the locomotor patterns and response times, we observed that the nociceptive behavior was manifestly different from the behavior induced by innoxious thermosensation. First, while flies walked against aversive warmth, they jumped to escape from noxious heat. Our result showed that flies did not jump even in the 34 °C air (see below). Secondly, it took flies several minutes to choose the preferred temperature (Sayeed & Benzer 1996; Zars 2001). In contrast, they responded to noxious heat within several seconds in our assays.

To further exclude the possibility that the jump response was elicited by innoxious thermosensation, we examined the mutant *biz* and flies with bilateral removal of the third antennal segments in the two assays. *biz* flies were not significantly different from wild-type flies in their jump latencies in the CLB assay (Fig. 2d). Two conclusions can be drawn from this observation. First, although *biz* flies are thermoblind (Sayeed & Benzer 1996), they are able to normally sense noxious thermal stimulus. Secondly, thermal nociception, rather than innoxious thermosensation, indeed induced the jump response in the CLB assay. Flies with the third antennal segments removed also exhibited normal nociceptive behavior in the CLB assay (Fig. 2e). This result suggested that thermal nociception in these flies was not influenced, although they were injured 4 h before (see *Materials and methods*).

However, the surgically manipulated flies exhibited significantly longer latencies than intact flies in the HP assay (Fig. 2e). We measured the temperature 1 mm above the 47 °C plate and found it was 32–33 °C. This suggested that the air temperature above the hot plate was able to activate the

two types of thermosensors. The two results raised the question whether innoxious thermosensation, rather than thermal nociception, elicits the jump response in the HP assay.



We designed a control experiment to test this possibility. We gently released wild-type flies into warm air, and its temperature measured by a thermocouple was 34 °C (in a conical flask bathed in 34 °C water). Instead of jumping, the flies just ran around, suggesting that innoxious thermosensation is not able to evoke the jump response. Thus, this possibility was excluded.

In summary, our results indicate that thermal nociception, rather than innoxious thermosensation, elicits the jump response in the two assays.

The painless gene is required for thermal nociception in adult Drosophila

The *painless* gene is essential for nociception in *Drosophila* larvae (Tracey *et al*. 2003). To further determine whether *painless* is also essential for nociception in adult *Drosophila*, we examined *painless*^{1} in the two assays.

We found that *painless*^{1} adults differed significantly from wild-type flies in their jump latencies in the CLB assay (Fig. 3a). No difference was observed in the 0.03-W laser

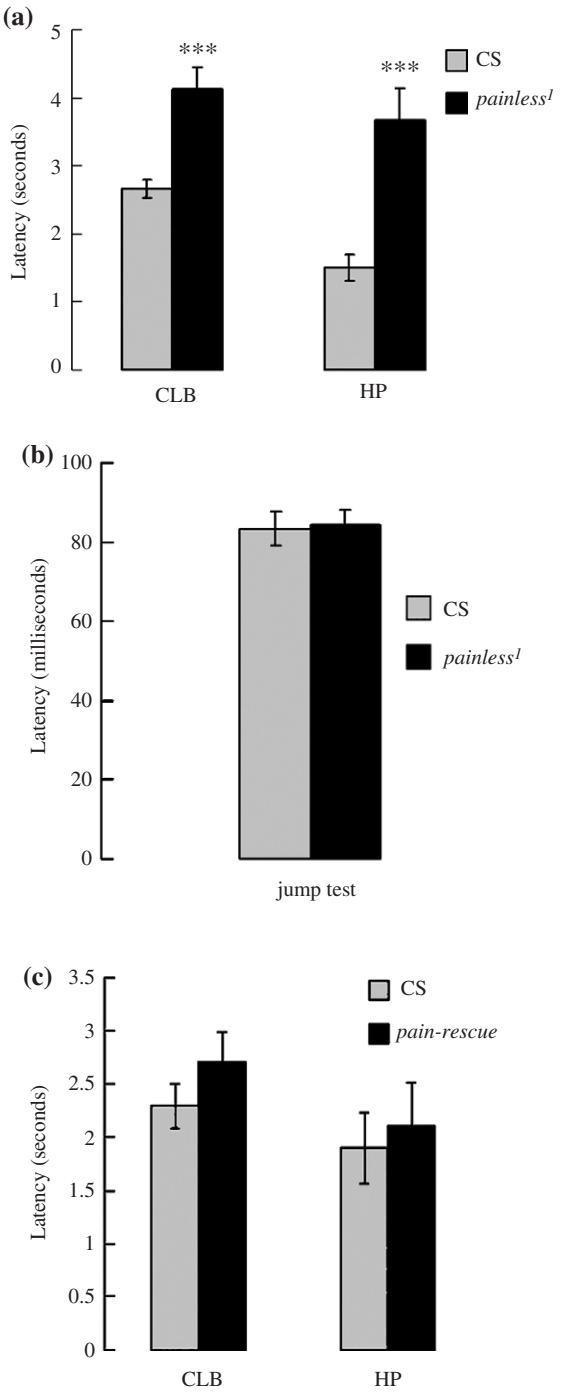
Figure 2: Thermal nociception elicited the jump response in the two assays (in all the results shown, bars represent mean values ± SEM and *n* represents the number of flies tested). (a) The average latencies of Canton Special (CS) flies tested with the lasers of different powers. Each $n = 35$. We considered that 0.02-W laser was suitable for exhibiting behavioral differences between mutants and wild-type controls. Thus, a 0.02-W laser was used to test all the flies in the CLB assay unless specifically indicated otherwise. (b) The average latencies of CS flies on the plates of different temperatures. Each $n = 30$. We considered that 47 °C plate was suitable for exhibiting behavioral differences between mutants and wild-type controls. Thus, a 47 °C plate was used to test all the flies in the HP assay unless specifically indicated otherwise. (c) The mean latencies in the first ($n = 36$), second ($n = 32$) and third ($n = 30$) tests of the same group of CS males were not significantly different in the CLB assay (ANOVA, $F_{[2,95]} = 0.6$, $P > 0.05$). (d) The average latency of *biz* flies ($n = 25$) was not different from that of CS flies ($n = 41$) in the CLB assay ($t = 1.7$, $P > 0.05$), and the latencies of *biz* flies ($n = 28$) were significantly different from those of CS flies ($n = 29$) in the HP assay ($Z = 3.0$, $^{**}P < 0.01$). *biz* is a *Drosophila* mutant that has a defect in innoxious thermosensation (see *Results*). *biz* flies are so sluggish that they cannot walk quickly (Zars 2001), and in our study, some of them cannot hold a cotton cord normally (data not shown). Thus, these mutant flies were not tested in the CLB assay. In the HP assay, 12 mutant flies were paralyzed compared with CS (one fly). (e) The average latency of CS flies ($n = 34$) whose antennae had been removed (denoted as 'ab-antenna' in the figure) was not different from that of intact CS flies ($n = 33$) in the CLB assay ($t = 0.05$, $P > 0.05$), and the latencies of surgically manipulated flies ($n = 30$) were significantly different from those of intact CS flies ($n = 34$) in the HP assay ($Z = 2.7$, $^{**}P < 0.01$). Seven surgically manipulated flies and two CS flies were paralyzed on the plate. The flies whose antennas have been removed lost their thermosensation at 27 °C (see *Results*).

beam test (see *Supplemental material* Fig. S1). Perhaps this power is too high to distinguish the difference between *painless*^{1} and CS flies.

It was noteworthy that 29 *painless*^{1} (35%) and four CS flies (6%) did not meet our criterion in the CLB assay (see *Material and methods*). A review of the video record revealed that most of them threw the cotton cord upward incompletely. We supposed that those flies also jumped, but their jump response was not obvious. Then we analyzed the latencies of the 29 *painless*^{1} flies. Their average latency was not significantly different from that of the *painless*^{1} flies exhibiting an obvious jump (data not shown). This suggests that the two groups of *painless*^{1} flies are not different in their nociceptive behavior. Although the flies failing to exhibit an obvious jump were excluded in our analysis, our criterion did not influence this result.

In the HP assay, *painless*^{1} flies displayed an obvious behavioral defect (Fig. 3a). We observed that they jumped in less than 1 second on the 52 °C plate (data not shown) and displayed normal jumping behavior in the jump test (Fig. 3b), indicating that the motor system of the jump response is not defective in *painless*^{1} mutant adults, and therefore the sensation to noxious heat is indeed defective in *painless*^{1}. Furthermore, we also tested *pain-rescue;painless*^{1}, which is created by transferring a genomic DNA rescue fragment containing the *painless* transcript unit into the *painless*^{1} strain (Tracey *et al.* 2003). The adults exhibited wild-type responses to noxious heat in the two assays (Fig. 3c). This confirmed that the *painless*^{1} mutation is responsible for the defective nociceptive behavior in *painless*^{1} flies.

Altogether, in the HP assay and in the CLB assay, *painless*^{1} adults exhibited a behavioral defect, which can be rescued by a transformed *painless* gene, and the motor pathway of the nociceptive behavior is normal in this mutant. Thus, our results proved that the *painless* gene is required for thermal nociception in adult *Drosophila*.



The expression pattern of painless in the legs, thoracic ganglia and brains of adult flies

To illustrate the pattern of *painless* expression in adult *Drosophila*, we crossed a GAL4 enhancer trap allele of *painless* (*pain*-GAL4) with UAS-EGFP (Tracey *et al.* 2003). We observed that in the legs, green fluorescence was obvious at

Figure 3: *painless* is required for thermal nociception in adult *Drosophila*. (a) The average latency of *painless*^{1} flies ($n = 47$) was significantly longer than that of Canton Special (CS) flies ($n = 58$) in the CLB assay ($t = 4.1$, $***P < 0.001$), and the latencies of *painless*^{1} flies ($n = 36$) were significantly different from those of CS flies ($n = 43$) in the HP assay ($Z = 4.7$, $***P < 0.001$). In the HP assay, 35 *painless*^{1} flies were paralyzed compared with the CS (two flies). (b) Comparison between wild-type CS ($n = 32$) and *painless*^{1} ($n = 22$) in the jump test ($t = 0.2$, $P > 0.05$). (C) The average latency of CS flies was not different from that of *painless-rescue*; *painless*^{1} flies in the CLB assay ($t = 1.3$, $P > 0.05$) nor were the latencies of *painless-rescue*; *painless*^{1} flies different from those of CS flies in the HP assay ($Z = 0.5$, $P > 0.05$). Each $n = 28$. Two mutant flies and one CS fly were paralyzed on the plate. The strain *painless-rescue;painless*^{1} is denoted as '*pain-rescue*' in the figure.

the root of the femurs (Fig. 4a, black arrows) and at the tarsal segments four and five (Fig. 4b, black arrows).

We observed green fluorescence in several fibers in the thoracic ganglions (TGs). Most of them projected into the prothoracic, mesothoracic and metathoracic neuromeres (Fig. 4c, red arrows). A few of them, which were usually parallel, connected the mesothoracic and metathoracic neuromeres (Fig. 4c, white arrows). Moreover, there was strong expression in several cell bodies in the metathoracic neuromere (Fig. 4c, yellow arrow).

In the brains, we observed green fluorescence mostly in the MBs (Fig. 4d, white arrow; the planes of several sections are shown in *Supplemental material* Fig. S2). Besides the MBs, green fluorescence was also observed invariably in three groups of small cells. One group was located between the two symmetrical α-lobes of the MBs (Fig. 4d, yellow arrow). The two others were under the β-lobes of the MBs (Fig. 4d, red arrows). In addition, green fluorescence was observed in scattered cells in the other parts of the brains, with different distributions in different flies.

Intact MBs are not required for thermal nociception

In adult *Drosophila*, MBs are brain structures essential for several behavioral functions, such as olfactory learning and choice behavior to contradictory visual cues (Tang & Guo 2001; Zars *et al*. 2000). We supposed that those structures might be important for thermal nociception, because *painless* is expressed obviously in the MBs. We then tested our hypothesis in the two assays.

Feeding newly hatched larvae with HU selectively deletes most of MBs (de Belle & Heisenberg 1994). We found that

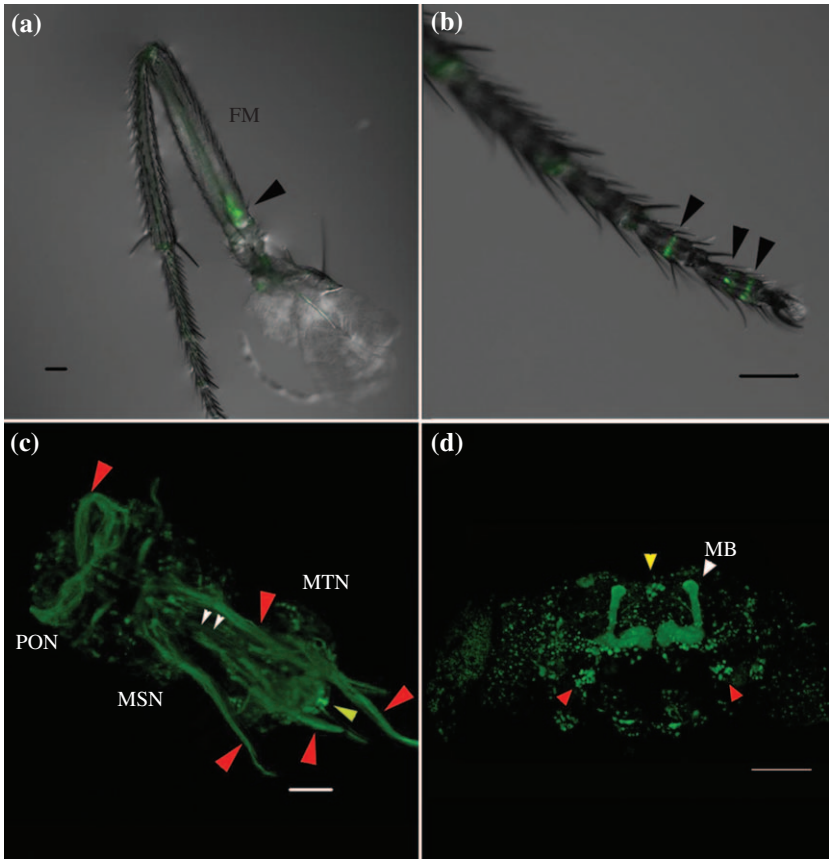


Figure 4: The expression of *painless* in the legs, thoracic ganglia and brains. (a) Green fluorescence was obvious at the root of the femur (black arrow). FM, femur. Scale bar, 100 μm; (b) Green fluorescence was obvious at tarsal segments four and five (black arrows). Scale bar, 100 μm; (c) Green fluorescence in the thoracic ganglion. Scale bar, 100 μm. Several fibers projected into the prothoracic, mesothoracic and metathoracic neuromeres (red arrows). A few parallel fibers connected the mesothoracic and metathoracic neuromeres (white arrows). There was strong expression in several cell bodies in the metathoracic neuromere (yellow arrow). PON, prothoracic neuromere; MSN, mesothoracic neuromere; MTN, metathoracic neuromere. (d) In the brain (dorsal view), green fluorescence was obvious in the mushroom bodies (white arrow) and in three groups of small cells. MB, mushroom body. Scale bar, 100 μm. One group was located between the α-lobes of the mushroom bodies (MBs) (yellow arrow). The other two were under the β-lobes of the MBs (red arrows). In addition, green fluorescence was distributed throughout other parts of the brain.

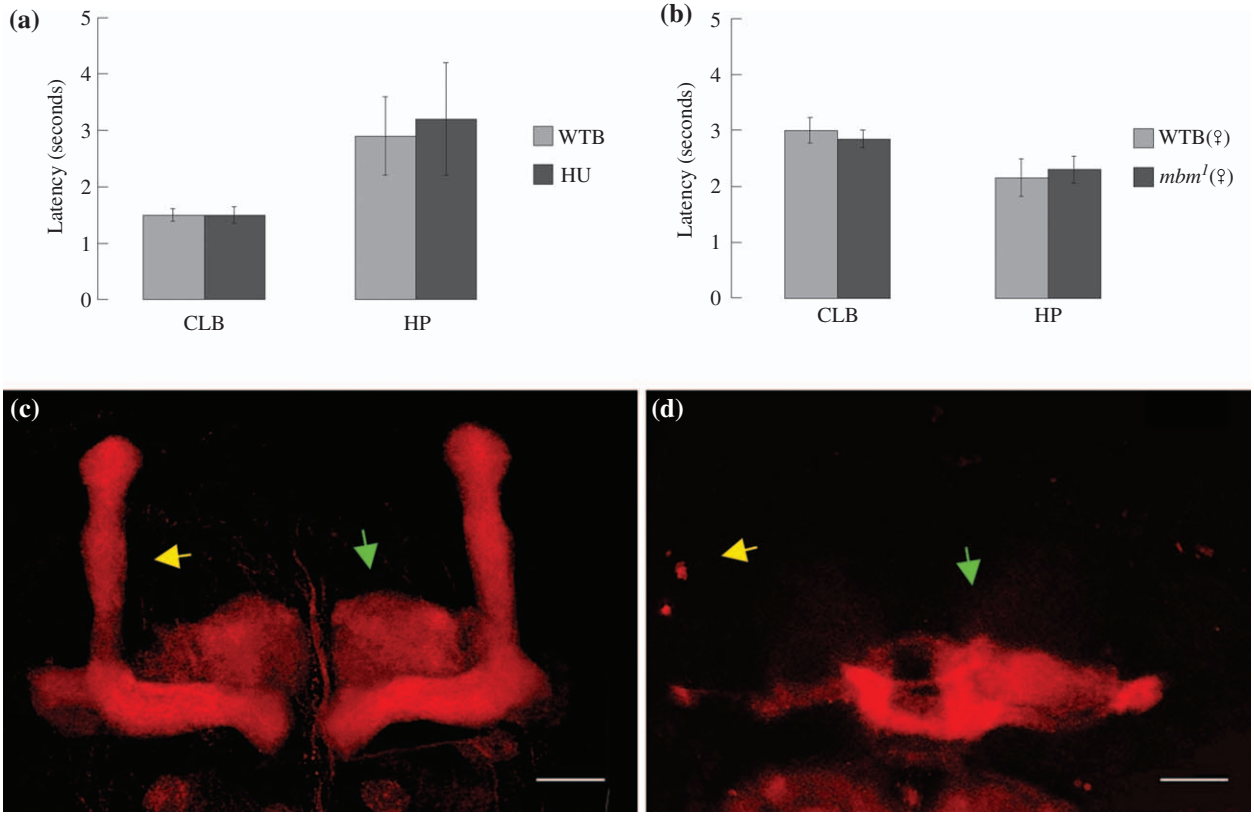


Figure 5: Intact mushroom bodies are not required for thermal nociception. (a) The average latency of untreated wild-type Berlin (WTB) flies ($n = 30$) was not different from that of hydroxyurea-treated flies ($n = 32$) in the CLB assay ($t = 0$, $P > 0.05$) nor were the latencies of untreated WTB flies ($n = 26$) different from those of hydroxyurea-treated flies ($n = 27$) in the HP assay ($Z = 0.1$, $P > 0.05$). One treated fly and one WTB fly were paralyzed on the plate. The hydroxyurea-treated group was denoted as HU in this figure. The larvae that were treated in hydroxyurea solution lost their mushroom bodies after eclosion (see *Results*). (b) The average latency of mbm^1 females ($n = 34$) was no different from that of WTB females ($n = 39$) in the CLB assay ($t = 0$, $P > 0.05$) nor were the latencies of mbm^1 females ($n = 27$) different from those of WTB females ($n = 27$) in the HP assay in which a 45 °C plate was used ($Z = 1.7$, $P > 0.05$). One mbm^1 fly and one WTB fly were paralyzed on the plate. (c) The mushroom bodies (MBs) in WTB females are intact. The α/α′-lobes (yellow arrow), β/β′-lobes and γ-lobes (green arrow) are complete. Scale bar, 20 μm. (d) The MBs in mbm^1 females are miniature. The α/α′-lobes nearly completely vanished (yellow arrow). The β/β′-lobes and γ-lobes are miniature (green arrow). Scale bar, 20 μm.

the HU-treated and mock-treated flies had no significant difference in the two assays (Fig. 5a). To assess the degree of MB ablation, we used a previously reported method (Tang & Guo 2001). When the larvae of WTB were being treated, we treated, in parallel, the larvae of *OK107:GFP*. Then we observed their MBs in fluorescence microscopy after eclosion. Of the 15 HU-treated fly brains analyzed, 12 exhibited nearly complete ablation and three exhibited partial ablation.

Miniature MBs in mbm^1 females have been reported (de Belle & Heisenberg 1996). Using an immunofluorescence method (see *Material and methods*), we ascertained that the mbm^1 females raised in our laboratory still had the reported structural defect in their MBs (Fig. 5c,d). In the two assays, mbm^1 females were not significantly different from WTB females in response to noxious heat (Fig. 5b).

Altogether, the results from the HU-treated flies and mbm^1 females indicated that intact MBs are not required for thermal nociception, suggesting MBs are unimportant for thermal nociception.

nob^{KS49} is defective in thermal nociception

To study the role of another brain structure, the central complex, in thermal nociception, we tested several mutants defective in the neural structures of the central complex and found that nob^{KS49} flies exhibited significantly longer latencies than WTB flies in the two assays (Fig. 6a), and they displayed normal behavior in the jump test (Fig. 6b). The results above indicate that this mutant is defective in thermal nociception. nob^{KS49} has a structural defect at the

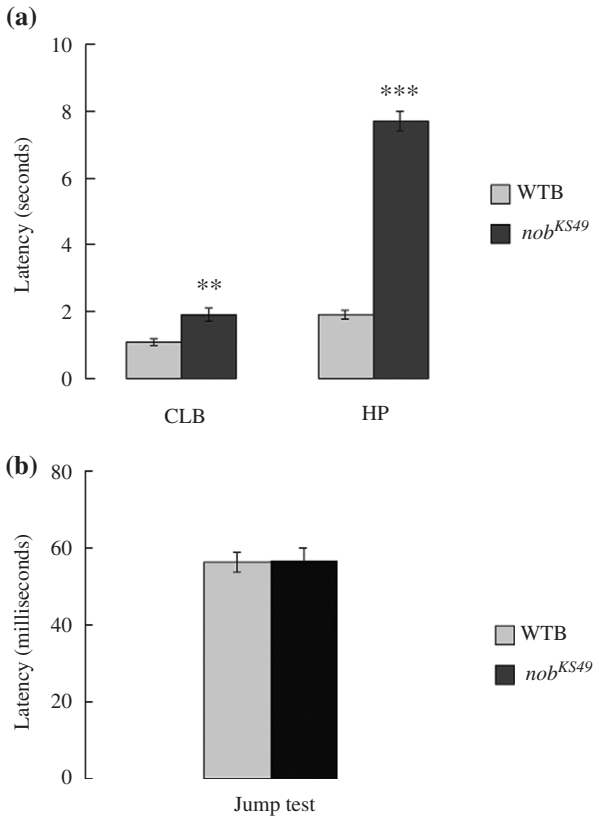


Figure 6: *nob*^{KS49} is defective in thermal nociception. (a) The average latency of wild-type Berlin (WTB) flies ($n = 25$) was significantly shorter than that of *nob*^{KS49} flies ($n = 25$) in the CLB assay in which a 0.03-W laser was used ($t = 2.9$, $^{**}P < 0.01$), and the latencies of WTB flies ($n = 40$) were significantly different from those of 44 *nob*^{KS49} flies ($n = 44$) in the HP assay ($Z = 7.6$, $^{***}P < 0.001$). Three *nob*^{ks49} flies and one WTB fly were paralyzed on the plate. (b) The comparison of WTB ($n = 23$) and *nob*^{KS49} ($n = 18$) in the jump test ($t = 0$, $P > 0.05$).

protocerebral bridge, the most dorso-posterior *neuropil* of the central complex (Strauss *et al*. 1992). Our results suggested that the protocerebral bridge participates in the processing of nociceptive information.

Discussion

The behavioral characteristics of thermal nociception in adult Drosophila

Vertebrates exhibit escape/avoidance behavior when they are exposed to painful stimuli. We observed a similar behavior, the jump response, when adult *Drosophila* was exposed to noxious heat. Therefore, we established two methods to measure this behavior. Our results indicate that the nociceptive behavior in adult flies has three distinct characteristics, which resemble those in mammals.

First, the results shown in Fig. 2(a,b) demonstrated that in adult flies the jump latency is inversely related to stimulus intensity. A similar phenomenon has been reported in the HP test in mice and the tail-flick test in rats (Ben-bassat *et al*. 1959; Woolfe & MacDonald 1944).

Second, adult flies are able to distinguish thermal nociception from innoxious thermosensation, which resembles mammals' ability to distinguish painful heat from warmth. Our studies demonstrated that the nociceptive behavior is significantly different from the behavior induced by innoxious thermosensation in locomotor pattern and response time (see *Results*). Moreover, the normal nociceptive behavior of *biz* flies (Fig. 3b) further confirms the difference between thermal nociception and innoxious thermosensation.

Third, adult *Drosophila* are able to respond to different kinds of noxious stimulus. We observed that when given a cotton cord wet with 1 M NaOH or HCl, each fly caught the cord (only touching it with its legs) for a few seconds, then released it suddenly and refused the cord for several minutes. This result suggests that adult flies also respond to noxious chemical stimuli in addition to noxious thermal stimuli. Additionally, strong electrical stimuli can elicit the jump response in adult flies (T. Tully, personal communication). The observations above suggest that the nociceptors in adult *Drosophila* can be activated by different kinds of noxious stimuli, as do the C-fibers in mammals (Lynn 1984).

Besides the three characteristics above, there may be another two shared by mammals and *Drosophila* adults. First, the threshold temperature for thermal nociception in mammals is approximately 45 °C (Vyklicky 1984). Our results suggest that the nociceptive threshold temperature in adult *Drosophila* is also approximately 45 °C. A previous study reported that *biz* flies distributed randomly across the temperature gradients from 23 to 36.5 °C, and even to 45 °C (Sayeed & Benzer 1996), but we found that *biz* flies behaved normally in the CLB assay (Fig. 2d), suggesting that the nociceptive threshold temperature in adult *Drosophila* is approximately 45 °C. This hypothesis is also supported by the result that the mean latencies decreased significantly with an increase of plate temperatures from 43 to 47 °C in the HP assay (Fig. 2b). However, we still lack a direct evidence to prove this hypothesis because of the technical difficulty in measuring the cuticle temperature of adult flies (see below).

Second, we tried to heat the different parts of thorax and abdomen with a focused laser beam. Adult flies were also able to jump away from the stimuli, suggesting that the nociceptors in adult flies are systemically located in their bodies, including the legs, the abdomens and the thoraxes. The systemic distribution of nociceptors may be a common characteristic shared by mammals and adult *Drosophila* (Lynn 1984).

The advantages and disadvantages of the two behavioral assays

Several nociceptive assays, such as the hot-plate, tail-flick and paw-withdraw assays, have been established in mammals for a long time (Ben-bassat *et al*. 1959; Hargreaves *et al*. 1988; Woolfe & MacDonald 1944). Recently, behavioral assays have also been established to assess nociception in invertebrates (Tracey *et al*. 2003; Wittenburg & Baumeister 1999). In comparison with the established assays, our assays have three obvious qualities.

First, similar to the nociceptive assays established for rodents (Ben-bassat *et al*. 1959; Hargreaves *et al*. 1988; Woolfe & MacDonald 1944), our assays provide two quantitative methods to analyze the nociceptive behavior in adult *Drosophila*.

Secondly, our results illustrated that the mean latency was stable in the CLB assay (Fig. 2c), as in the paw-withdraw test in rats (Hargreaves *et al*. 1988), despite repeated stimuli. This advantage is considered to be an important criterion for a well-developed nociceptive assay (Vyklicky 1984).

Thirdly, similar to the rotarod treadmill test, which served as the control for the HP test in mice (Chen *et al*. 2001), the jump test was established to serve as the control for the two assays. Although similar methods have been established (Elkins & Ganetzky 1990; Kaplan & Trout 1974; Snowball & Holmqvist 1994), our assay is simple and effective. The results observed in glc^1 flies demonstrate its effectiveness. glc^1 is a mutant of a glutamate-gated chloride channel subunit gene (*DmGlula*) (Kane *et al*. 2000). glc^1 flies exhibited a behavioral defect in response to noxious heat in the two assays (see *Supplemental material* Fig. S1). However, their mean latency in the jump test was significantly longer than that of wild-type flies (see *Supplemental material* Fig. S1), suggesting that glc^1 is defective in the output pathway of the jump response. Thus, the abnormal behavior of glc^1 in the two assays does not prove that glc^1 is indeed defective in thermal nociception.

Despite the three advantages above, our assays also have two major disadvantages. First, it is difficult to find an available method to measure the temperature of local cuticle directly because of the small body size of adult flies. In contrast, this parameter is easily measured in rats (Hargreaves *et al*. 1988). Secondly, the surgically manipulated flies and *biz* flies responded slower than wild-type flies in the HP assay (Fig. 2d,e), suggesting that innoxious thermosensation sensitizes the jump response, although thermal nociception elicits it in this assay. Similarly, one of the behavior patterns of rats in the HP test was a response to a mixture of warmth and noxious heat (Espejo & Mir 1993). Finding a way to avoid the sensitization will be a goal of our future studies.

The role of the painless gene in thermal nociception

We examined $painless^1$ flies in the two assays and the jump test, and further tested *pain-rescue;*$painless^1$ flies (Fig. 3a,b,c). The results indicated that *painless* is required for thermal nociception in adults, as in larvae (Tracey *et al*. 2003), suggesting that this gene is expressed in the neurons of the nociceptive sensory pathway. Thus, we further investigated the expression pattern of *painless* in adult flies.

The *pain*-Gal4-driven expression of UAS-EGFP showed green fluorescence in the cells where *painless* was expressed (Tracey *et al*. 2003). By this method, we found the expression of this gene at the root of the femurs (Fig. 4a, black arrows) and at the tarsal segments four and five (Fig. 4b, black arrows). This suggested that *painless* is expressed in the nociceptors at the ends of legs, which were in contact with the copper plate. Therefore, we hypothesized that the behavioral defect of $painless^1$ in the HP assay may have been caused by the abnormal function of those nociceptors.

In the TGs, most of the *painless*-expressing fibers, projected from the peripheral tissues into the prothoracic, mesothoracic and metathoracic neuromeres (Fig. 4c, red arrows), suggested that *painless* is expressed in the neural pathway that sends nociceptive information from the peripheral nervous system to the TG. Additionally, we did not observe obvious fiber projecting from the TGs to the brains, suggesting that *painless* is not expressed in the neural pathway that sends nociceptive information from the TG to the brain. The two suggestions above, plus the complex expression pattern of *painless* in the TGs (Fig. 4c), imply that the TG might be a primary nerve center that processes nociceptive information coming from the peripheral nervous system.

In the brains, green fluorescence invariably was found in three groups of small cells and in the MBs (Fig. 4d), implying that MBs play an important role in thermal nociception. However, the HU-treated flies and mbm^1 females, whose MBs were very incomplete, exhibited a normal nociceptive behavior (Fig. 5a,b). This indicates that intact MBs are not required for thermal nociception. The result is not surprising, because mbm^1 females and HU-treated flies exhibited normal learning scores and avoidance scores in a visual learning paradigm, in which heat punishment was used (S. Tang, personal communication; de Belle & Heisenberg 1994; Tang & Guo 2001). Considering that MBs may be unimportant for thermal nociception although *painless* is expressed in MBs, we hypothesized that, first, the expression of *painless* in the TG and the peripheral nervous system, rather than its expression in MBs, might be necessary for thermal nociception; secondly, the expression of *painless* in MBs might participate in other functions and consequently *painless* might be a pleiotropic gene.

The central complex is another brain structure in adult *Drosophila*. Several mutants (ccd^{KS135}, ccb^{KS145}, cbd^{KS96}, ebo^{678}, ebo^{KS263}, agn^{X1} and nob^{KS49}), defective in the neural structure of central complex (Strauss & Heisenberg 1993), exhibited defective behavior in a visual learning paradigm, in which heat punishment was used (Li Liu, personal communication). The evidences implied that the mutants might also be defective in sensing noxious heat. Thus, we tried the

mutants and found that none of them were obviously defective in the nociceptive behavior (data not shown), except nob^{KS49} (Fig. 6a,b). nob^{KS49} has a structural defect at the protocerebral bridge, the most dorso-posterior neuropil of the central complex (Strauss *et al*. 1992). Important for walking and flight behavior, this structure may be a part of the highly regulatory center for locomotion in adult *Drosophila* (Strauss *et al*. 1992). In our study, the abnormal nociceptive behavior of nob^{KS49} suggests that the protocerebral bridge also participates in the processing of nociceptive information (Fig. 5e), although what role this structure plays in thermal nociception needs further study.

In summary, the CLB assay, the HP assay and the jump test compose a behavioral testing system to assess thermal nociception in *Drosophila*. We demonstrated the behavioral comparability between mammals and adult flies, suggesting that adult *Drosophila* is a useful model for the study of nociception. The various genetic tools available in *Drosophila*, together with this behavioral testing system, will facilitate the identification of novel genes and neural pathways involved in thermal nociception.

References

de Belle, J.S. & Heisenberg, M. (1994) Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science* **263**, 692–695.

de Belle, J.S. & Heisenberg, M. (1996) Expression of *Drosophila* mushroom body mutations in alternative genetic backgrounds: a case study of the *mushroom body miniature* gene (*mbm*). *Proc Natl Acad Sci USA* **93**, 9875–9880.

Ben-bassat, J., Peretz, E. & Sulman, F.G. (1959) Analgesimetry and ranking of analgesic drugs by the receptacle method. *Arch Int Pharmacodyn Ther* **122**, 434–447.

Benzer, S. (1967) Behavioral mutants of *Drosophila* isolated by countercurrent distribution. *Proc Natl Acad Sci USA* **58**, 1112–1119.

Campbell, J.L. & Nash, H.A. (1998) The visually-induced jump response of *Drosophila melanogaster* is sensitive to volatile anesthetics. *J Neurogenet* **12**, 241–251.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J. & Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816–824.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J. & Julius, D. (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* **398**, 436–441.

Chen, Z.F., Rebelo, S., White, F., Malmberg, A.B., Baba, H., Lima, D., Woolf, C.J., Basbaum, A.I. & Anderson, D.J. (2001) The paired homeodomain protein DRG11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. *Neuron* **31**, 59–73.

Dickenson, A.H. & Sullivan, A.F. (1987) Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurons. *Neurosci Lett* **83**, 207–211.

Elkins, T. & Ganetzky, B. (1990) Conduction in the giant nerve fiber pathway in temperature-sensitive paralytic mutants of *Drosophila*. *J Neurogenet* **6**, 207–219.

Engel, J.E. & Wu, C.F. (1996) Altered habituation of an identified escape circuit in *Drosophila* memory mutants. *J Neurosci* **16**, 3486–3499.

Engel, J.E. & Wu, C.F. (1998) Genetic dissection of functional contributions of specific potassium channel subunits in habituation of an escape circuit in *Drosophila*. *J Neurosci* **18**, 2254–2267.

Espejo, E.F. & Mir, D. (1993) Structure of the rat's behavior in the hot plate test. *Behav Brain Res* **56**, 171–176.

Gao, Y.J., Ren, W.H., Zhang, Y.Q. & Zhao, Z.Q. (2004) Contributions of the anterior cingulate cortex and amygdala to pain- and fear-conditioned place avoidance in rats. *Pain* **110**, 343–353.

Gilbert, S.F. (2000) *Developmental Biology*, 6th edn. Sinauer Associates, Sunderland.

Guo, A., Liu, L., Xia, S., Feng, C., Wolf, R. & Heisenberg, M. (1996) Conditioned visual flight orientation in *Drosophila*: dependence on age, practice, and diet. *Learn Mem* **3**, 49–59.

Guo, J., Wang, H., Zhang, Y.Q. & Zhao, Z.Q. (2005) Alterations of membrane properties and effects of D-serine on NMDA-induced current in rat anterior cingulate cortex neurons after monoarthritis. *Neurosci Lett* **384**, 245–249.

Hargreaves, K., Dubner, R., Brown, F., Flores, C. & Joris, J. (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**, 77–88.

Heisenberg, M. & Bohl, K. (1979) Isolation of anatomical brain mutants of *Drosophila* by histological means. *Z Naturforsch* **34c**, 143–147.

Heisenberg, M. & Wolf, R. (1984) *Vision in Drosophila*. Springer-Verlag, Berlin.

Kane, N.S., Hirschberg, B., Qian, S., Hunt, D., Thomas, B., Brochu, R., Ludmerer, S.W., Zheng, Y., Smith, M., Arena, J.P., Cohen, C.J., Schmatz, D., Warmke, J. & Cully, D.F. (2000) Drug-resistant *Drosophila* indicate glutamate-gated chloride channels are targets for the antiparasitics nodulisporic acid and ivermectin. *Proc Natl Acad Sci USA* **97**, 13949–13954.

Kaplan, W.D. & Trout, W.E. (1974) Genetic manipulation of an abnormal jump response in *Drosophila*. *Genetics* **77**, 721–739.

Lee, T. & Luo, L. (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451–461.

Lee, R., Sawin, E., Chalfie, M., Horvitz, H. & Avery, L. (1999) EAT-4, a homolog of a mammalian sodium-dependent inorganic phosphate cotransporter, is necessary for glutamatergic neurotransmission in *Caenorhabditis elegans*. *J Neurosci* **19**, 159–167.

Lynn, B. (1984) The detection of injury and tissue damage. In Wall, P. D. & Melzack, R. (eds) *Textbook of pain*, Churchill Livingstone, New York, pp. 19–33.

Nelson, L., Rosoff, M. & Li, C. (1998) Disruption of a neuropeptide gene, *flp-1*, causes multiple behavioral defects in *Caenorhabditis elegans*. *Science* **281**, 1686–1690.

Sayeed, O. & Benzer, S. (1996) Behavioral genetics of thermosensation and hygrosensation in *Drosophila*. *Proc Natl Acad Sci USA* **93**, 6079–6084.

Smith, C.U.M. (2000) *Biology of Sensory Systems*. John Wiley & Sons Ltd, UK.

Snowball, M. & Holmqvist, M. (1994) An electronic device for monitoring escape behaviour in *Musca* and *Drosophila*. *J Neurosci Methods* **51**, 91–94.

Strauss, R. & Heisenberg, M. (1993) A higher control center of locomotor behavior in the *Drosophila* brain. *J Neurosci* **13**, 1852–1861.

Strauss, R., Hanesch, U., Kinkelin, M., Wolf, R. & Heisenberg, M. (1992) No-bridge of *Drosophila melanogaster*: portrait of a structural brain mutant of the central complex. *J Neurogenet* **8**, 125–155.

Tang, S.M. & Guo, A.K. (2001) Choice behavior of Drosophila facing contradictory visual cues. *Science* **294**, 1543–1547.

Thoms, J.B. & Wyman, R.J. (1984) Mutations altering synaptic connectivity between identified neurons in Drosophila. *J Neurosci* **4**, 530–538.

Tracey, W., Wilson, R., Laurent, G. & Benzer, S. (2003) *Painless*, a *Drosophila* gene essential for nociception. *Cell* **113**, 261–273.

Tully, T. & Koss, S. (1992) Habituation of the jump reflex to olfactory cues in normal and mutant *Drosophila*. *Soc Neurosci Abstr* **18**, 942.

Vyklicky, L. (1984) Methods of testing pain mechanisms in animals. In Wall, P.D. & Melzack, R. (eds) *Textbook of Pain*, Churchill Livingstone, New York, pp. 178–185.

Wittenburg, N. & Baumeister, R. (1999) Thermal avoidance in *Caenorhabditis elegans*: an approach to the study of nociception. *Proc Natl Acad Sci USA* **96**, 10477–10482.

Wolf, R. & Heisenberg, M. (1991) Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *J Comp Physiol [A]* **169**, 699–705.

Woolfe, G. & MacDonald, A.D. (1944) The evaluation of analgesic action of pethidine hydrochloride (Demerol). *J Pharmacol Exp Ther* **80**, 300–307.

Zars, T. (2001) Two thermosensors in *Drosophila* have different behavioral functions. *J Comp Physiol [A]* **187**, 235–242.

Zars, T., Fischer, M., Schulz, R. & Heisenberg, M. (2000) Localization of short-term memory in *Drosophila*. *Science* **288**, 672–675.

Acknowledgments

We thank W. Daniel Tracey, Jr for *painless*^{1}, *pain*-Gal4, *pain-rescue;painless*^{1} and CS; Martin Heisenberg and Reinhard Wolf for *mbm*^{1}, *nob*^{KS49} and WTB; Troy Zars for *biz*; Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences) for mAb1D4; Chun-Fang Wu, Xu Zhang and Yu-Qiu Zhang for reading preliminary versions of the manuscript; Jennifer Heacock and Xian-Jue Zhou for editing the manuscript; Zuo-Ren Wang, Min-Kui Zhang, Chun-Yan Yuan and Li-Xia Chen for technical assistance; and Dupont Co. for the nylon thread freely. This research was supported by the National Science Foundation of China (Grant 30270341), the Multidisciplinary Research Program (Brain and Mind) of the Chinese Academy of Sciences and the Major State Basic Research Program (G2000077800), the Precedent Project of Important Intersectional Disciplines in the Knowledge Innovation Engineering of the Chinese Academy of Sciences (Grant No. KJCX1-09-03).

Supplementary material

Video S1. A fly throwing the cotton cord upward after being heated by the CO_2 laser.

Video S2. A fly jumping from the rim of the chamber in response to the fast approach of a shadow produced by a fan.

Figure S1. (a) The mean latency of *painless*^{1} flies ($n = 26$) was not different from that of CS flies ($n = 29$) in the CLB assay in which the power of the laser was 0.03 W ($t = 0.1$, $P > 0.05$). (b) The mean latency of *glc*^{1} flies ($n = 30$) was significantly different from that of wild-type Oregon-R flies ($n = 35$) in the CLB assay ($t = 2.8$, $^{**}P < 0.01$). The power of the laser was 0.03 W. (c) The jump latencies of *glc*^{1} flies ($n = 37$) were significantly different from those of wild-type Oregon-R flies ($n = 35$) in the HP assay ($Z = 5.1$, $^{***}P < 0.001$). (d) The mean latency of *glc*^{1} flies ($n = 18$) was significantly different from that of wild-type Oregon-R flies ($n = 17$) in the jump test ($t = 2.4$, $^{*}P < 0.05$). The wild-type Oregon-R strain was denoted as 'OR' in the figures.

Figure S2. A fly brain was observed using two-photon fluorescence microscopy. The eight figures show a series of optical dissections of the whole fly brain with a distance of 6 μm between each dissection.

These materials are available as part of the online article from http://www.blackwell-synergy.com