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painless, a *Drosophila* Gene Essential for Nociception

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Summary

We describe a paradigm for nociception in *Drosophila.* In response to the touch of a probe heated above 38°C, *Drosophila* larvae produce a stereotypical rolling behavior, unlike the response to an unheated probe. In a genetic screen for mutants defective in this noxious heat response, we identified the *painless* gene. Recordings from wild-type larval nerves identified neurons that initiated strong spiking above 38°C, and this activity was absent in the *painless* mutant. The *painless* mRNA encodes a protein of the transient receptor potential ion channel family. Painless is required for both thermal and mechanical nociception, but not for sensing light touch. *painless* is expressed in peripheral neurons that extend multiple branched dendrites beneath the larval epidermis, similar to vertebrate pain receptors. An antibody to Painless binds to localized dendritic structures that we hypothesize are involved in nociceptive signaling.

Introduction

In humans and other vertebrates, painful stimuli are sensed by specialized neurons known as nociceptors (Sherrington, 1906), which fire in response to noxious heat, mechanical, or chemical stimuli that have the potential to cause tissue damage (Bessou and Perl, 1969). The signals are in turn processed by the central nervous system and perceived as pain, which serves an indispensable, protective role. Nociceptors are also involved in pathological pain states caused by inflammation, nerve damage, or cancer. An increased understanding of nociception therefore is of wide interest, and model systems for molecular genetic analysis are desirable.

Great progress in understanding the molecular mechanisms underlying pain signaling was made with the cloning and identification of the vanilloid receptor (TRPV1), an ion channel of the transient receptor potential (TRP) ion channel family, which has been proposed to function at the transduction step in nociceptive pathways (Caterina et al., 1997). In *Xenopus* oocytes and human embryonic kidney (HEK) cells, TRPV1-dependent currents are gated by stimuli that are also capable of activating nociceptors, including heat in the noxious temperature range, protons, and capsaicin, the spicy ingredient in chili peppers (Tominaga et al., 1998; Caterina et al., 1997). In these systems, the TRPV1 channel is gated by heat in the range of 42°C–48°C, and a related channel, TRPV2, was found to be activated at still higher temperatures (Caterina et al., 1999). More recently, another channel of the TRP family was shown to be activated by menthol and cool temperatures, leading to the suggestion that the ion channels in the TRP family might play a central role in sensing temperature (McKemy et al., 2002; Peier et al., 2002). Although the vertebrate TRPV1 channel has been shown to be activated by heat and protons in heterologous systems, mice mutant for TRPV1 still show a marked behavioral response to noxious heat and no defect in mechanical nociception, suggesting that other genes are involved in these processes (Caterina et al., 2000). This may be due to functional redundancy, as several other genes closely related to TRPV1 are expressed in dorsal root ganglia and are activated by heat in heterologous expression systems (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002).

The TRP gene family has been subdivided into major groups based on sequence homology (Montell et al., 2002). For example, the TRPC class is closely related to the canonical TRPs identified for their role in *Drosophila* phototransduction, the TRPV class are closely related to the vanilloid receptor, and the TRPN class is closely related to *no mechanoreceptor potential-C,* a *Drosophila* gene suspected to participate in mechanosensation (Walker et al., 2000).

It has been suggested that the role of TRPV channels in sensing noxious stimuli is an ancient one, as a TRPV1-related gene in *C. elegans*, *osm-9*, has been shown to be required for avoidance of chemicals and high osmolarity (Roayaie et al., 1998). A family of genes related to *osm-9* and the capsaicin receptor (the *ocr* gene family) has been identified in *C. elegans*. These studies have led to the proposal that the transduction mechanisms for TRPs, which sense physical stimuli, depend on combinatorial expression patterns of different TRP genes, allowing a single TRP gene to play a polymodal sensory role (Tobin et al., 2002). However, *osm-9* mutants have also been tested in *C. elegans* thermal avoidance and showed a strong response to noxious heat (Wittenburg and Baumeister, 1999). Invertebrate molecular and cellular mechanisms underlying thermal nociception are unknown.

Here, we describe a striking *Drosophila* behavior that occurs in response to a variety of noxious stimuli. In a forward genetic screen, we identified the *Drosophila* mutant *painless,* which is defective in sensing both noxious mechanical and thermal stimuli. The *painless* gene is expressed in multidendritic and chordotonal sensory neurons and is required for the activation of sensory neurons by noxious heat.

Results

In models of nociception, noxious heat is often used as the stimulus to elicit a defensive motor output, as in the tail flick response of the rat. Since heat has also been shown to be an effective negatively reinforcing stimulus

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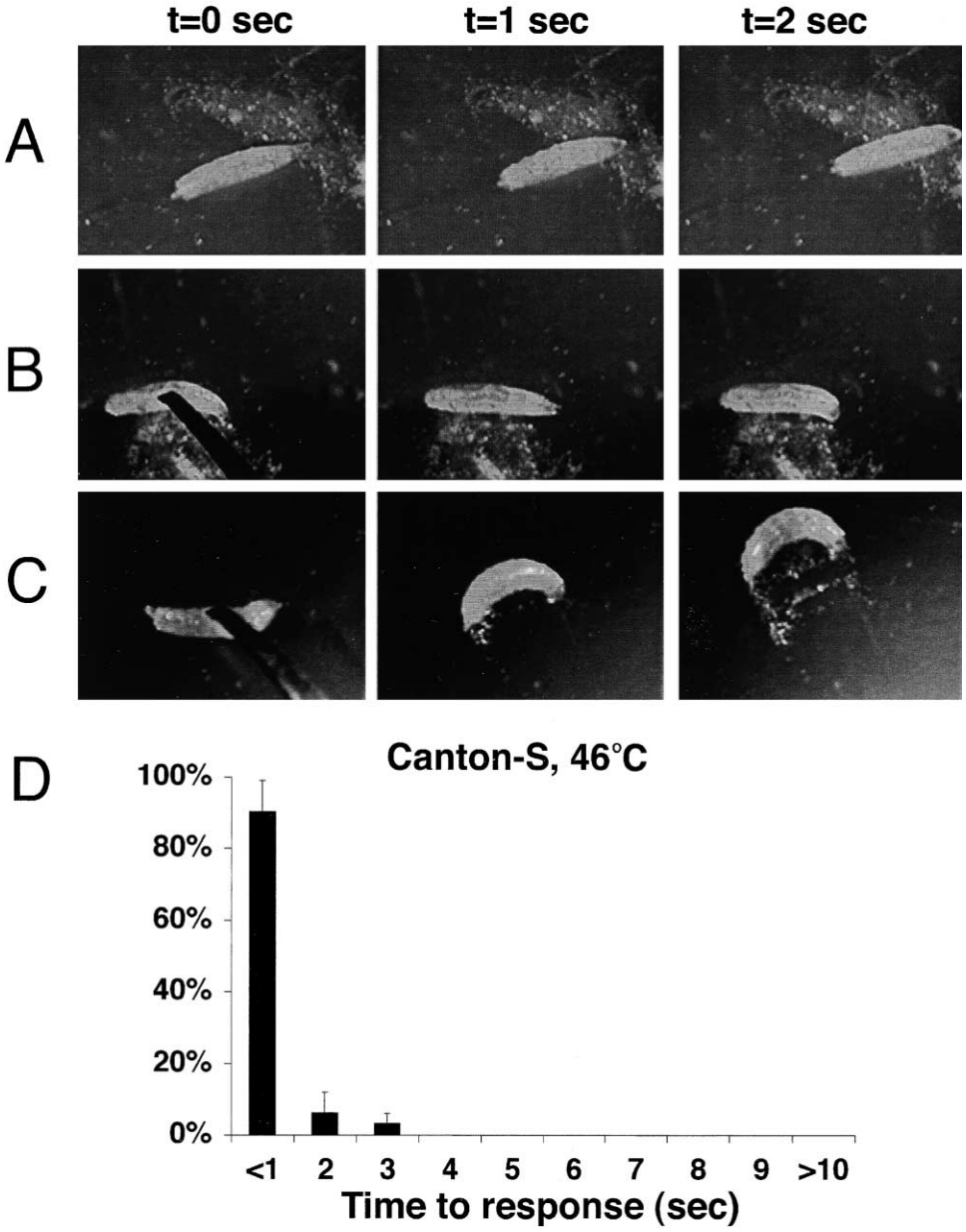

Figure 1. Noxious Heat Produces a Stereotypical Behavioral Output in a *Drosophila* Larva

Images extracted from digital video recordings. (A) Normal larval movement. (B) In response to a light touch, the larva pauses. (C) When touched with a probe heated to a noxious temperature (left), the larva responds by rolling laterally in a corkscrew-like motion. (D) The behavioral output quantitatively analyzed by measuring the delay in response time.

in adult *Drosophila* learning (Brembs and Heisenberg, 2000; Mariath, 1985), we conjectured that heat might also be used to study nociception per se. A normal, undisturbed *Drosophila* larva moves through its environment with a rhythmic motion (Figure 1A). In response to light touch with a probe, a larva will pause (Figure 1B) or make one or more contractile waves, moving away from the stimulus (Kernan et al., 1994).

In contrast, when touched with the same probe heated above a threshold temperature, larvae are seen to vigorously roll sideways in a corkscrew-like motion (Figure 1C). The threshold probe temperature for eliciting this behavior is 39°C–41°C (noxious heat), at which temperature several seconds of stimulation are required to induce rolling, but at 42°C or higher, the response occurs in as little as 0.4 s (Figure 1D). Importantly, the temperature threshold for firing of nociceptors in vertebrates, including primates, is similar, 39°C–41°C (Tillman et al., 1995). Since vertebrate nociceptors also respond to noxious mechanical stimuli (Beck et al., 1974; Bessou and Perl, 1969; Van Hees and Gybels, 1981), we examined the response of *Drosophila* larvae to strong punctate stimuli or pinching of the cuticle with forceps, and these elicited the same rolling behavior as noxious heat.

In vertebrates, the cell bodies of nociceptors are located in sensory ganglia. These cells have projections

to the periphery, where profuse branching of naked dendrites occurs beneath the skin. In contrast to other sensory modalities of the epidermis, which utilize specialized receptor cells to transduce signals, the naked dendrites of nociceptors themselves are thought to contain the transducing machinery for noxious stimuli.

Similar distinctions prevail in the peripheral nervous system of *Drosophila*. Type I sensory neurons have a single dendrite as part of a specialized sensillum (Hartenstein, 1988). Type II, or multidendritic sensory neurons, do not appear to be associated with specialized receptor cells but utilize naked dendrites. Although mutations affecting their developmental biology and branching patterns are under active investigation (Gao et al., 1999; Grueber et al., 2002), the function of these cells was previously unknown.

Kernan, Cowan, and Zuker (1994) isolated *Drosophila* mutants that were insensitive to a light touch on the nose. Several of the mutants isolated in that screen have now been cloned, and each has been found to be expressed in ciliated Type I sensory organs, but not in the multidendritic neurons (Chung et al., 2001; Walker et al., 2000). Because the md-da neurons beneath the cuticle project a plexus of naked dendrites similar to the pain-sensing neurons in mammalian skin and because they were of unknown function, it seemed possible that they might function as the nociceptors.

To test that hypothesis, we used a cell-specific "driver" strain to selectively disrupt the function of these cells. The tetanus toxin light chain (TeTxLC) blocks calcium-dependent evoked synaptic vesicle release through proteolytic cleavage of the v-SNARE synaptobrevin (Sweeney et al., 1995). To create larvae expressing TeTxLC in md neurons, we used the enhancer trap driver strain GAL4109(2)80 (md-GAL4), in which GAL4 is expressed in all md-da neurons of the larval peripheral nervous system (Gao et al., 1999) and in approximately 100 cells of the central nervous system (W.D.T. and S.B., unpublished data). When crossed to lines bearing UAS binding sites for GAL4 upstream of a gene of interest, md-GAL4 thus causes expression of this gene specifically in the above pattern (Brand and Perrimon, 1993; Gao et al., 1999). We crossed md-GAL4 to a line carrying UAS-TeTxLC and to a negative control line containing UAS-IMPTNT-V, which contains an inactivating point mutation (Sweeney et al., 1995).

To quantify the response, we used third instar larvae from vials seeded by adult flies for 7 days at room temperature. The noxious heat probe (a soldering iron sharpened to a chisel tip shape 0.6 mm wide) was set to maintain a temperature of 46°C, and the stimulus was delivered by gently touching the larvae laterally, in abdominal segments four, five, or six. At that temperature, wild-type larvae performed the rolling avoidance behavior within less than 1 s from the initiation of contact (Figure 1D, average response time, 0.4 $\pm$ 0.06 s).

In contrast, third instar larvae expressing the tetanus toxin light chain in the expression pattern of md-GAL4 were strikingly unresponsive to noxious heat. Indeed, the majority of md-GAL4/UAS-TeTxLC larvae did not respond at all, even after 10 s of stimulation (Figure 2B). These larvae also failed to roll in response to strong mechanical stimuli. However, control larvae with the driver alone or expressing the tetanus toxin with the mutated catalytic domain showed a normal rolling response (Figures 2A and 2C). Therefore, the neurons targeted by md-GAL4 are essential for nociception. Although one can not exclude a role for the central neurons expressing this driver, this result suggested that the multidendritic neurons, or a subset of them, might be the ones that function as nociceptors. The evidence described below supports this hypothesis.

Forward Genetic Analysis of Nociception

To identify genes important for nociception, we performed a genetic screen for mutations that cause insensitivity to noxious heat. We screened through a collection of fly lines, carrying randomly inserted EP transposable elements (Rorth et al., 1998). We considered a line to have impaired sensitivity to noxious heat if stimulation longer than 3 s was required to produce the rolling response.

Among the 1500 EP lines screened, we identified 49 with reproducibly decreased sensitivity to noxious heat. EP(2)2451, among the most insensitive and carrying an insertion on the second chromosome, was chosen for further study. Larvae homozygous for EP(2)2451 had a defective response to noxious heat, some failing to roll even after 10 s (Figure 2D). To reflect this phenotype, we named the mutant *painless*^{1} (*pain*^{1}). Although *painless*^{1} larvae showed a defect in the writhing response, they still showed a normal response to a light touch on the nose (see below). Also, they did not display the highly uncoordinated movement and poor adult viability typical of the touch-insensitive mutants having defects in mechanosensory external sensillae (Kernan et al., 1994).

Nociceptors in vertebrates have been found to be divisible into several classes. Low threshold, polymodal nociceptors respond to noxious heat in the range of 42°C–48°C and to noxious mechanical stimuli, while high-threshold nociceptors respond at even higher temperatures. To test whether the *painless*^{1} mutation blocks all nociception, we examined the response of the mutant larvae over a range of temperatures. The larvae also showed a delayed response to a 48°C stimulus, but 52°C or higher elicited a rapid response, similar to that of normal larvae (Figure 2E). As the response to high temperature is seen even in putative null alleles of *painless* (W.D.T., unpublished data), this result may indicate that moderate and intense levels of noxious heat are processed via separate pathways. In addition, these data imply that the motor system needed for a rapid response is not abolished by mutations in *painless*; the defect is at the sensory level.

We also examined *painless* for the rolling response seen in wild-type with strong mechanical stimuli. To do this, we stimulated the larvae with calibrated Von Frey filaments (0.2 mm diameter). In the wild-type Canton S strain, rolling was not observed when larvae were stimulated with a 10 mN filament (n = 19); instead touch responses described above occurred. When stimulated with a 45 mN fiber, vigorous rolling was observed in 92% of the larvae (n = 36). In contrast, only 13% of *painless*^{1} mutant larvae (n = 31) rolled in response to the 45 mN fiber. Nevertheless, with an even stronger mechanical stimulus (100 mN), a high proportion (81%) of *painless*^{1} mutant larvae (n = 43) responded by rolling.

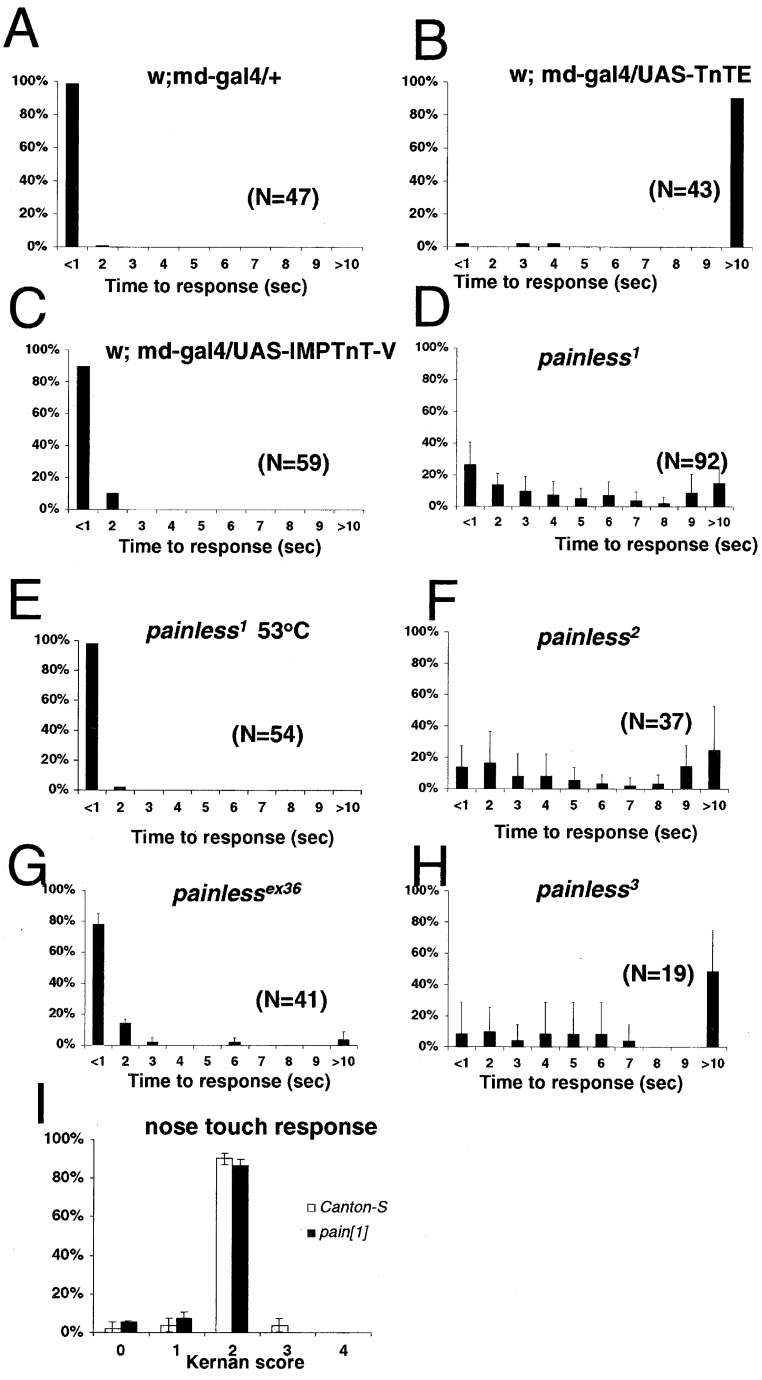

Figure 2. Genetic Alterations to Larval Nociception Behavior

(A–C) Targeted expression of tetanus toxin blocks nociception. (A) Noxious heat response of larvae heterozygous for the GAL4109(2)80 (md-GAL4) driver alone (in a white background). (B) Same driver, activating expression of tetanus toxin, blocked the response. (C) Control for (B); inactive tetanus toxin, with a mutation in the catalytic domain, is ineffective.

(D–I) Behavior of *painless* mutants. Analysis of videotaped behavior was used to generate frequency distributions of the response times of larvae stimulated with a noxious probe heated to 46°C Bars indicate standard error of the mean (SEM). (D) Homozygous *painless*^{1} mutant. (E) The *painless*^{1} mutant larvae do respond to a higher temperature 53°C stimulus, showing that the motor output is intact. (G) Reversion of the *painless* phenotype in precise excision allele *painless*^{ex36}. (F and H) Two independent alleles of *painless* display the same insensitive phenotype as *painless*^{1}. (I) The response to nose touch is unaffected by *pain*^{1} (*pain*^{1}, n = 70; Canton-S, n = 80). For a description of the Kernan score, see Experimental Procedures.

These data demonstrate that the *painless*^{1} mutation results in an increased threshold for both thermal and mechanical nociception.

The *painless*^{1} larvae that did not roll with the 45 mN fiber were not completely insensitive to the stimulus; instead, they responded by pausing their feeding movement, as in the wild-type response to light touch. Although thus defective in responding to strong mechanical stimuli, the response to light touch on the nose was unaffected in *painless*^{1} (Figure 2I). The *painless*^{1} mutation therefore genetically separates mechanical nociception from mechanosensation.

Temperature-Dependent Spiking of Sensory Afferents Is Eliminated in *painless*^{1}

We next tested whether the behavioral defect exhibited by *painless*^{1} mutants could be correlated with a specific defect in the response of abdominal primary afferents. We performed suction-electrode recordings from sectioned abdominal nerves in third instar larvae containing axons of peripheral sensory neurons. All recordings were done blind to the genotype. At room temperature, the mean bulk spiking frequencies of nerves from wild-type and *painless*^{1} larvae were not significantly different (wild-type 8 ± 2 Hz, *pain*^{1} 11 ± 4 Hz) (Figures 3A and

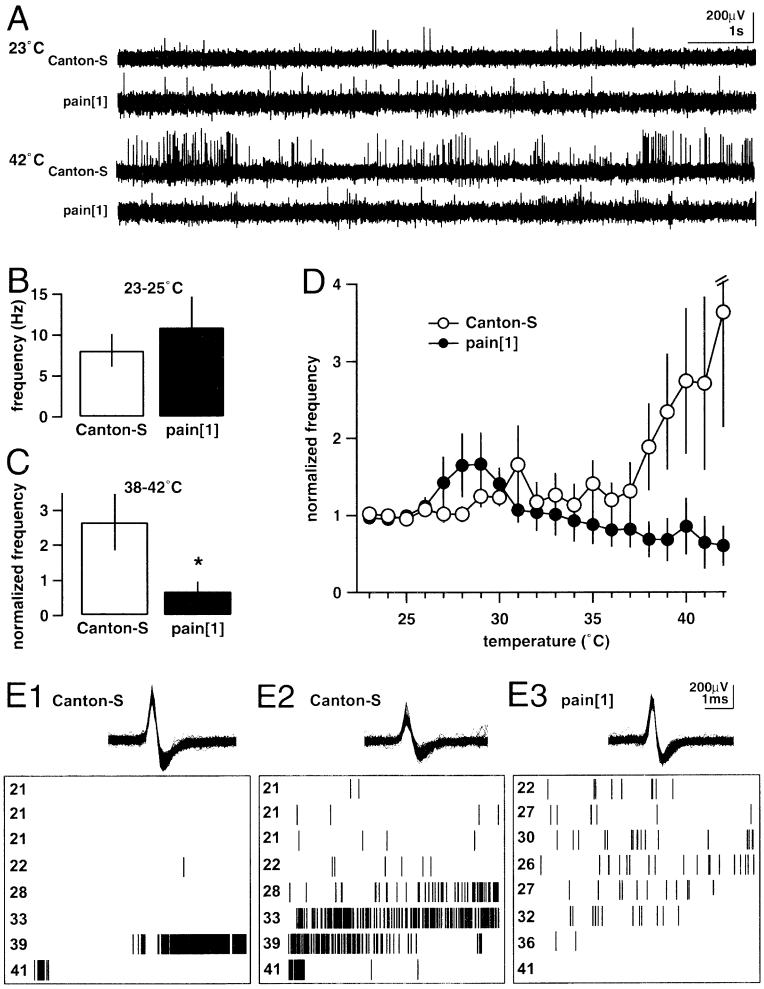

Figure 3. Sensory Nerves in *pain*^{1} Larvae Show Diminished Responses to High Temperatures

(A) Representative raw traces show spikes recorded from abdominal nerves at room temperature and at a noxious temperature (42°C) (n = 6).

(B) Average spike frequency at room temperature was not significantly different in Canton-S and *pain*^{1} nerves ($p > 0.5$, t test) (n = 6).

(C) Average spike frequency at noxious temperatures was significantly greater in wild-type nerves than in *pain*^{1} nerves ($p < 0.05$, t test; normalized to frequency at 23°C–25°C).

(D) Temperature dependence of spiking frequency in Canton-S and *pain*^{1} nerves.

(E) Rasters (E1–E3) represent spikes originating from three individual neurons, two from the same Canton-S nerve and one from a *pain*^{1} nerve. The spike waveforms of these three neurons were sufficiently distinct to permit them to be sorted from spikes generated by other neurons in the nerve. Boxes: each row represents 1 min of data; each block represents eight continuous minutes of recording as temperature is increased. The average bath temperature (in °C) is indicated to the left of each row. Waveforms corresponding to each spike are extracted from the raw traces, aligned at the peak, overlaid, and displayed above the corresponding raster. The depressed firing rate observed at very high temperatures in these two Canton-S neurons (E1 and E2) was common, but not invariable. In other units, firing continued to increase at temperatures higher than 41°C.

3B). Data were continuously recorded from the nerve as the temperature of the saline bathing the larvae was gradually increased. The bulk spiking rate of wild-type nerves increased more than 2-fold at the temperatures that elicited rolling behavior (Figures 3C and 3D). The temperature threshold for the increase in firing rate was near 38°C; frequency at 38°C–42°C was 2.6 ± 0.8 times greater than at room temperature (Figure 3D). By contrast, the firing rate of *painless*^{1} nerves did not increase in the noxious temperature range (Figures 3C and 3D).

Some of these recordings contained a variety of sufficiently distinctive spike waveforms to permit separation of spikes originating from different individual neurons (Figure 3E) (Pouzat et al., 2002). In wild-type nerves, many neurons showed a marked firing rate increase near 38°C–40°C but little spontaneous spiking activity below that temperature, as expected for thermal nociceptors (Figure 3, E1). Other wild-type neurons had a lower temperature threshold (Figure 3, E2). Neurons relatively insensitive to temperature were recorded simultaneously in most wild-type nerves, implying that the thermoresponses of neurons like E1 and E2 are not a nonspecific general property of insect sensory neurons. Importantly, the spiking rate of individual *painless*^{1} neurons almost never increased at elevated temperatures (Figure 3, E3). Thus, Painless is required for the excitatory response of abdominal sensory neurons to noxious heat.

The *painless* Gene Encodes a Transient Receptor Potential Ion Channel

The *EP(2)2451* insertion is located 3.6 kb upstream of the predicted gene CG15860 (Figure 4A). As shown below, alterations to CG15860 indeed resulted from the *painless* mutations. The closest protein relatives of CG15860 are ion channels of the transient receptor potential family. Consistent with this, the predicted protein contains eight ankyrin repeats at its N terminus and a TRP-like ion channel domain near its C terminus (Figure 4B). While Painless (CG15860) is distantly related to the TRPV genes previously implicated in nociception, it represents a distinct member of the TRP ion channel family. The protein of known function most closely related to Painless is NOMP-C. Painless thus belongs in the TRPN class of ion channels (Montell et al., 2002). When the predicted channel region was used for the purpose of comparison, the closest vertebrate homolog found was the human gene ANKTM1 (Jaquemar et al., 1999) (Figure 4C).

To determine whether the insensitivity of *painless*^{1} was due to the presence of the EP element insertion, we crossed *painless*^{1} to a transposase line to mobilize

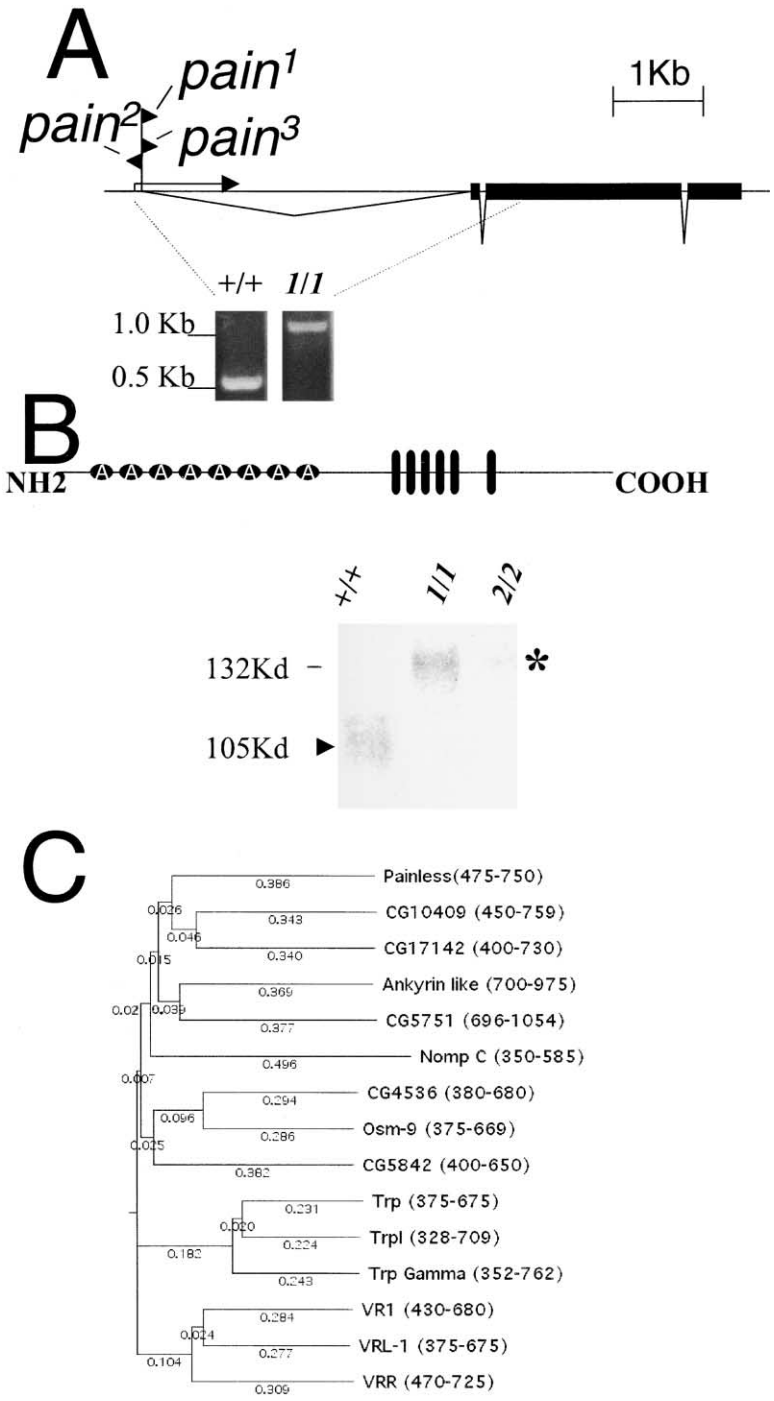

Figure 4. The *painless* Gene

(A) Genomic structure. P element insertions indicated by flags. Inset is RT-PCR against *pain*$^{+}$ and *pain*^{1} larval cDNA. The mutant product was cloned and sequenced (see text).

(B) The predicted Painless protein (914 amino acids), Ankyrin repeats are represented by black ovals, predicted transmembrane segments by bars. Western blots of SDS-PAGE separated larval extracts probed with affinity-purified antisera. GN6620 detects a protein of the correct predicted molecular weight (arrowhead) in wild-type extracts (+/+); in *pain*^{1}(1/1) and *pain*^{2} (2/2) this band is absent, while a higher molecular weight species appears (asterisk).

(C) Dendrogram of TRP-like channel peptide sequences encoding their respective six transmembrane segments; brackets indicate the precise residues used. *Drosophila*: Painless, NompC (Walker et al., 2000), CG10409 (Adams et al., 2000), CG17142 (Adams et al., 2000), CG5751 (Adams et al., 2000), Trp (Montell and Rubin, 1989), Trpl (Phillips et al., 1992), Trp Gamma (Xu et al., 2000), CG4536 (Adams et al., 2000), and CG5842 (Adams et al., 2000). Mammal: Ankyrin like (ANKTM1) (Jaquemar et al., 1999), VRL-1 (Caterina et al., 1999), VR-1(Caterina et al., 1997), and VRR (Strotmann et al., 2000). *C. elegans*: Osm-9 (Colbert et al., 1997). Data analysis was performed using ClustalW identity matrix within the MacVector software package.

the EP element. Of 80 excision alleles obtained, 73 were homozygous viable, and 29 of those were tested as third instar larvae for the *painless* phenotype. Among them, 22 alleles showed reversion of the larval response to noxious heat (Figure 2G). Therefore, the *painless* phenotype can be reverted by excision of the P element.

In addition to EP(2)2451, three other P element insertions have been identified within 16 bp of EP(2)2451 by Berkeley *Drosophila* Genome Project (BDGP) (Figure 4A) (BDGP, 2002). Larvae homozygous for EP(2)2621 (*painless*^{2}) and EP(2)2251 (*painless*^{3}) were found to be strongly insensitive to noxious heat in the nociception paradigm (Figures 2F and 2H). The fourth insertion, EP(2)2462 (*painless*^{4}), showed reduced viability, but those larvae that did survive to third instar displayed the same insensitive phenotype as did the other alleles. All of the alleles failed to complement each other for the nociception defect when tested in *trans*, indicating that the behavioral defects of the lines are due to mutations in the same gene. *pain*^{3}, like *pain*^{1}, was recessive. *pain*^{2} and *pain*^{4} were semidominant and showed mild nociception defects when heterozygous.

Using the reverse transcriptase polymerase chain reaction (RT-PCR) with primers designed across the region, we determined the structure of a cDNA from the *painless* locus, encoding a 105 kDa protein identical to that predicted by BDGP for CG15860 (Painless). Several features of this cDNA were not predicted by BDGP, namely a small, 54 bp noncoding exon 3.6 kb upstream of CG15860, and a 260 bp 3′ untranslated region. All four of the *painless* P element insertions disrupt the 5′ noncoding exon of this transcript (Figure 4A). A Northern blot showed that the 3.0 kb transcript of *painless*^{1} mutant larvae migrated at approximately 3.5 kb. The Northern blot also indicated smaller, approximately 2.0 kb and 1.0 kb transcripts that were unaffected by the mutation.

Using RT-PCR, we found that the 3.0 kb *painless* transcript encoding Painless/CG15860 was indeed altered as a result of splicing of P element derived sequences into the wild-type message. In one mutant transcript, 441 base pairs of EP element sequence are spliced into the second exon of the wild-type transcript (Figure 4A). This mutant stretch of 441 nucleotides contains four start codons (ATGs), each shortly followed by an in-frame stop codon. As upstream small open reading frames greatly reduce the efficiency of translational initiation from those downstream, this transcript is unlikely to produce a Painless protein. Another mutant transcript utilizes the 3′ splice donor site of the noncoding *painless* 5′UTR but contains an upstream in-frame mutant ATG encoded by sequences from within the 3′ end of the P element. This transcript is thus predicted to encode a mutant Painless protein that has 65 additional amino acids at its N terminus. From *pain*^{3}, we isolated a similar mutant transcript, which also contained an upstream, in-frame ATG that was predicted to add 24 amino acids to the N terminus of Painless.

To examine effects of the mutations on the Painless protein, we raised rabbit antisera against peptides from the Painless sequence. The affinity purified anti-serum GN6620, raised against an intracellular loop in the six-transmembrane region (Experimental Procedures), stained sense organs of the peripheral nervous system, which also expressed the *painless* mRNA (see below). The antibody also detected alterations on Western blots of extracts from *painless*^{1} and *painless* ^{2} (Figure 4B).

In wild-type extracts, the serum detected a band of 105 kd, consistent with the predicted molecular weight of Painless. That band was absent in the *painless*^{1} and *painless*^{2} strains. However, an abnormal, higher molecular weight species was detected in both mutants (Figure 4B). The presence of this band was consistent with our finding that an upstream in-frame ATG from the *painless*^{1} mutant transcript was predicted to add 65 amino acids (18 kd) to the Painless N terminus. The data also suggest that a mutant Painless protein is produced in the *painless*^{2} strain.

By P element transformation, we created transgenic flies containing a genomic DNA rescue fragment (P-*painrescue*). This fragment consisted of the *painless* transcription unit, as well as 2.0 kb of upstream genomic DNA, which we hypothesized might contain critical cis-regulatory sequences. The results indicate that sequences sufficient to restore the response to noxious heat were present in the 8.5 kb of rescue DNA (Figures 5A–5C).

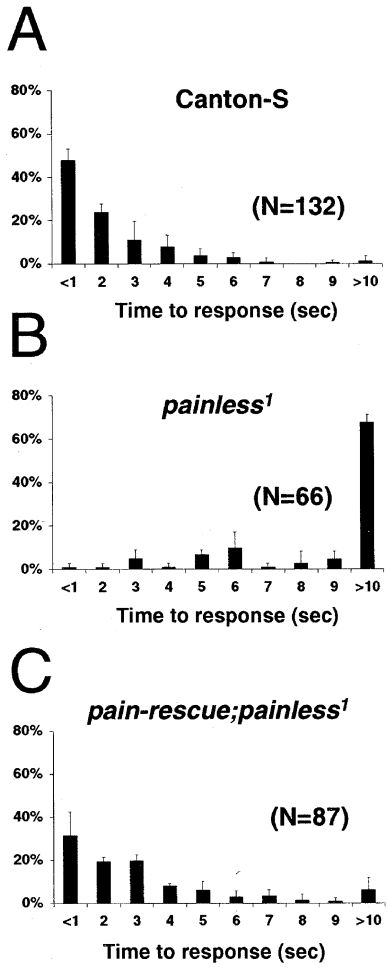

Figure 5. Rescue of the *painless* Mutant Phenotype, Larvae Stimulated with a Noxious Probe Heated to 44°C

(A) Wild-type Canton-S strain. (B) *painless*^{1}. (C) P-*pain-rescue*; *painless*^{1}.

In all panels, bars indicate SEM.

painless Is Expressed in a Subset of Sensory Neurons

To determine the pattern of *painless* mRNA expression, we performed whole-mount in situ hybridization on embryos with anti-sense RNA probes. Beginning at stage 13 of embryonic development, *painless* mRNA was detected in a small number of cells in the central nervous system and in a subset of neurons of the peripheral nervous system (Figure 6A). The cell bodies of these latter neurons were in positions suggesting that they might include sensory precursors of multidendritic (md) neurons, which we confirmed in double labeling experiments (Figurse 6C–6I). At embryonic stage 16, prior to when dendritic process are elaborated, the *painless* mRNA appeared to be distributed in the cytoplasm of the multidendritic neurons in a polarized manner (Figures 6C–6I). At stage 17 of development, when the md neurons first initiate dendritogenesis, the *painless* RNA be-

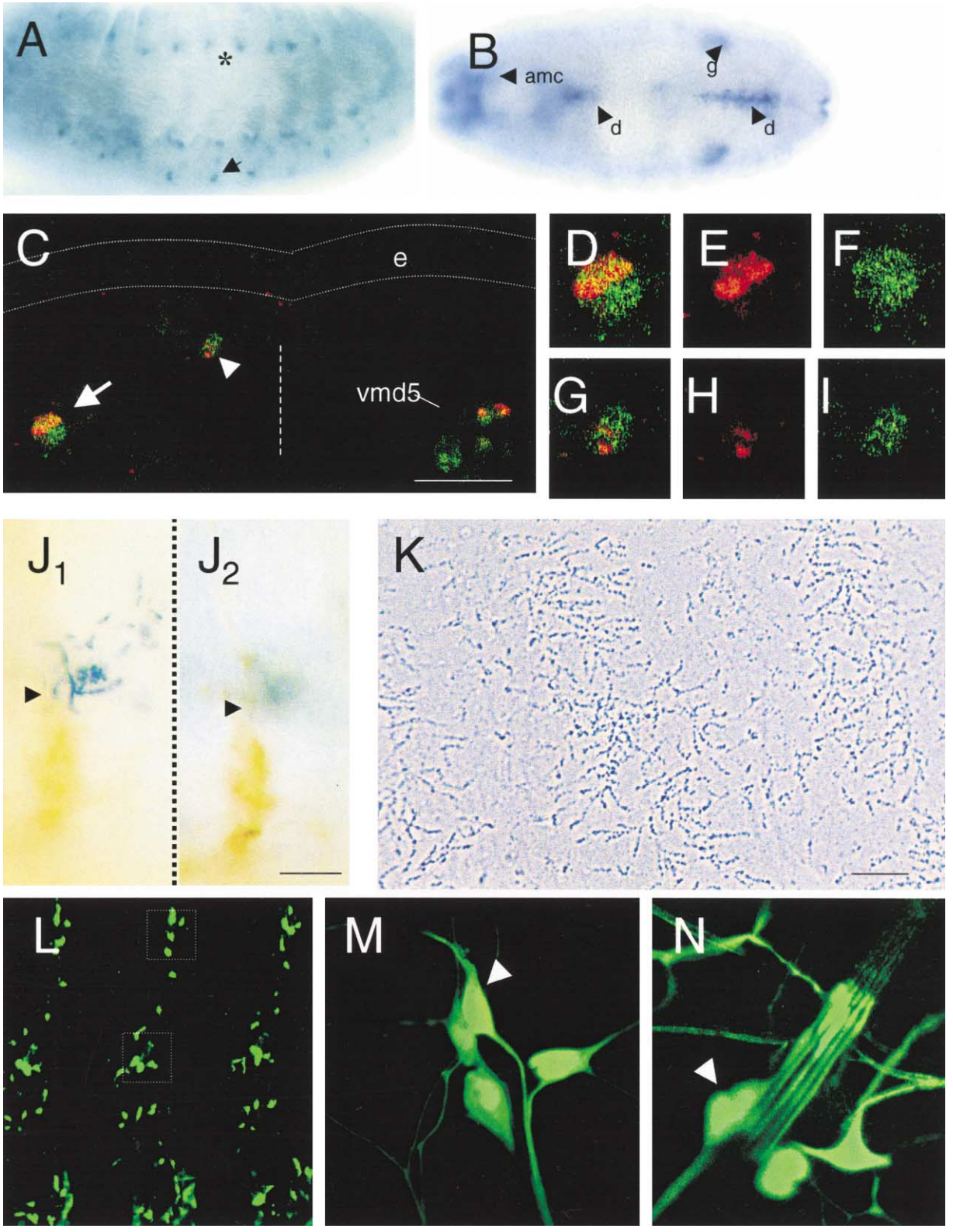

Figure 6. Expression Pattern of *painless*

(A) Expression of *painless* (dark blue) in a stage 14 embryo is seen in a small number of cells the CNS (arrow) and in cells of the peripheral nervous system (asterisk).

(B) Expression in the dorsal vessel (d), antennal maxillary complex (amc), and gonad (g) are also seen.

(C–I) Double labeling of *painless* with an md neuron reporter. (C) In md-GAL4/UAS-mCD8-GFP, *painless* expression (red) is colocalized with the mouse CD8 gene (green) in the cell bodies of md neurons. Confocal optical section (0.9 μm) through two hemisegments of a stage 16

comes localized to branched projections (Figure 6J$_1$) initiating from clusters of multidendritic neurons (Figure 6J$_2$). The structures projecting from the neurons in Figure 6J$_1$ project dorsally, consistent with the previously described initial dendritic projections from the dorsal cluster of multidendritic neurons (Gao et al. 1999). In still older embryos with a more developed dendritic arbor, the pattern of expression evolves into a more elaborate subepidermal plexus of staining (Figure 6K). Combined, these data suggest that the *painless* mRNA is present in md neuron precursors at early stages and becomes localized to their dendrites at later stages. However, further experimentation will be needed to formally demonstrate the latter. In addition, strong expression was also observed in sensory neurons of the antennal maxillary complex (Figure 6B). Weak expression of *painless* mRNA was also observed in chordotonal neurons. Outside of the nervous system, *painless* was expressed in the embryonic gonad and in the dorsal vessel, the insect heart equivalent (Figure 6B).

We also generated a GAL4 enhancer trap allele of *painless* (*pain*-GAL4) by P element replacement (Sepp and Auld, 1999). The *pain*-GAL4 insertion is within the *painless* transcription unit at an identical site to the *pain^1* EP insertion. Consistent with our in situ hybridization results, *pain*-GAL4 driven expression of UAS-GFP showed fluorescence in multidendritic neurons (Figures 6L and 6M), chordotonal neurons (Figures 6L and 6N), a subset of cells in the central nervous system, and a subset of sensory neurons in the antennal-maxillary complex. Unlike for *painless* mRNA, we did not detect expression of UAS-GFP driven by *pain*-GAL4 in the dorsal vessel or in the gonad. Although chordotonal neurons express *painless*, these neurons are not required for nociception since *atonal^1* mutant larvae, which lack most chordotonal organs, show rapid, though uncoordinated, responses in both the thermal and mechanical nociception paradigms (W.D.T., unpublished data). Thus, we hypothesize that the md-da neurons include the primary nociceptors in the larval abdominal segments.

Immunostaining of embryos using the anti-Painless antibody showed strong staining in chordotonal organs (Figure 7A). Consistent with the results of our Western blots, which showed the presence of novel bands rather than a reduction in protein levels in *pain^1*, *pain^1* mutant embryos showed apparently normal staining. Staining in *pain^2* mutants, however, was greatly reduced (Figure 7B), again consistent with the Western analysis. In addition to the staining seen in embryonic chordotonal organs, punctate staining was seen beneath the embryonic epidermis. Given the expression pattern of *pain*-GAL4, we reasoned that this staining might correspond to structures associated with the fine dendrites of the multidendritic neurons. We therefore examined staining of the md neuron arbors in filleted preparations of third instar larvae.

Anti-painless immunoreactivity was tightly associated with the dendritic arbors. The most intense anti-Painless staining was present in bright puncta that were juxtaposed with the dendritic arbor (Figures 7C and 7D), but Painless was not strongly detected throughout the main branches of the arbor (compare Figures 7D and 7E). The anti-Painless staining was highly localized and often clearly seen to be attached to the dendrite (Figure 7F). The highly localized nature of Painless immunoreactivity may indicate a specialized region of the dendrite used for nociceptive signaling.

Discussion

We have developed a sensitive paradigm for studying nociception in *Drosophila* and propose that it can be used as a model to uncover molecules homologous to those important in vertebrate pain pathways. Indeed, in a screen for mutants insensitive to noxious heat, we have identified mutations in a fly gene encoding a molecule distantly related to the human pain-processing vanilloid receptor. However, *painless* does not encode a *Drosophila* ortholog of TRPV1 but instead represents a second member of the TRPN gene family whose founding member, *no mechanoreceptor potential-C* (NOMP-C), is essential for *Drosophila* mechanosensation. Indeed, the closest vertebrate homolog of *painless*, ANKTM1, has recently been shown to be expressed in a subset of pain sensing neurons of the mouse (Story et al., 2003).

We hypothesized that the multidendritic neurons might include nociceptors in *Drosophila*, and we have presented several lines of evidence to support this hypothesis. First, we found that driving tetanus toxin in all of the md neurons using the GAL4109(2)80 driver completely blocked the response to noxious heat as well as the response to noxious mechanical stimuli. In a forward genetic screen to identify mutants defective in

embryo stained with anti-sense *painless* (red) and an antibody to the mouse CD8 gene (green). Arrow indicates a neuron from the ventral (vmd5) cluster of multidendritic neurons (magnified in [D]–[F]), and arrowhead indicates vpda (magnified in [G]–[I]). The epidermis (e) is outlined for reference and the segmental boundary is shown by a dotted line. Due to the polarized nature of *painless* localization, not all md neurons within this optical section show *painless* staining. In other planes, each of the neurons was indeed seen to be positive for *painless*. (D–F) The anterior most neuron of an abdominal vmd5 cluster indicated by the arrow in (C). (G–I) The ventral posterior dendritic arborizing md neuron expresses *painless* mRNA, indicated by the arrowhead in (C).

(J$_1$ and J$_2$) When embryos first initiate dendritic processes, *painless* RNA (blue) is detected in dorsally directed branched structures that project from the dorsal cluster of multidendritic neurons (the latter detected with monoclonal AB22C10 [brown]). In J$_1$, these somata are beneath the focal plane, which is adjusted in J$_2$ so that they can be seen. The arrowhead indicates the ventral most structure stained for *painless*.

(K) Later in stage 17, expression is seen in the subepidermal plexus of md neuron dendrites.

(L–N) Expression of GFP under control of *pain-GAL4*. (L) Three hemisegments of a first instar larva. Boxes indicate clusters of neurons corresponding to those shown in (M) and (N) for third instar. (M) Confocal micrographs of the dorsal cluster of neurons in an abdominal segment of third instar larva shows *pain-GAL4* driven UAS-GFP in the dorsal cluster of md neurons (arrowhead indicates the dorsal most cell, ddaF). (N) Confocal micrograph of *pain-GAL4* driven UAS-GFP in the neurons of the lateral pentascolopidial chordotonal organ; arrowhead indicates a cell body of one of these neurons.

In all panels, dorsal is up and anterior is to the left. Scale bars are 10 μm.

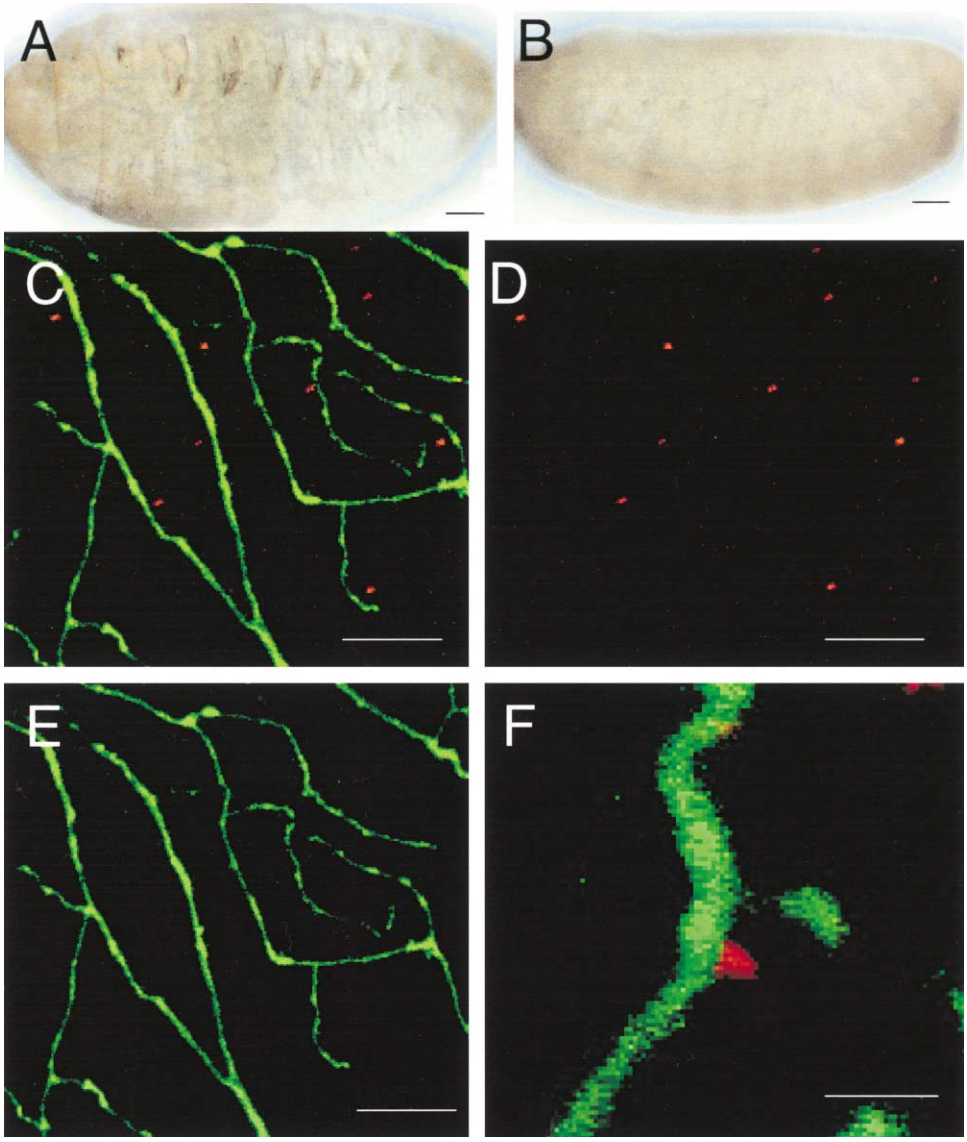

Figure 7. Staining with Anti-Painless Antibody

(A and B) In stage 17 embryos, strong Painless expression is detected in chordotonal organs. (B) Staining of chordotonal organs is greatly reduced in *pain*^{2}.

(C–F) Dendritic field in a lateral abdominal hemisegment in a third instar larva stained with anti-HRP (green) and anti-Painless (red). (C) Low magnification view. (D) Red channel alone. Anti-Painless stains bright puncta, but not the main branches of the dendritic arbor (compare to [E]) (E) Green channel alone. (F) High resolution view.

Scale bars: 20 μm in (A) and (B), 10 μm in (C)–(E), and 2 μm in (F).

sensing noxious stimuli, we identified mutations in the *painless* gene, which we found to be expressed in these same cells. To our knowledge, our data provides the first genetic evidence for the sensory function of the multidendritic neurons. The effects of the *painless* mutations on producing nociception defects were more specific than blocking evoked synaptic vesicle release in the multidendritic neurons with tetanus toxin. In addition to nociception defects, md-GAL4/UAS-TeTLx larvae appeared mildly uncoordinated. They did, however, respond to light touch. The more general effect of tetanus toxin driven by md-GAL4 may indicate other roles for multidendritic neurons, or alternatively, it might be due to the cells in the central nervous system that express this driver.

It will be interesting to determine whether *pickpocket,* a degenerin-like ion channel expressed in three of the fourteen md neurons per hemisegment, is required for mechanical and/or thermal nociception (Adams et al., 1998). However, the *pickpocket* expressing cells may serve a non-nociceptive sensory function. Indeed, it has been proposed that the md neurons be classified into four morphological subtypes, which may relate to as many functional subclasses (Grueber et al., 2002). Our

data suggest that the md neurons, or a subset of them, include nociceptors, but our data do not rule out additional sensory functions for these cells.

In our electrophysiological recordings, we also found evidence for multiple types of heat sensitive units in larval nerves. Some units showed strong activation at temperatures that elicited rolling behavior (Figure 3, E_1). Others showed markedly increased firing at high temperatures but with a temperature threshold below that for rolling (Figure 3, E_2). Not all units in identified in our nerve recordings were activated by heat, indicating that increased firing is not a nonspecific property of insect neurons. As temperature-dependent increases were not seen in *painless*^{1} mutant larvae (Figure 3) and because Painless is expressed in the chordotonal and multidendritic neurons, the heat-sensitive units must be either chordotonal or multidendritic. As *atonal*^{1} larvae showed a rapid response to noxious heat, chordotonal neurons are not required for this sensory modality. Final demonstration that the heat-sensitive units are multidendritic will require an electrophysiological preparation that allows monitoring of individual identified neurons. A recent study using a genetically encoded calcium sensor observed temperature-dependent calcium increases in a *painless*-expressing md neuron (dda-B) at high temperatures (Liu et al., 2003) but relatively small changes in chordotonal neurons.

In addition to the requirement of Painless for sensing high temperature, we have found that *painless* is required for the rolling response to strong mechanical stimuli. The detection of strong mechanical stimuli may also be mediated by the multidendritic neurons, as it was blocked by expression of tetanus toxin in these cells. Furthermore, in larval *Manduca sexta*, mechanical stimuli resulted in spiking from multidendritic neurons that were proposed to be homologous to those of larval *Drosophila* (Grueber et al., 2002).

Anti-Painless staining was highly localized within the dendritic arbor (Figures 7C–7F). In contrast, an ion channel important for intrinsic excitability, the Na^+/K^+ ATPase (which is detected by anti-HRP [Sun and Salvaterra, 1995]), was present throughout the main branches of the dendritic arbor (Figures 7C and 7E) and did not overlap with anti-Painless (Figures 7C–7E). Although nonoverlapping with anti-HRP, anti-Painless staining was indeed dendritic as it directly contacted the anti-HRP stained dendrites (Figure 7F). The highly localized nature of Painless immunoreactivity suggests compartmentalization of these dendrites into domains that contain distinct ion channel populations. The lack of overlap with anti-HRP suggests that Painless and the Na^+/K^+ ATPase occupy mutually exclusive domains of the multidendritic membrane.

We hypothesize that the biophysical mechanism underlying the requirement for Painless in both thermal and mechanical nociception might be explained under the combinatorial model for TRP function (Tobin et al., 2002). The TRPV genes osm-9 and ocr-2 were shown to be mutually required for subcellular localization, suggesting that they might form a complex. It was proposed that combinatorial action of TRPV genes might explain the requirement of a single TRPV in different sensory pathways (Tobin et al., 2002). In the case of Painless, heteromeric ion channel partners may be the closely related TRPN genes CG10409 and CG17142 and/or the *osm-9* related TRPV genes CG5842 and CG4536, all of which are of unknown function (Figure 4C). The need for partners in nociceptive transduction mechanisms is suggested by analogy to *Drosophila* phototransduction, where three closely related TRP genes, TRP, TRPL, and TRPγ, participate (Xu et al., 2000).

Isolation of the *painless* gene provides long-awaited evidence that ion channels in the transient receptor potential family have an ancient heat-sensing function likely present in a common ancestor of vertebrates and insects. Nociception is to pain as phototransduction is to vision. *painless* is the first of the genes from our screen to be analyzed in detail. Others will lead to a genetic dissection of the pathways involved.

Experimental Procedures

Fly Strains

The EP collection was generated by Pernille Rorth; all the available homozygous viable insertions on the second and third chromosomes were examined. Other fly strains were *GAL4109(2)80*, *UAS-TnTE*, *UAS-IMPTnT*, *pain*^{1}(EP(2)2452), *pain*^{2} (EP(2)2621), *pain*^{3} (EP(2)2251), and *pain*^{4}(EP(2)2462). To identify homozygous mutant *pain*^{2,3,4} larvae, these mutations were balanced by T(2:3)*K87 SM6a TM6b Tb* (a gift from Barry Ganetzky), in which the *Tb* mutation conveniently segregates with the second chromosome. To create the *pain*^{1} excision alleles, single *w; EP(2)2451/CyO w+Hop2.1* males were crossed to *w;Sco/CyO* or *w; al dp p th sp/SM6a* and *w-* male progeny were used to establish stocks. Transformants were produced in a *w*^{1118} background by standard techniques, and genetic crosses were used to create a strain that was homozygous for both the X linked insertion of rescue-4 and *pain*^{1}. As a control for *pain*^{1} outcrossing, a *pain*^{1} strain that did not contain the rescue-4 chromosome was also generated and found to retain the *painless* phenotype. *pain-GAL4* was produced with P element replacement of the *painless*^{1} EP element with P{GawB}. Insertion of *pain-GAL4* at the *painless* locus was verified by PCR.

Behavior

To perform the nociception paradigm, six mated female flies were allowed to seed vials for 6 days at room temperature (21°C–23°C). On the seventh day, the females were cleared from the vials, and 1.75 ml of double distilled water was added to soften the food. After a 15 min incubation period, the softened food along with the larvae was gently poured into 35 mm plastic petri dishes. We found that for the behavioral response to be robust, it was necessary for the water in the dish to be shallow enough that the ventral cuticle of the larvae made contact with the petri dish. If the water was too deep, such that the larvae floated, they were reluctant to perform the rolling response and attempted other means of escape. The probe was a soldering iron with a copper tip that was filed to a chisel shape 0.6 mm wide. A variac was used to adjust the voltage, being calibrated with a fine thermocouple. Larvae were touched laterally in abdominal segments four through six. To measure the response times, the behavioral responses were videotaped and later analyzed.

For nose touch experiments, the larvae were touched with an eyebrow hair affixed to the tip of a dissecting needle. The scoring system of Kernan et al. (1994) is as follows: 0 = no response to touch, 1 = a response of pausing mouth-hook movement, 2 = responding by withdrawing the anterior or turning away from the touch, 3 = a single reverse peristaltic wave away from the touch, and 4 = multiple peristaltic waves away from the touch (Kernan et al., 1994).

Electrophysiology

Larvae were fixed ventral side down on a glass cover slip using cyanoacrylate glue (Duro "Super Glue") and bathed with saline con-

taining 120 mM NaCl, 3 mM KCl, 5 mM TES, 10 mM $NaHCO_3$, 10 mM glucose, 10 mM trehalose, 10 mM sucrose, 1.5 mM $CaCl_2$, and 4 mM $MgCl_2$. The saline, bubbled with O_2, was perfused over the preparation during recording (2.0 ml/min). An incision was made in the dorsal surface above the ventral abdominal ganglion, the gut and fat bodies were removed, and the abdominal nerves were cut near the ganglion. While visualizing the preparation with a 40× water immersion objective, the distal end of a nerve corresponding to one of the segments, A3–A7, was pulled into a glass suction electrode (tip diameter approximately 5 μm) filled with saline. Only nerves which generated spontaneous spikes at room temperature were accepted for recording. Data were acquired at 10 kHz with an Axopatch 1-D amplifier and bandpass filtered at 100 Hz–2 kHz. The temperature of the saline was controlled using a TC-324B temperature controller with a SH-27B in-line solution heater (Warner Instrument Corp.), with the thermistor output continuously acquired. After a stable recording at room temperature was achieved, the heater was turned on until 43°C was reached, generally within 6–7 min. Spikes were detected off-line. Raw traces were divided into 1 s windows, and average temperature and spike frequency were calculated within each window. The data was then binned by temperature in 1°C increments and averaged within bins within each experiment. The plots of temperature versus spike frequency were then averaged across experiments to obtain Figure 3D. Spike detection and sorting was performed using custom software written in Igor Pro. It was not possible to assign all spikes to a well-separated cluster. The units illustrated in Figure 3E represent separated clusters, but the spike waveforms were more variable than the background noise (Pouzat et al., 2002). Thus, we cannot exclude the possibility that each block in E1–E3 contains spikes originating from two or more neurons with similar spike waveforms.

Molecular Biology

To produce larval extracts for protein and RNA analyses, adult flies were allowed to lay eggs on yeasted apple juice plates for 24 hr at 25°C. The plates were then aged for a further 24 hr. The mixed first and second instar larvae were collected in egg baskets and washed extensively. For RNA, the larvae were ground in Trizol reagent, and RNA was obtained using the instructions of the manufacturer (Invitrogen). For protein extracts, larvae were ground in phosphate-buffered saline, 0.1% Tween 20 containing protease inhibitors (Complete, Roche). Insoluble material was removed by centrifugation on a tabletop centrifuge for 10 min at 13,000 rpm. The supernatant was then boiled for 5 min in 1× sample buffer followed by SDS-PAGE on 5.0% ready-gels (BioRad) in Laemmle buffer. Proteins were transferred to 0.45 μm PROTRAN nitrocellulose membranes (Shchleicher + Schuell) by electrophoresis in Towbin's buffer (25 mM Tris, 192 mM Glycine, 20% MeOH [pH 8.3]). The blots were probed with affinity-purified GN6620 at 1:1000 dilution.

The primers used for Figure 2B were the forward primer 5′-TGCGGTCGCTTTCACGGATC-3′ and reverse primer 5′CACATGGT GCAAGGGTGTGAACTCGC-3′. 8.5 kb of genomic rescue DNA was amplified with high-fidelity PCR from BACR08I14 using the forward primer 5′-TATACACATTTCGCCAACTGCGCCGT-3′ and the reverse primer 5′AACATTTGAAATTAAATATTTACTCGGG-3′. The resulting PCR product was cloned into TOPO-pCR-XL3.1 (Invitrogen) to give pRescue-45. The coding region of *painless* in pRescue-45 was sequenced to be sure that no mutations were introduced during cloning. The pRescue-45 vector was linearized with Not1, and ligated into the Not1 digested P element transformation vector P-INDY-4 (gift of L. Seroude). The GN6620 antiserum was prepared in rabbits against the peptide antigen NH2-CPPLGKKEGKDEEQ-COOH conjugated to KLH, and affinity purified against columns containing the immobilized peptide antigen (Sigma-Genosys). Anti-sense RNA probes were generated from the 3′ end of Spe1 digested partial cDNA clone 1742-2478R (W.D.T., unpublished data).

Histochemistry

Samples were probed with antisense *painless* digoxigenin-labeled RNA by standard techniques. Detection was with anti-dig-AP and NBT/BCIP in Figures 6A, 6B, and 6J. For dendritic staining, embryos were permeabilized by sonication and proteinase K treatment, and extensive washing was performed. The 22C10 antibody in Figure 6J was detected with anti-mouse Elite ABC (Vector Labs). For fluorescent detection, sheep anti-digoxigenin was detected with donkey anti-sheep AlexaFluor 555 (Molecular Probes). Whole-mount immunohistochemistry on embryos was performed according to standard techniques. Larval preparations were as follows: GAL4109(2)80GFP/UAS-mCD8GFP larvae were filleted by opening on the ventral side in 0.2 M phosphate buffer (0.2 M Na_2HPO_4 [pH 7.4]), followed by fixation for 3 hr in 4% paraformaldehyde, 12.5 mM EGTA, 1X PBS (135 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 [pH 7.4]). The larvae were then permeablized by incubation for 1 hr in 0.2M phosphate buffer (0.5% NP-40). Antibody incubations were in 10% goat serum, and dilutions were: affinity-purified GN6620 1:1000, anti-Rabbit-CY3 (1:1000), goat anti-HRP-FITC (Cappel) 1:1000, and rat-anti-mCD8-FITC 1:1000. The larvae were incubated with the primary antibody for 1 hr, rinsed three times, and washed three times for 20 min with gentle shaking in 0.2 M phosphate buffer. Secondary antibodies were diluted 1:1000 in 10% goat or heat inactivated horse serum, incubated with the samples overnight, and the samples washed in the same fashion as for the primary antibodies.

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Accession Numbers

The GenBank accession number for the *painless* cDNA and the Painless protein sequence is AY268106.