A CONSERVED SIGNALING NETWORK FOR NOCICEPTION AND SOCIAL BEHAVIORS IN *DROSOPHILA*

by

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(Under the Direction of Ping Shen)

ABSTRACT

Nociception is critical for protecting animals from tissue damage. It is also involved in many biological and pathological processes. However, the underlying molecular and cellular mechanisms are underexplored. *Drosophila* larvae display food-averse behaviors at the end of third-instar stage, providing a unique opportunity to delineate the neurobiological basis of age-restricted response to aversive / stressful environmental stimuli. Here we provided evidences for a fructose-responsive chemosensory pathway that modulates food-averse migratory and social behaviors. *painless* (*pain*), a transient receptor potential (TRP) channel that is responsive to noxious stimuli, is required for the fructose response. A subset of *pain*-expressing sensory neurons have been identified that show PAIN-dependent excitation by fructose.

We also found that the developmental switch from food attraction to aversion is regulated by Neuropeptide F, a neuropeptide Y (NPY)-related signaling peptide. NPF, like mammalian opioids and endocannabinoids, suppresses peripheral noxious stimulation through its G-protein coupled receptor NPFR1. NPF / NPFR1 signaling negatively modulates different subtypes of fly and mammalian TRP-family ion channels expressed in larval sensory neurons. In human cells, NPFR1 attenuates TRPV1-mediated Ca^{2+} influx and its enhancement by cAMP analogs.

Similarly, the NPF / NPFR1 pathway also blocks sensitization of larval aversive response by cAMP-dependent protein kinase (PKA).

Drosophila post-feeding larvae also display food-averse social burrowing to through food proper to migrate towards food-free pupation sites. We show that the social burrowing is comprised of three genetically separable behaviors, seeking, clumping and burrowing. The PKA activities in a subset of *pain*-expressing neurons are essential for the onset of seeking, clumping and burrowing behaviors. Meanwhile, PKA in *atonal*-expressing neurons and a subset of Va neurons differentially regulate clumping but not seeking and burrowing activity.

Taken together, we provided the first molecular and genetic evidences for a complex neuronal network for developmental regulation of sugar-averse behaviors in post-feeding *Drosophila* larvae, comprised of a conserved nociceptive module containing TRPA channel PAIN, and a temporal control module containing conserved NPY family peptide NPF. In the long term, our *Drosophila* larva model will not only serve as an excellent model for studying molecular and cellular mechanisms of nociception, but also help in identifying novel nociceptive genes and gaining insights into pain mechanisms of higher organisms.

INDEX WORDS: *Drosophila melanogaster*, Transient receptor potential channels, Neuropeptide Y, Neuropeptide F, NPF receptor, PAINLESS, Nociception, Aversive stimuli, Food-averse behaviors, Social Behaviors

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW 1

OVERVIEW OF PAIN AND NOCICEPTION 1

THE TRANSIENT RECEPTOR POTENTIAL (TRP) CHANNEL FAMILY 3

THE NEUROPEPTIDE Y-LIKE SIGNALING SYSTEM 10

OBJECTIVES 16

2 *DROSOPHILA* TRPA CHANNEL MODULATES SUGAR-STIMULATED NEURAL EXCITATION, AVOIDANCE AND SOCIAL RESPONSE 23

ABSTRACT 24

INTRODUCTION 24

RESULTS 27

DISCUSSION 33

METHODS 35

ACKNOWLEDGEMENT 40

3 *DROSOPHILA* NEUROPEPTIDE Y-LIKE PATHWAY DEFINES A CONSERVED ANTINOCICEPTIVE MECHANISM THA SUPPRESSES TRP CHANNEL-MEDIATED STRESSFUL STIMULATION 63
ABSTRACT 64
INTRODUCTION 64
RESULTS 67
DISCUSSION 72
METHODS 75
ACKNOWLEDGEMENT 79
4 GENETIC DISSECTION OF MOLECULAR AND NEURONAL PATHWAYS MEDIATING STRESS-INDUCED SOCIAL BEHAVIORS IN *DROSOPHILA* LARVAE 94
ABSTRACT 95
INTRODUCTION 95
RESULTS 97
DISCUSSION 102
METHODS 104
ACKNOWLEDGEMENT 106
5 CONCLUSIONS AND DISCUSSION 119
REFERENCES 124

LIST OF TABLES

Page

Table 2.1: Summary of the calcium imaging data 62

LIST OF FIGURES

Page

Figure 1.1: The structural features of TRP superfamily 19

Figure 1.2: Figure 1.2 Sequence comparisons of *Drosophila* NPF and human NPY family peptides 21

Figure 2.1: Behavioral procedures for larval response to aversive food chemicals 41

Figure 2.2: *pain* is involved in larval aversion to fruit juice and fructose 43

Figure 2.3: Conditional disruption of *pain*-expressing neuronal signaling attenuates larval food aversion 45

Figure 2.4: Larvae expressing a mammalian vanilloid receptor display capsaicin-averse behaviors 47

Figure 2.5: The ventral PAIN neurons of the three thoracic segments (T1 to T3) project to the thoracic ganglia and denticle belts 49

Figure 2.6: Imaging and SOARS analysis of excitation of thoracic PAIN neurons by fructose with the cameleon Ca^{2+} indicator 51

Figure 2.7: The responses of larval sensory neurons to sugar stimulation 54

Figure 2.8: Ablation of fructose-responsive PAIN neurons in the thoracic segments disrupts larval food aversion 56

Figure 2.9: Localization of NPFR1-positive PAIN neurons 58

Figure 2.10: The affinity purified anti-NPFR1 antibody selectively stains cells near Keilin's organs in larval ventral epidermis 60

Figure 3.1: Suppression of PAIN neuron-mediated food-averse migration by the NPF/NPFR1 pathway 80
Figure 3.2: Regulation of sugar-stimulated social response by increased or decreased NPFR1 signaling 82
Figure 3.3: Imaging and SOARS analysis of excitation of thoracic PAIN neurons by fructose with the cameleon Ca^{2+} indicator 84
Figure 3.4: NPFR1 suppresses PAINLESS-mediated thermal nociception in larvae and chemical nociception in adults 86
Figure 3.5: NPFR1 suppresses larval avoidance to capsaicin induced by ectopically expressed mammalian TRPV1 88
Figure 3.6: NPFR1 suppresses Ca^{2+} influx mediated by rat TRPV1 in human cells 90
Figure 3.7: Effects of cyclic nucleotides, PKA and NPFR1 on TRP channel activities 92
Figure 4.1: Characterization of cooperative burrowing activity in post-feeding larvae 107
Figure 4.2: PKA activity in *pain*-expressing neurons regulates clumping activity in post-feeding larvae 109
Figure 4.3: PKA activity in *ato*-expressing neurons mediates larval clumping activity 111
Figure 4.4: A genetic mutation disrupts larval clumping but not seeking and burrowing activities 113
Figure 4.5: The insertion site of the UAS-*PKAc* transgene 115
Figure 4.6: The pattern of Leaky PKAc expression 117

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

OVERVIEW OF PAIN AND NOCICEPTION

As defined by the International Association for the Study of Pain (IASP), pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Merskey, 1994). It is comprised of a physiological sensation in a part or parts of the body, and a psychological interpretation by the brain. To be considered as pain, both the physiological and psychological components must be present. For instance, activities induced in the nociceptive sensory neurons by a noxious stimulus are not always pain, which must be a psychological state. Unpleasant abnormal experiences (dysesthesias) are not necessarily pain since, subjectively, they may not have the usual sensory qualities of pain (Merskey, 1994).

As far as the primary mechanism of production is considered, pain can be grouped into two main categories, nociceptive pain and neuropathic pain. Nociceptive pain is caused by stimulation of nociceptive neurons due to chemical, thermal, or mechanical events that have the potential to cause tissue damage. Neuropathic pain is caused by damage to the nervous system itself, due to disease or trauma (Treede et al., 2008). A third kind of pain is sometimes mentioned as psychogenic pain, which is the physical pain that is caused, increased, or prolonged by mental, emotional, or behavioral factors (Merskey et al., 1967). In this review, I will focus on nociceptive pain.

Nociceptive pain starts in the peripheral nervous system (PNS), where the sensory fibers fire in response to various noxious stimuli. In humans there are four main types of sensory fibers in the PNS, Aα-fibers, Aβ-fibers, Aδ-fibers and C-fibers. Each has different properties allowing them to respond to and transmit different types of sensory information. The large-diameter and highly myelinated Aβ-fibers conduct action potentials at velocity. These fibers have low activation thresholds and normally respond to light touch and are responsible for conveying tactile information. The small-diameter and thinly myelinated Aδ-fibers are slower-conducting than Aβ-fibers, and they also possess higher activation thresholds. They respond to both thermal and mechanical stimuli. C-fibers are the smallest type of primary afferents and are unmyelinated, thus making them the slowest conducting. They have the highest thresholds for activation and therefore detect selectively noxious stimuli.

The term 'nociceptor' usually refers to the free endings of Aδ- and C-fibers. They respond to an array of noxious stimuli in various natures, including noxious mechanical, chemical or thermal stimuli. They also fire action potential in response to molecules released by injured tissue, e.g., substance P, prostaglandin, serotonin, etc. Nociceptors are distinguished by their relatively high thresholds for activation. They can be activated by intense stimuli that are damaging or potentially damaging to the tissues, but not by innocuous stimuli, such as warming or touch (Djouhri and Lawson, 2004). Many nociceptive neurons respond to multiple stimulus modalities, hence the term 'polymodal'.

Nociceptors compose the majority of dorsal root ganglia (DRG) neurons in the spinal cord and the majority of trigeminal ganglia neurons. The cell bodies of these neurons are located close to central nervous system (CNS) structures and send long

processes that terminate throughout peripheral tissues, including the skin, muscle, and viscera (Patapoutian, 2004). The central terminals of primary afferent fibers terminate in the dorsal horn of the spinal cord, and the output from the dorsal horn to the brain is carried by spinal projection neurons along ascending pathways to CNS. The pathways and mechanisms of processing and modulating the signals in the brain are reviewed elsewhere (D'Mello and Dickenson, 2008).

The nociceptive system is an indispensable part of the animal sensory system. The unpleasant signals from the nociceptive system serve as early warnings, prompting the animal to avoid potential tissue damage (Melzack and Wall, 1965). The nociceptive system is also involved in various pathological processes including inflammation and cancer. For instance, mice with tumors in the hind paw induced by local injection of a carcinoma display significant reduction in withdrawal thresholds in response to mechanical stimulation (Guerrero et al., 2008). Recent study showed that the mice with defects in sensing environmental irritants have reduced respiratory depression and airway inflammation, indicating that the nociceptive system is important for maintaining inflammation state (Bessac and Jordt, 2008). Further understanding of nociception, especially its molecular and cellular mechanisms, is of wide interest.

THE TRANSIENT RECEPTOR POTENTIAL (TRP) CHANNEL FAMILY

Great advances have been made in identifying the molecular identities of nociceptors. The conserved TRP channels have emerged as important molecules for nociception. The TRP channels are a large family of ion channels distinguished by their common structure and permeability. The first TRP channel was identified in *Drosophila,*

in which it was required for visual transduction. The fly *trp* mutants respond to continuous light with transient rather than sustained potential in electroretinogram (Montell and Rubin, 1989), hence the name *transient receptor potential*. The TRP channels have six membrane-spanning domains with a pore domain between the fifth and sixth transmembrane domains (TM) (Figure 1.1), and resemble voltage-gated K^+ channels (K_v), cyclic-nucleotide-gated channels (HCN and CNG) and the Polycystic Kidney Disease Protein (Clapham et al., 2001). Similar to other 6-TM channels, they probably form homo- or heterotetramers (Ramsey et al., 2006). All TRP channels are cation-permeable and most of them are non-selective, with exceptions of TRPM4 / 5 (Ca^{2+} impermeable) and TRPV5 / 6 (highly Ca^{2+} permeable) (Flockerzi, 2007; Montell et al., 2002).

TRP channels are among the largest family of ion channels known, with representative members in many species. Based on the amino acid sequence homology, TRP channels may be grouped into 7 subfamilies: the classic TRPs (TRPCs); the melastatin TRPs (TRPMs); the vanilloid receptor TRPs (TRPVs); the ankyrin transmembrane protein (TRPAs); the polycystins (TRPPs), the mucolipins (TRPMLs) and TRPNs (Montell et al., 2002). The structure and molecular features of different families of TRP channels are shown in Figure 1.1 (Venkatachalam and Montell, 2007). Within one subfamily the amino acid sequence similarity can reach up to 90%, but the similarities between different subfamilies are low. As a result, only members from one subfamily may account for heteromeric TRPs *in vivo* (Flockerzi, 2007).

Activation modes of TRP channels

TRP channels can be activated by a variety of stimuli. There are three general categories of modes of activation: ligand activation, receptor activation and direct activation. Ligands that can activate TRP channels include inorganic ions (Ca^{2+}, Mg^{2+}), small organic molecules (capsaicin, icilin), lipids or product of lipid metabolism (phosphoinositides, diacylglycerols, anandamide) and purine nucleotides and their metabolites (adenosine diphosphoribose, βNAD+) (Montell, 2005b; Ramsey et al., 2006). Receptors that can activate TRP channels include G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) involved in phospholipase C (PLC) pathway (Montell, 2005b; Venkatachalam and Montell, 2007). Direct activation of TRP channels can be achieved by temperature changes or mechanical stimulations (Caterina et al., 1997; Christensen and Corey, 2007). Many TRP channels are polymodal. The classic example is the TRPV1, which can be activated by capsaicin, protons, and high temperature (Caterina and Julius, 2001). This suggests that the panel of stimuli that can activate a given TRP channel is dependent on the cellular context (Ramsey et al., 2006).

Sensory functions of mammalian TRP channels

Over the past two decades, numerous studies have established that TRP channels can function as cellular sensors. TRP channels have been reported to be involved in sensing various stimuli from outside as well as inside the cell. Among the seven subfamilies, the TRPV, TRPM and TRPA families are especially important for sensing exogenous stimuli.

Four out of six members of the TRPV subfamily (TRPV1-V4) respond to changing temperature (thermal TRPs). The well-characterized mammalian vanilloid receptor TRPV1 has been shown to respond to noxious heat, protons and capsaicin, as well as endocannabinoid lipids such as 2APB and anandamide (Caterina and Julius, 2001; Caterina et al., 1997). In mammals, the TRPV1 is expressed in dorsal root ganglion (DRG), trigeminal ganglion, suggesting a likely role in pain pathway. However it is also expressed in many other tissues such as kidney, pancreas, spleen, etc., indicating other physiological functions beyond thermal sensation. The TRPV1 knockout mice show reduced sensitivity in nociception assays (Caterina et al., 2000). TRPV2 channels have been reported to mediate high-threshold heat sensation in cultured Human Embryonic Kidney (HEK) cells (Leffler et al., 2007). Interestingly, in Chinese Hamster Ovary (CHO) cells and smooth muscle cells, TRPV2 appears to be stretch-activated (Muraki et al., 2003). TRPV3 responds to non-noxious heat, 2-APB and camphor (Chung et al., 2004; Hu et al., 2004; Xu et al., 2002). It is widely expressed in humans, but only exists in the skin of mice (Ramsey et al., 2006). Cultured mouse keratinocytes expressing TRPV3 show heat-induced currents and $TRPV3^{-/-}$ mice show different thermal preference than wild type (Moqrich et al., 2005). TRPV4 is activated by moderate heat and also by hypotonic challenge, and $TRPV4^{-/-}$ mice show altered thermal preference, reduced pressure and osmotic sensitivity as well as hearing loss (Ramsey et al., 2006).

The TRPM subfamily is genetically and functionally diverse. Among all eight members, the TRPM2, M5 and M8 have been implicated in sensory functions. The non-selective TRPM2 is responsive to oxidative stress (reactive oxygen species H_2O_2) in immune cells, suggesting that TRPM2 functions as a cellular redox sensor (Perraud et al.,

2005). The TRPM5 is involved in gustatory sensation (Sweet, Bitter, Umami) via T1R, T2R and PLCβ (Zhang et al., 2003). The TRPM8 is considered as a thermal TRP. It is activated by low temperature and menthol in cultured CHO cells (Peier et al., 2002). TRPM8 is expressed in sensory neurons, in which it may function as a cold sensor. $TRPM8^{-/-}$ mice exhibit strikingly reduced avoidance of cold temperatures. They also lack behavioral response to the application of cold-inducing icilin and display an attenuated response to acetone (Dhaka et al., 2007).

The TRPA1 is the sole member of mammalian TRPA family. Its characteristic feature is the 14 ankyrin repeats located at the N terminus. It is a cold-sensitive and ligand-activated non-selective channel (Story et al., 2003). The ligands that activate TRPA1 are mostly pungent compounds, including allyl isothionate (ingredient of mustard oil) and allicin (ingredient of garlic) (Bandell et al., 2004; Jordt et al., 2004; Macpherson et al., 2005). It is also shown that TRPA1 is responsive to environmental irritants such as chorine, and tear gas. Together with TRPV1, it is important for maintaining the inflammation state in various pathological conditions including asthma and COPD (Bessac and Jordt, 2008). These evidences all point to the nociception function of TRPA1. In addition, it has been implicated in mechanosensory functions in worms (Kindt et al., 2007). Interestingly $TRPA1^{-/-}$ mice do not show any hear defect (Bautista et al., 2006; Kwan et al., 2006).

Drosophila TRP channels

The *Drosophila* genome contains 13 TRP channel family members. Similar to mammalian TRPs, the fly TRPs can be grouped into 7 subfamilies based on amino acid

sequence homology: TRPC, TRPM, TRPN, TRPV, TRPA, TRPP and TRPML (Montell, 2005a). The range of functions of fly TRPs are strikingly similar to mammalian counterparts, although the functions of members from corresponding families across the species are not exactly the same. To date, 11 out of the 13 *Drosophila* TRPs have been characterized in various depths. Most *Drosophila* TRP channels are involved in sensory functions.

The founding members of the TRP family, fly TRPC channels, are responsible for photo transduction (Montell, 2005c; Montell and Rubin, 1989). Flies with mutation in the *trp* gene show transient response to continuous light stimulation. Further study showed that in the *trp* mutants, the light and Ca^{2+} dependent movement of pigment granule is transient, and the light-induced Ca^{2+} influx is drastically reduced (Hardie and Minke, 1992; Lo and Pak, 1981). Later it was demonstrated in transfected insect Sf9 cells and HEK cells that the *trp* gene encodes Ca^{2+} permeable channel (Vaca et al., 1994; Xu et al., 1997). However the TRP is not considered as a light-sensing channel. The light response in fly photoreceptor cells is dependent on phospholipase C (PLC) (Bloomquist et al., 1988). Recently a diacylglycerol lipase (INAE), acting downstream of PLC, is identified to be an essential molecule for opening of TRP (Leung et al., 2008).

Unlike mammalian TRPV channels, which are mostly thermal TRPs, the *Drosophila* TRPV channels are responsible for hearing. The two genes of this family, *nanchung* (*nan*) and *inactive* (*iav*), are highly expressed in chordotonal organs located on the antenna (Johnston's Organ), which are responsible for hearing. The *nan* and *iav* mutants have no response to auditory stimulations (Kim et al., 2003). In transfected CHO

cells, both of them can be activated by hypoosmolarity (Gong et al., 2004). However, whether they can be activated directly by mechanical stimulation *in vivo* is not clear.

The *Drosophila* TRPN family currently has one member, the No Mechanoreceptor Potential C (NOMPC). It is expressed in mechanosensory organs and *nompC* mutants showed abolished mechanosensory functions and uncoordination (Walker et al., 2000). It has a distinct 29 ankyrin repeats located at the N-terminus and it is proposed that the motif constitutes a spring gating mechanism to control channel opening (Howard and Bechstedt, 2004).

The *Drosophila* TRPA family has four members, dTRPA1 (also ANKTM1), dTRPA2 (PYREXIA), dTRPA3 (WATERWITCH) and PAINLESS (PAIN). This family has the most diverse functions, ranging from thermal and chemical sensation to moisture sensation. The dTRPA1 is activated by warm heat (>24 °C) when expressed in CHO cells (Viswanath et al., 2003). Consistently the dTRPA1 mutant fly display defects in thermotaxis (Rosenzweig et al., 2005). The dTRPA2 is activated by noxious heat (>40 °C) when expressed in Xenopus oocytes and HEK cells. The majority of *dTrpA2* mutant flies can be paralyzed within 3 min when exposed to 40 °C, indicating the dTRPA2 may protect flies from heat stress (Lee et al., 2005). The dTRPA3 is functionally required to detect moist air by the fly antenna, while the mechanism is not yet clear (Liu et al., 2007). The fourth member, PAIN, responds to noxious heat and mechanical stimuli. The *pain* mutants were identified in a behavioral screen, in which the larvae show significant longer delay to respond to noxious heat (42 °C). The mutants also showed significant lower spiking rate by sectioned abdominal nerve when bathing in higher temperature buffer (Tracey et al., 2003). It was also shown that PAIN is also involved in sensing

benzyl-isothiocyanate (BITC). The wild type flies reject BITC-containing medium while the *pain* mutants show no preference to either BITC-containing or BITC-free medium (Al-Anzi et al., 2006).

THE NEUROPEPTIDE Y-LIKE SIGNALING SYSTEM

In the past two decades, many studies have associated Neuropeptide Y (NPY), a 36-amino-acid neuromodulator, with pain modulation. For instance, NPY blocks nociceptive signals to the brain and induces a calming effect in laboratory animals exposed to stressful situations (Brumovsky et al., 2007; Flood and Morley, 1991; Heilig, 2004). NPY is substantially upregulated in dorsal root ganglia after peripheral nerve injury (Zhang et al., 1993). In rats, intrathecal administration of NPY raise threshold of the paw-withdraw in hot plate assays (Hua et al., 1991). All these evidences indicate that NPY is a prominent regulator of nociception.

NPY and NPY receptors in mammals

NPY was first discovered in the porcine brain. It is widely distributed throughout the central and peripheral nervous system (Dumont et al., 1992). NPY bears structural similarities to the peptide YY (PYY) and pancreatic polypeptide (PP) (Tatemoto et al., 1982). These three molecules belong to a large family characterized by a polyproline helix folded onto an α-helix with a C-terminal tyrosine (Silva et al., 2002). NPY is one of the most abundant neuropeptides in the mammalian CNS (Williams et al., 2001). It is mainly synthesized in the neurons localized in the hypothalamus, particularly paraventricuar nucleus (PVN), arcuate nucleus (ARC), superachiasmatic nucleus (SCN),

and dorsomedial nucleus (VMH) (Silva et al., 2002). In addition to its central localization, NPY is expressed in many peripheral tissues, including adrenal medulla, liver, heart, and endothelial cells of blood vessels. NPY is highly expressed in the sympathetic nervous system. It can also be detected in a subpopulation of parasympathetic neurons (Silva et al., 2002).

NPY interacts with a family of G-protein coupled receptors belonging to the rhodopsin like superfamily (class 1) of receptors (Larhammar et al., 2001). Five NPY receptors have been cloned from mammals (Y1, Y2, Y4, Y5, and y6). In addition, the Y3 have been postulated based on pharmacological profiles using various tissue preparations (Berglund et al., 2003). The y6 receptor is truncated in most mammals including humans, hence the lower case designation. The NPY receptors share the common character of seven transmembrane domains. Their distribution and physiological functions, however, vary considerably based on many anatomical, pharmacological and behavioral studies (Thorsell and Heilig, 2002). All of them have been cloned and are encoded by individual genes at different loci (Inui, 1999). Research during the past two decades points to a complex role for two of NPY receptors, the Y_1 and the Y_2, in the modulation of pain, in addition to regeneration and survival mechanisms at the spinal level.

The Y1 receptor was the first cloned NPY receptor in the family. Across all species, the Y1 receptors displayed greater than 95% amino acid sequence identity in the transmembrane regions (Larhammar et al., 2001). The tissue expression of the Y1 receptors can be differentially regulated by alternative splicing (Ball et al., 1995). In the CNS, Y1 receptors are expressed in various brain regions including cerebral cortex, basolateral amygdala, dentate gyrus and paraventricular nucleus (Silva et al., 2005). They

mediate several NPY-activated functions, including feeding behaviors, anxiety, neuronal excitability and hormone secretion. In the periphery, Y1 receptors are expressed in a large portion of DRG neurons and superficial dorsal horn neurons, where they mediate regulation of nociception (Brumovsky et al., 2007). Intracerebroventricular injections of a Y1 receptor agonist induced antinociception and a dose-dependent inhibition of acetic acid-induced writhing in mice (Broqua et al., 1996). The Y1 receptor knockout mice develop hyperalgesia to acute thermal, cutaneous and visceral chemical pain, and exhibit mechanical hypersensitivity (Naveilhan et al., 2001; Shi et al., 2006).

The Y2 receptor was first proposed based on the activity of amino-terminally truncated fragments of NPY and PYY. The Y2 receptor is highly conserved between species with more than 90% identity between orders of mammals. It is found in the hippocampus and acts to decrease postsynaptic excitability by the inhibition of neurotransmitter release. The Y2 receptors are also expressed in the PNS (mainly in dorsal horn, DRG, and nerve endings in the skin), intestine and certain blood vessels (Hokfelt et al., 2007). It has diverse biological functions in regulating feeding and appetite, seizures and epilepsy, angiogenesis, wound healing, anxiety, pain, stress, fear and aggression, etc. Recent research reveals that the Y2 receptor is an important player in pain modulation. Y2 receptors in the nerve endings in the skin receive nociceptive projections from the dorsal horn, and could be involved in the mechanisms of pain induction (Brumovsky et al., 2005). The expression of the receptors in DRG is upregulated proximally upon spinal nerve injury, and this could be connected to pain stimuli. Interestingly, Tracey and colleagues have shown a facilitating effect of NPY on thermal hyperalgesia through the activation of Y2Rs (Tracey et al., 1995).

NPY receptors other than the Y1 and Y2 might also have a role in pain modulation at the DRG level. For instance, Y5 receptors have been found in rat DRGs and show a modest up-regulation after nerve injury (Xiao et al., 2002). Furthermore, the y6 receptor has been detected in the developing nervous system. Although no evidence of its presence in mouse primary afferent neurons has yet been published, it is nonetheless possible that it may also be involved in nociception.

In summary, NPY may exert its physiological effects differentially via signaling through various receptors. In fact, NPY has been shown to both cause and reduce pain, depending on its actions at different sites (axon vs. cell body), through different receptors (Y1 vs. Y2) and/or types of neuron (ganglion neurons vs. interneurons vs. projection neurons). It is accepted that NPY generally exerts an inhibitory effect when acting on neurons expressing the Y1 receptor, whereas binding to the Y2 receptor increases neuronal excitability. However, the Y2 receptor also exerts an inhibitory role by reducing the release of excitatory neurotransmitters such as glutamate (Colmers and Bleakman, 1994; Smith et al., 2007). Thus, pain transmission through nociceptive sensory neurons expressing several types of NPY receptors might be modulated in different ways (Brumovsky et al., 2007).

Intracellular signaling pathway of NPY

NPY exerts its diverse physiological functions through its binding with one of the 5 subtypes of G-protein coupled receptors (Silva et al., 2002). These G-proteins are all identified as pertussis toxin (PTX)-sensitive G-proteins (G_i and G_q). Cloned Y1, Y2, Y4 and Y5 receptors have all been shown to transduce the signal by inhibition of adenylyl

cyclase (AC) to decrease the synthesis of cAMP (Larhammar et al., 2001). Activation of NPY receptors also involves PTX-sensitive phosphorylation of extracellular signal regulated protein kinase 1 and 2 in CHO cells, indicating that these receptors couple to G_i / G_o. While protein kinase C (PKC) mediates the phosphorylation of the Y5 receptor, a PKC independent pathway may also be involved in Y1, Y2, and Y4 signaling (Mullins et al., 2000). NPY receptors also couple to phospholipase C to induce release of Ca^{2+} from intracellular stores (Gehlert, 2004). Moreover, NPY receptor modulates calcium and potassium channels by the coupled G_q-protein at the plasma membrane (Lemos and Takeda, 1995). Finally, NPY is shown to activate a mitogen-activated protein kinase (MAPK) through the cascade of Y1 receptor, PTX-sensitive G-protein, and phosphatidylinositol-3-kinase (PI3K) (Keffel et al., 1999).

Another signaling pathway in which NPY may be involved is nitric oxide (NO). It has been reported that Y1 receptor mediates vasodilation of human subcutaneous arteries through an NO- dependent pathway (Nilsson et al., 2000; You et al., 1995). Furthermore, *in vitro* assays have demonstrated that NPY-induced vasodilation can be abolished by nitro-L-arginine, a potent NO synthase inhibitor (You et al., 1995).

The NPY-like signaling system in *Drosophila*

Neuropeptide F (NPF) is the sole *Drosophila* member of the NPY family. It was isolated based on a radioimmunoassay for a gut-derived peptide from the corn earworm, *Helicoverpa zea* (Brown et al., 1999). NPF is considered as the first insect neuropeptide that belongs to the NPY family (Larhammar, 1996). The NPF transcripts are localized only in four neurons in the protocerebral region of the CNS and a number of endocrine

cells in midguts of larvae and adults as revealed by whole mount *in situ* hybridization (Brown et al., 1999; Shen and Cai, 2001). Such remarkably definitive expression pattern of *Drosophila* NPF is reminiscent of that of NPY (enriched in the brain) and PYY/PP (enriched in the gut).

The receptor of NPF, NPFR1 (CG1147), was identified recently in the *Drosophila* genome database (Garczynski et al., 2002). When expressed in CHO cells, the cloned NPFR1 could be activated by NPF in a peptide binding assay. CHO cells stably transfected with NPFR1 bound radiolabeled ^{125}I- NPF specifically, and this binding could be displaced in a graded manner by the addition of unlabled NPF. Moreover, activation of the NPFR1 with NPF led to inhibition of forskolin-stimulated [^{3}H]adenine incorporation into cAMP. Thus, the NPFR1 inhibits AC activity, similar to the properties shown by the *Lymnaea* NPY receptor homolog when activated by the respective endogenous ligands *in vitro* (Garczynski et al., 2002). NPFR1 RNA transcripts were detected in neurons located in the brain lobes and ventral nerve cord as well as scattered isolated cells of the midgut in *Drosophila* larvae (Garczynski et al., 2002). Taken together, these results strongly argued that NPFR1 is a functional endogenous receptor for NPF.

NPF signaling may be involved in the food intake and digestion. The NPF expression in the CNS of feeding larvae can be modulated by exposure to fructose and glucose, suggesting that the dNPF neuronal circuit is an integral part of the sensory system that mediates food signaling (Shen and Cai, 2001). NPF is also shown to be important for regulating the onset of wandering behavior in third-instar *Drosophila* larvae. Ectopically overexpressing NPF in larvae abolishes the food-avoidance and migration toward food-free habitat (Wu et al., 2003). Considering the fact that NPF level

in feeding larvae is high and decreases rapidly upon exiting the feeding phase, it is suggested that NPF is an important modulator of onset of wandering behaviors and habitat switch.

OBJECTIVES

Nociception is not only important for protecting animals from tissue damage, but also for mediating various biological processes. Over the years, pharmacological studies have been widely employed and are essential for discoveries of modulators of nociception. In addition, the tremendous progress in mouse genetics allowed transgenic studies to uncover the underlying neural mechanisms of nociception. However, due to the complex yet ubiquitous compensatory mechanisms in the mammalian nervous system, there are many discrepancies between the pharmacological evidences and transgenic data. It is often complicated and difficult to investigate the basic molecular and cellular mechanisms for nociception and its modulation. The genetically-tractable *Drosophila melanogaster* has emerged as a simple yet robust model organism for neural and behavioral studies. *Drosophila* has a relatively simpler nervous system, allowing easier identification and functional characterization of neurons and neural circuits underlying complex biological processes. Moreover, thanks to the large repertoire of molecular and genetic tools, various transgenic flies can be conveniently generated in order to investigate the underlying basic molecular and cellular mechanisms of diverse biological processes.

Drosophila display food-averse behaviors at the end of third-instar stage (usually 96 h *A*fter *E*gg *L*aying), providing an excellent opportunity to study neural and molecular

mechanisms underlying the avoidance behaviors induced by aversive / noxious stimuli. These behaviors include migration toward food-free surface for pupation, and social burrowing through food proper upon on exiting feeding habitat. It is shown that these behaviors are dependent on the external stimulation of chemicals in the food and the internal stimulation of NPF signaling (Wu et al., 2003). In addition, *Drosophila* shares a conserved nociceptive system with higher organisms, comprised of the TRP channel proteins and NPY-like signaling system, with similar set of regulatory pathways including PKA and PKC pathways. Thus the genetically tractable *Drosophila* can potentially be a powerful model to study the molecular mechanisms of nociception. In this study, I would like to establish the postfeeding *Drosophila* larva model for study of the molecular mechanisms of nociception and the role of nociceptive system in the developmental control of food-averse behavior. In the long term, the *Drosophila* model will help in identifying novel genes involved in nociception and gaining insights into conserved nociception of higher organisms. The following are the three major objectives of this dissertation:

Objective 1. Delineate the neurobiological basis of age-restricted response to environmental stimuli, and identify molecules and neurons important for sensation of aversive chemicals present in the food. The results of these studies are presented in Chapter 2 of this dissertation in manuscript format and have been published in *Nature Neuroscience* (Xu et al., 2008).

Objective 2. Elucidate the mechanism of developmental regulation of the food-averse behaviors by the NPF / NPFR1 signaling system, investigate the role of NPF / NPFR1 in suppression of activity of nociceptive neurons, and reveal other regulatory

pathways involved in the process. The results of these studies are presented in Chapter 3 of this dissertation in manuscript format.

Objective 3. Dissect the molecular and neuronal pathways mediating the social burrowing in postfeeding larvae, and investigate the role of central and peripheral neuronal pathways in regulating the seeking, clumping and burrowing activities. The results of these studies are presented in Chapter 4 of this dissertation in manuscript format.

Figure 1.1. The structural features of TRP superfamily.

Single members from each of the seven subfamilies. The following domains are indicated: A, ankyrin repeats; cc, coiled-coil domain; protein kinase domain; TRP domain. Also shown are transmembrane segments (vertical rectangles) and pore loop (P), allowing the passage of cations (+++). Reprinted, with permission, from C. Montell and the Annual Review of Biochemistry, Volume 76 © 2007 by Annual Reviews. www.annualreviews.org

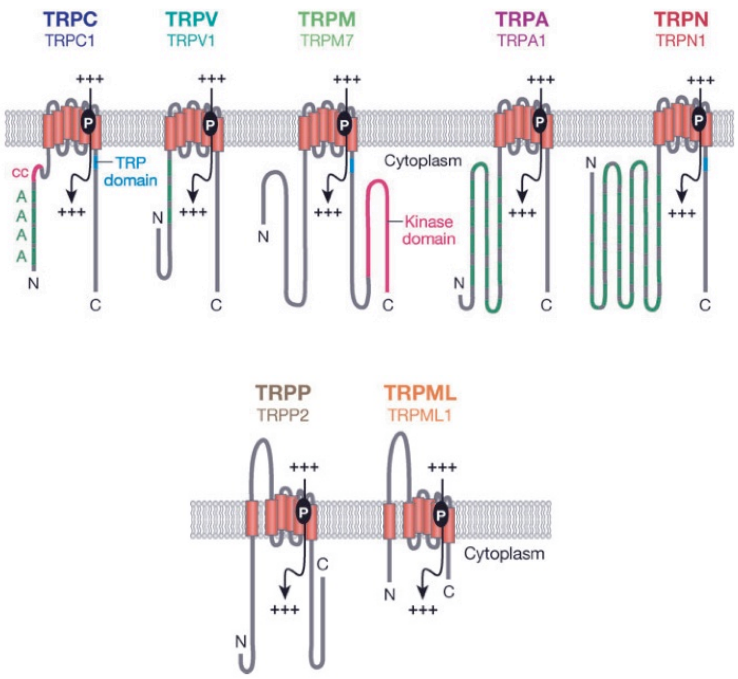
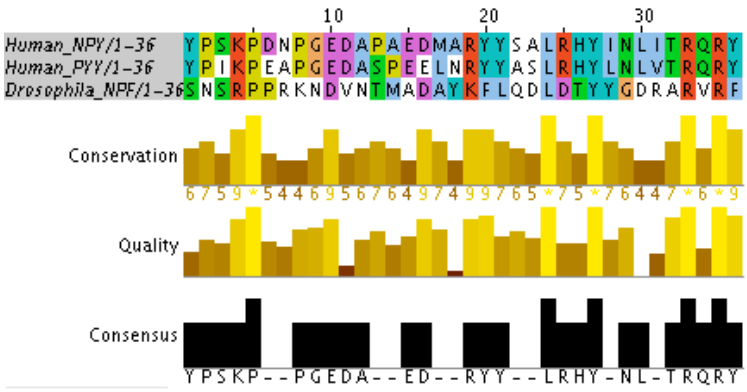

TRPC
TRPC1
TRPV
TRPV1
TRPM
TRPM7
TRPA
TRPA1
TRPN
TRPN1
TRP domain
Cytoplasm
Kinase domain
cc
A
A
A
A
N
C
TRPP
TRPP2
TRPML
TRPML1
Cytoplasm

Figure 1.2. Sequence comparisons of *Drosophila* NPF and human NPY family peptides

Abbreviations: NPF, neuropeptide F; NPY, neuropeptide Y; PYY, peptide YY. Multiple-sequence alignment analysis was performed using ClustalW2. For amino acid color schemes, see http://www.ebi.ac.uk/Tools/clustalw2/help.html#color.


10
20
30
Human_NPY/1-36 YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY
Human_PYY/1-36 YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY
Drosophila_NPF/1-36 SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRF
Conservation
6 7 5 9 * 5 4 4 6 9 5 6 7 6 4 9 7 4 9 9 7 6 5 * 7 5 * 7 6 4 4 7 * 6 * 9
Quality
Consensus
YPSKP--PGEDA--ED--RYY--LRHY-NL-TRQRY

CHAPTER 2

DROSOPHILA TRPA CHANNEL MODULATES SUGAR-STIMULATED NEURAL EXCITATION, AVOIDANCE AND SOCIAL RESPONSE[1]

[1]Xu, J., Sornborger, A.T., Lee, J. K. & Shen, P. 2008. *Nature Neuroscience* **11**, 676-682.

Permission not required for dissertation purpose.

ABSTRACT

Drosophila melanogaster postfeeding larvae show food-averse migration toward food-free habitats before metamorphosis. This developmental switching from food attraction to aversion is regulated by a neuropeptide Y (NPY)-related brain signaling peptide. We used the fly larva model to delineate the neurobiological basis of age-restricted response to environmental stimuli. Here we provide evidence for a fructose-responsive chemosensory pathway that modulates food-averse migratory and social behaviors. We found that fructose potently elicited larval food-averse behaviors, and PAINLESS (PAIN), a transient receptor potential channel that is responsive to noxious stimuli, was required for the fructose response. A subset of *pain*-expressing sensory neurons have been identified that show PAIN-dependent excitation by fructose. Although evolutionarily conserved avoidance mechanisms are widely appreciated for their roles in stress coping and survival, their biological importance in animal physiology and development remains unknown. Our findings demonstrate how an avoidance mechanism is recruited to facilitate animal development.

INTRODUCTION

Sensory systems, which define an animal's perception of its own world, are of primary importance to behavioral development and adaptation. It has been widely observed in both vertebrate and invertebrate species that an organism may restrict or modify its behavioral response to a particular sensory stimulus in an age-dependent manner (Bolhuis and Gahr, 2006; Sokolowski, 2003). However, regulatory mechanisms

underlying developmentally programmed modifications of natural behaviors remain to be better understood.

The genetically tractable *D. melanogaster* larva offers a useful model to investigate how an animal modulates its chemosensory properties and behaviors in coordination with development (Wu et al., 2003). Third-instar fly larvae display two opposing food responses: younger larvae live mostly inside aqueous food media such as overripe fruits and apple juice-agar paste; in contrast, older postfeeding larvae avoid food media and display migration (also known as wandering) towards food-free sites such as soils or plastic surface for pupation. New postfeeding larvae also display a social response to aversive food stimuli; these larvae instinctively aggregate on harder apple juice-agar media and dig cooperatively through the food proper. These food-conditioned migratory and social behaviors are likely beneficial to the survival of pupae by minimizing their exposure to harmful microorganisms and drowning in the feeding habitat such as rotten fruits (Ashburner, 1989; Chiang, 1950).

Neuropeptide Y (NPY), an abundant signaling peptide in the brain of mammals, has been implicated in diverse physiological processes and behaviors including food and alcohol response and the suppression of anxiety and pain (Brumovsky et al., 2007; Thorsell and Heilig, 2002). NPY family signaling peptides have been found in organisms ranging from humans to worms (Brown et al., 1999; Larhammar, 1996; McVeigh et al., 2005). The genome of *D. melanogaster* encodes a single member of the NPY family, neuropeptide F (NPF) (Brown et al., 1999). We previously showed that the brain expression of NPF is high in younger third instars that live mostly inside food media, but rapidly downregulated in new postfeeding larvae (Shen and Cai, 2001; Wu et al., 2003).

Prolonged NPF expression in older larvae is sufficient to block the developmental onset of food-averse migratory and social behaviors and extend the feeding phase. Conversely, attenuated NPF signaling in younger feeding larvae triggers precocious display of food-averse behaviors normally associated with wandering larvae. These findings suggest the *Drosophila* NPY-like system may be a developmental regulator of larval food aversion.

The conserved transient receptor potential (TRP) family ion channel proteins are polymodal receptors capable of responding to diverse stressful stimuli including noxious chemicals, light, heat and touch (Montell, 2005a; Montell et al., 2002; Moran et al., 2004; Ramsey et al., 2006). The well-characterized mammalian vanilloid receptor TRPV1 has been shown to respond to noxious heat, protons and capsaicin, a spicy substance in hot chili peppers (Caterina and Julius, 2001; Caterina et al., 1997). The *D. melanogaster* genome contains at least 13 TRP family members including the *pain* gene, which mediates sensation of noxious heat and mechanical touch in fly larvae (Montell, 2005a; Tracey et al., 2003). These findings have led us to postulate that TRP channel proteins may play a role in larval sensation of aversive food chemicals.

In this report, we show that the developmental onset of food-averse migratory and social behaviors in *D. melanogaster* larvae is regulated by a chemosensory neuronal pathway responsive to fructose. A TRPA channel protein, PAIN, is essential for larval chemosensory response to fruit juice or fructose. Furthermore, we have identified a subset of larval sensory neurons that display PAIN-dependent excitation by fructose, and targeted ablation of these neurons abolished larval food aversion. Our findings from this and previous studies suggest that the larval behavioral switch from food attraction to

aversion may require modulation of a PAIN-dependent peripheral sensory module by a temporal control module involving brain NPF signaling.

RESULTS

Behavioral procedures for larval food aversion

Under controlled laboratory conditions, new postfeeding larvae (ca. 96 hr *a*fter *e*gg *l*aying, 96hr AEL) display migration towards food-free plastic surfaces prior to metamorphosis (Figure 2.1a). We employed two behavioral paradigms to quantitatively assess larval behavioral response to food media. In the first assay, we measured larval migratory response by placing 25 new postfeeding larvae on the center surface of apple juice-containing soft agar media. A majority of normal w^{1118} larvae (~85%) moved away from the apple juice medium within two hours, and they became less mobile and subsequently pupated on a dry plastic surface. In one set of experiments, apple juice was replaced with 10% fructose in the agar paste. Again, almost 80% of larvae displayed the wandering behavior and selected pupation sites outside of the fructose medium (Figure 2.1b). In contrast, on water agar paste, larvae browsed randomly and remained in the medium. Consequently, about 80% larvae were found to form pupae that were embedded in the surface layer (Figure 2.1c). Moreover, 10% lactose or sorbitol was not effective in triggering larval wandering (Figure 2.1d), suggesting that larvae are responsive to external gustatory stimulation by fructose, the most abundant sugar in many overripe fruits, rather than to osmotic pressure.

We showed previously that on a harder apple juice-agar surface, new postfeeding larvae exhibited rapid aggregation, which provides the synergy that enables larvae to dig

efficiently through a hard food medium (Shen and Cai, 2001; Wu et al., 2003). Since fruit fly larvae exiting rotten fruits display a strong preference to quickly burrow into the pupation habitat (moist soil) (Alyokhin et al., 2001; Thomas, 1995), this instinctive cooperative behavior may conceivably facilitate larval penetration of fruit juice-stained compact soil near the fallen fruits. We have also found that postfeeding larvae (96hr AEL) displayed aggregation and cooperative burrowing on solid 10% fructose but not water agar media (Figure 2.1e-g). These results suggest that fructose can also trigger the grouping behavior of larvae on harder surfaces.

pain mediation of larval aversion to fruit juice/fructose

The *pain* gene is required for the sensation of noxious heat and mechanical touch in third-instar larvae of *D. melanogaster* (Tracey et al., 2003). We investigated whether *pain* may play a role in larval food-averse migration. The effects of four mutant alleles of *pain* on larval wandering behavior were tested. For example, we found that $pain^3$ larvae were largely insensitive to the apple juice medium, with about 75% remaining on the medium (Figure 2.2a). Also, $pain^1$ larvae showed smaller but significant deficits in migration. The trans-heterozygous larvae (e.g., $pain^1/pain^3$) exhibited an intermediate food-averse response. These results are consistent with the previous finding that $pain^3$ larvae displayed stronger defects in noxious heat response relative to $pain^1$ (Tracey et al., 2003). Other trans-heterozygous larvae also exhibited significant deficits in migration. The $pain^3$ larvae were also largely insensitive to 10% fructose and other sugar media (Figure 2.2b), and a transgenic construct containing an 8.5-kb genomic sequence of *pain* rescued the mutant phenotype (Figure 2.2c).

We further tested the behavioral response of *pain*^{3} larvae to solid 10% fructose agar media. These larvae browsed randomly on both 10% fructose and water agar media, and no aggregation and burrowing activities were observed (Figure 2.2d and e). Importantly, *pain*^{3} and wild type larvae showed comparable locomotor activities on the water agar medium (Figure 2.2f). For example, *pain*^{3} larvae crawled at a speed of about 0.5 mm /sec which could allow a larva to move across an assay plate in 3 min, suggesting that the mutant phenotypes of *pain*^{3} larvae are unlikely due to a locomotor defect. Taken together, these results indicate that the TRP channel protein PAIN is essential for larval chemosensory response to aversive fructose in the feeding habitat.

Conditional disruption of PAIN neuronal signaling by *shibire*^{ts1}

The *pain*^{gal4} (also referred as *pain-gal4*) allele contains a GAL4 coding sequence inserted immediately downstream of the *pain* promoter (Tracey et al., 2003). This *pain-gal4* driver has been shown to direct reporter expression in the PAIN cells of the central and peripheral nervous system (CNS and PNS (Figure 2.3a). To conditionally disrupt the activity of PAIN neurons, we used *pain-gal4* to express a temperature-sensitive allele of *shibire* (*shi*^{ts1}), which encodes a semi-dominant negative form of dynamin capable of blocking neurotransmission at a restrictive temperature (> 29 °C) (Kitamoto, 2002). At 23 °C, both experimental (*pain-gal4* X UAS-*shi*^{ts1}) and control larvae (e.g., UAS-*shi*^{ts1} alone) displayed similar wandering activities on apple juice media. However, a majority of experimental larvae remained in the food medium at 30 °C, while most of control larvae migrated away (Figure 2.3b). The locomotor activity of *pain-gal4* X UAS-*shi*^{ts1} larvae was similar to those of controls (Figure 2.3c). These findings suggest that the

signaling activity of PAIN neurons is acutely required to maintain the food-averse response in wandering larvae.

Larval aversion to capsaicin triggered by mammalian TRPV1

To provide further evidence that larval wandering behavior represents an aversive response to fructose, we used *pain-gal4* to express a mammalian TRP channel protein VR1E600K, a variant of vanilloid receptor TRPV1 responsive to a spicy substance capsaicin from chili peppers (Caterina et al., 1997; Marella et al., 2006; Tobin et al., 2002). The majority of control larvae (e.g., VR1E600K alone) browsed and eventually pupated on the agar paste containing 25 μM capsaicin, showing no aversive response to the medium (Figure 2.4a). However, virtually all of the postfeeding larvae expressing VR1E600K migrated away from the capsaicin-agar medium (Figure 2.4b and c). Moreover, in a two-choice assay, postfeeding VR1E600K-expressing larvae displayed preference for capsaicin-free media while younger feeding VR1E600K-expressing larvae showed no such preference (Figure 2.4d). These data strongly support the notion that fructose is an aversive chemical to postfeeding larvae, and peripheral PAIN sensory neurons are responsible for the migratory response to aversive chemical cues.

Loss of neuronal excitation by sugar in *pain* mutants

PAIN neurons are present widely in the larval PNS. Since larvae crawling on a flat surface of apple-juice agar display the aversive response, we postulated that *pain* may mediate sugar stimulation in those sensory neurons located in the ventral side of the larval body. Of particular interest are six clusters of *pain*-expressing ventral neurons in

the three thoracic segments (see Figure 2.5 and Figure 2.8a). The somata of these neurons are located near the Keilin's organs (the presumed primordial legs of larvae) (Cohen et al., 1991; Lakes-Harlan et al., 1991), and their processes are projected directly into the thoracic ganglia and the ventral denticle belts. We imaged neuronal excitation with a Ca^{2+}-sensitive fluorescent protein, yellow cameleon 2.1 (YC2.1) (Liu et al., 2003). Initial imaging studies provided evidence for fructose-stimulated intracellular Ca^{2+} increases in three pairs of clustered neurons from each of the thoracic segments in normal third-instar larvae (*pain-gal4*/UAS-*YC2.1*; 96h AEL). We subsequently focused our analysis on the PAIN neurons in the second and third thoracic segments. The imaging results of PAIN neurons from the third thoracic segment are shown as an example in Figure 2.6a-e. We found that these neurons in *pain-gal4*/UAS-*YC2.1* larvae were stimulated by lactose-to-fructose but not lactose-to-lactose switch. Moreover the nearby *pain*-expressing chordotonal neurons were not responsive to the fructose treatment nor the thoracic Pain neurons from younger feeding larvae (76h AEL; see Table 2.1 and Figure 2.7). On the other hand, the same thoracic PAIN neurons in *pain* mutants ($pain^{gal4}/pain^{3}$; UAS-*YC2.1*) showed no significant Ca^{2+} increases upon fructose stimulation (Figure 2.6f-h and Table 2.1). These results indicate that *pain* is essential for the excitation of these thoracic sensory neurons by fructose.

Disruption of food aversion by ablating thoracic PAIN neurons

We also tested whether selective ablation of the fructose-responsive PAIN neurons could abolish food-averse behaviors. Simultaneous ablation of all six clusters of ventral PAIN neurons in the three thoracic segments (T1 to T3) effectively disrupted

larval aversion to apple-juice media (Figure 2.8e). Moreover, partial ablation of two of the six clusters in the second thoracic segment was sufficient to severely disrupt larval food aversion. However, asymmetric ablation of one of the two clusters in two or all three thoracic segments had at most marginal effect on larval food aversion. These results indicate that the fructose-responsive PAIN neurons in the thoracic segments, especially those in the T2 segment, are essential for larval food aversion.

NPFR1 expression in peripheral PAIN neurons

To determine whether PAIN neurons could potentially respond to NPF directly, we examined where NPFR1 is expressed in the nervous system. The intact CNS and epidermis tissues of larvae (96h AEL) expressing DsRed driven by *pain-gal4* were immunostained with both anti-DsRed and anti-NPFR1 peptide antibodies. We found that NPFR1 immunoreactivity co-localizes with DsRed in three pairs of clustered *pain*-expressing neurons near the Keilin's organs in the ventral epidermis of the thoracic segments (see Figure 2.9 and Figure 2.10). These results raise the possibility that fructose-responsive PAIN neurons may be directly regulated by NPF. NPFR1 immunoreactivity was also detected in the somata and neuropils of the brain lobes, subesophageal ganglia and ventral nerve cord. However, it does not appear to overlap with *pain*-expressing cells in the CNS (Figure 2.9e). Importantly, mammalian Y1 and Y2, which are most closely related to NPFR1, have also been found in the nociceptors of the dorsal root ganglia and spinal neurons (Brumovsky et al., 2007; Ji et al., 1994). Thus, our findings suggest another potential parallel activity between NPF and NPY in the suppression of stressful sensation.

DISCUSSION

We have provided both neuroanatomical and functional evidence that the developmental onset of migratory and social behaviors in the wandering larvae of *D. melanogaster* requires aversive stimulants (e.g., fructose) from the feeding habitat, and the conserved nociceptive gene *pain* is essential for fructose stimulation of thoracic sensory neurons and fructose-conditioned aversive behaviors. In a previous study, we found that NPF, the sole fly homolog of human NPY, is highly expressed in the brain of feeding larvae but downregulated in new wandering larvae; it potently suppresses larval aversion to apple juice media (Wu et al., 2003). Together, our studies indicate that the developmental switch from food attraction to aversion of wandering larvae is regulated by a conserved neural signaling network involving a TRPA-like peripheral sensory module and an NPY-like central module for temporal control. Our results also provide a rare example of how an avoidance mechanism has been recruited during evolution to facilitate animal development.

Our results have revealed that a subset of thoracic PAIN sensory neurons that directly projects to thoracic ganglia and the areas near the bristles of the ventral denticle belts. Loss-of-function *pain* mutations completely abolished the fructose-responsive intracellular calcium increase in these neurons. Therefore, the ventral projections of these fructose-responsive neurons are like to allow them to be in direct contact with the medium surface that wandering larvae crawl on, making them well suited to mediate larval food-averse response. Our finding also suggests that fly larvae use two separate chemosensory pathways for sensing sugars. The appetitive response to sugar is likely to be mediated by maxillary or terminal organs, whereas the aversive response to sugar may

be mediated by sensory organs on the ventral thoracic surface. We noticed that although thoracic PAIN neurons expressing DsRed in each of the clusters showed comparable red fluorescence levels, some of the neurons in the cluster showed significantly less immunofluorescent signals when stained with anti-DsRed antibodies. Therefore, it is possible that some PNS neurons may be less accessible to antibodies, and the actual number of NPFR1-positive neurons could also be higher.

It remains unclear how fructose triggers excitation of PAIN neurons in the thoracic neurons. The PAIN ion channel could act as a promiscuous receptor for diverse chemicals such as fructose and isothiocyanate (Al-Anzi et al., 2006). Alternatively, it may mediate the signaling activity of a yet uncharacterized member of the seven-transmembrane gustatory receptor family. Interestingly, A significant number of gustatory receptor-expressing neurons (e.g., Gr66a neurons) in the fly have been found to express *pain* (Al-Anzi et al., 2006). The functional significance of PAIN in gustatory neurons for food tasting remains to be determined.

NPF suppresses food-averse behaviors in feeding larvae, and its neural signaling activity is regulated developmentally (Wu et al., 2003). Consistent with our previous observations, we have now shown that in feeding larvae, *pain*-expressing ventral neurons in the thoracic segments do not respond to fructose. We have also observed that NPFR1 overexpression directed by *pain-gal4* suppressed the onset of larval food-averse behaviors as well as other sensory functions of abdominal PAIN neurons that normally do not express NPFR1 (unpublished data). Our finding that fructose-responsive thoracic PAIN neurons are positive for NPFR1 immunoreactivity suggests that NPF may act directly upon these primary sensory neurons. We postulate that NPF could act as an end

effector of a temporal control module that may suppress PAIN channel activity or render PAIN neurons incompetent in the sensation of aversive stimuli.

In mammalian models, NPY and its two receptors, Y1 and Y2, have been implicated in the suppression of fear and anxiety (Greco and Carli, 2006; Heilig, 2004), and most published data suggest that NPY has an anti-nociceptive role (Brumovsky et al., 2007; Tatemoto, 2004). For example, NPY injected into the forebrain of rats significantly elevated the nociceptive threshold in a paw-withdraw test (Li et al., 2005). NPY Y1 receptor knockout mice develop hyperalgesia to acute thermal and chemical pain, and exhibit mechanical hypersensitivity (Naveilhan et al., 2001). In addition, Y1 and nociceptive TRPV1 channel protein are both strongly expressed in the nociceptors of the DRG, further supporting an inhibitory role of Y1 in TRP family protein-mediated pain sensitivity (Gibbs et al., 2004). It is conceivable that the action of NPF on peripheral PAIN neurons may be parallel to that of NPY on the DRG neurons. We suggest that the NPF system may be a useful platform for elucidating the molecular and cellular underpinnings of neuropeptide-mediated pain suppression.

METHODS

Flies, media and larval growth

Conditions for rearing adult flies and egg collection were described previously (Roberts, 1986; Shen and Cai, 2001; Wen et al., 2005). The larvae were raised at 25 °C with exposure to natural lighting. Synchronized eggs were collected within a 2 h interval, and late second instars were transferred to a fresh apple-juice plate with yeast paste (< 80 larvae per plate). The *pain-rescue, pain*^{1}, *pain*^{3}, *pain*^{gal4}, *Gr66a-gal4* and UAS-*shi*^{ts1},

UAS-*VR1E600K,* UAS-*YC2.1* lines are in *w* background (Kitamoto, 2002; Liu et al., 2003; Marella et al., 2006; Scott et al., 2001; Tracey et al., 2003).

Behavioral Assays

The food aversion assay was performed in plastic petri dishes (60 mm in diameter). Each apple juice-agar plate contains a mix (ca. 7 ml) of 1g of *Drosophila* agar powder (USB, Swampscott, Massachusetts) and 6 ml apple-juice solution (0.1g carbohydrates per ml, equivalent to 20% of frozen concentrate). Other soft agar media were made by mixing 1 g agar powder with 6.5 ml of distilled water or solution containing 10% fructose, 10% lactose, 10% sorbitol or 50 μM capsaicin, respectively. The amount of agar powder may require adjustment depending on agar quality. Twenty-five new postfeeding larvae (96h AEL) were transferred onto a plate. The larvae were allowed to move freely on the medium, and those that crawled onto the plastic surface became less mobile and eventually formed pupae there. The percent of pupae on agar media was scored after 24 hours. All experiments were performed at room temperature except for the temperature shift assay. In these experiments, larvae and food media were pre-incubated at 30 °C for 60 min before the assay. The larvae were subsequently transferred onto the medium and kept at 30 °C. All assays were performed in the dark. At least three separate trials were performed per assay.

The locomotion test was performed in a plate (87 mm in diameter) containing 1.3 g agarose powder mixed with 8.7 ml of distilled water. Larvae were rinsed repeatedly to remove visible food particles. Eight larvae were allowed to crawl on the medium, and their locomotion activities were recorded by a SONY DCR-HC36 video camera. The

video clip was converted to 1 frame s^{-1} by iMovie HD, and imported into the Image J software. The track lengths were calculated using the MTrack2 plug-in and converted to speed. At least 20 individual larvae were tested for each data point. All data were analyzed using one-way ANOVA, followed by the Student-Newman-Keuls analysis.

The two-choice medium preference assay was performed on a 2.5 % agar plate. The apple juice or water agar paste is the same as described above. 10 μl of 5 mM capsaicin stock solution (in 100% ethanol) or 10 μl ethanol was added to each ml of the agar media. 30 larvae were washed extensively to remove any food particles. They were then placed between the two piles of capsaicin and capsaicin-free media (1 cm in diameter), which are located 4 mm apart. The number of larvae in each medium was recorded after 20 min. A preference index was defined as the fraction of larvae choosing the capsaicin-free medium, minus the fraction of larvae choosing the capsaicin medium. A preference index close to +1 indicates that the larvae are attracted to the tested medium, whereas −1 indicates strong rejection. The larvae outside of both media (typically 5 %) were excluded from the calculation.

Laser ablation

Selected *pain*-expressing sensory neurons were ablated using a 337 nm nitrogen laser unit (Spectra-Physics, Model VSL337NDS, Irvine, CA). Power calibration was performed by focusing laser beam onto a mirror. The graduated neutral density filter was adjusted until the reflective layer of the mirror can be penetrated by a single shot. The filter was then moved 4 stops toward the clear end. Early third instar larvae (*pain-gal4* X UAS-*YC 2.1*, ca. 78h AEL) were rinsed briefly and placed onto a coverslip, and then

anesthetized by CO_2 for 10 min. The immobilized larvae were transferred onto a microscope slide with larval ventral side facing straight up. 100 µl of ether was added to a piece of absorbent paper sandwiched between the slide and cover slip to keep the larvae immobile during laser ablation, which typically takes about 10 min.

To ablate the neurons, the laser beam was focused to the nucleus. 4 bursts of 10 shots were fired at a repetition rate of 10 shots/s. Ablated neurons showed reduced GFP intensity, and became invisible after 24h. After ablation, each individual larva was allowed to recover on a 35 mm soft apple juice agar plate with 30 µl yeast paste on the surface. The pupation site was recorded after 48 h. Larvae from the mock group were handled and anesthetized in the same manner as experimental larvae except without laser treatment. The mortality rates of the control and experimental groups were similar (32.4% vs. 36.7%). The survived larvae developed into adults normally. Data was analyzed using an unpaired Student t-test.

Immunohistochemistry

Larval epidermis was filleted from the dorsal side. The CNS and epidermal tissues were fixed according to a previously published protocol with some modifications (Wu et al., 2003). The fixation time was 35 min. Tissues were washed in PBS with 0.4% TritonX-100, and permeabilized with Proteinase K digestion and post-fixation. Tissues blocked in 10% BSA were incubated overnight at 4˚C with mouse anti-DsRed (1:250, BD Biosciences, San Jose, CA) and affinity-purified rabbit anti-NPFR1 antibodies (1:100). The NPFR1 peptide antibodies were raised and purified using two peptide antigens (CMTGHHEGGLRSAIT and SSNSVRYLDDRHPLC). Alexa 488-conjugated

anti-rabbit IgG and Alexa 568-conjugated anti-mouse IgG secondary antibodies were diluted to 1:2,000.

Imaging

The live images of second instar larvae (ca. 48h AEL) expressing DsRed driven by *pain-gal4* were obtained using a Leica epifluorescence microscope. The immunofluorescence images were taken by a Zeiss LSM 510 Meta confocal microscope and processed by Zeiss AIM Image Examiner. Calcium imaging of sensory neurons was performed using third instar larvae (ca. 96 h AEL) transgenic for the genetically encoded yellow cameleon Ca^{2+} indicator (UAS-*YC2.1*) under the *pain* promoter. Larvae were dissected in a modified HL6 solution containing lactose (HL6-Lac, 23.7 mM NaCl, 15 mM $MgCl_2$, 24.8 mM KCl, 0.5 mM $CaCl_2$, 10 mM $NaHCO_3$, 5 mM HEPES, 240 mM lactose) (Macleod et al., 2002). An incision was made along the dorsal midline, and the gut and the fat bodies were removed. The tissue was placed ventral side up in HL6 solution on a silicon-coated coverslip (Sylgard 184, DOW Corning Corp., Midland, MI) in a Dvorak-Stotler perfusion chamber (Lucas Highland, Chantilly, VA). A minute amount of cyanoacrylate glue (Nexaband S/C, Abott Laboratories, North Chicago, IL) was applied to the edge of the cuticle using a glass micropipette to immobilize the tissue. The imaging was performed on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Microimaging, Thornwood, NY). YC2.1 was excited at 458 nm with an argon laser. Emission fluorescence was filtered by a BP 475-525 filter (cyan) and an LP 530 filter (yellow). 256 X 256 pixel images for ratiometric analysis were collected at 1 frame s^{-1}. The tissue was perfused at a rate of 8.3 ul s^{-1} with HL6-Lac, periodically alternating with

a modified HL6 solution containing fructose (HL6-Fru, with 240 mM fructose substituting 240 mM lactose in HL6-Lac), switching at 120-second intervals for a total of 800 seconds. The images were subsequently analyzed with the SOARS method (Statistical Optimization for the Analysis of Ratiometric Signals, Version 1.1, Broder et al., 2007; Fan et al., 2007), using Matlab (MathWorks, Natick, MA; also see http://www.engr.uga.edu/research/groups/atslab/Software.html). In brief, SOARS is a multivariate statistical optimization procedure performed on both CFP and YFP channels of the FRET imaging data. The analysis results in a set of eigenimages and associated time courses that represent the part of the imaged signal displaying statistically significant spatial correlation and temporal anti-correlation (i.e. the aspects of the signal that are demonstrable due to FRET). These eigenimages represent the spatial distribution of anti-correlated FRET changes in response to sugar stimulation. Because the stimulus was periodic, we tested the time courses for the presence of stimulus-locked activity. To quantify the significance of the periodic part of the FRET response, we calculated p-values for a periodic (sinusoidal) response at the stimulus frequency using multitaper harmonic analysis (a common method for the detection of sinusoids in noisy data, Mitra and Bokil, 2008; Mitra and Pesaran, 1999; Sornborger et al., 2003; Thomson, 1982).

ACKNOWLEDGEMENTS

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Figure 2.1. Behavioral procedures for larval response to aversive food chemicals

(**a**) Postfeeding larvae instinctively migrate away from feeding sites and pupate on the plastic surface of a bottle. (**b** and **c**) Most of w^{1118} postfeeding larvae (ca. 96h AEL, n=25 per plate) moved out of 10% fructose but not water agar paste. Pictures were taken after the larvae pupated. Scale bars, 10 mm. (**d**) Quantification of larval aversive responses to different media containing 10% fructose, lactose or sorbitol. (**e** and **f**) Most of w^{1118} postfeeding larvae (ca. 96h AEL, n=30 per plate) had a sequential display of dispersing, clumping and cooperative burrowing on the solid 10% fructose agar medium within a 30-min period (Wu et al., 2003). The same larvae showed no clumping or burrowing on water agar medium even after 1.5 hr. Pictures were taken 30 min after the larvae were transferred onto the medium. Scale bars, 10 mm. (**g**) Quantification of larval grouping behavior. The feeding larvae do not show clumping and burrowing activity on solid apple juice agar medium. In all figures, unless otherwise stated, error bars represent standard deviations. At least 3 separate trials were performed for each experiment. Asterisks indicate statistically significant differences from paired controls. $P<0.001$. ANOVA followed by Student-Newman-Keuls analysis.

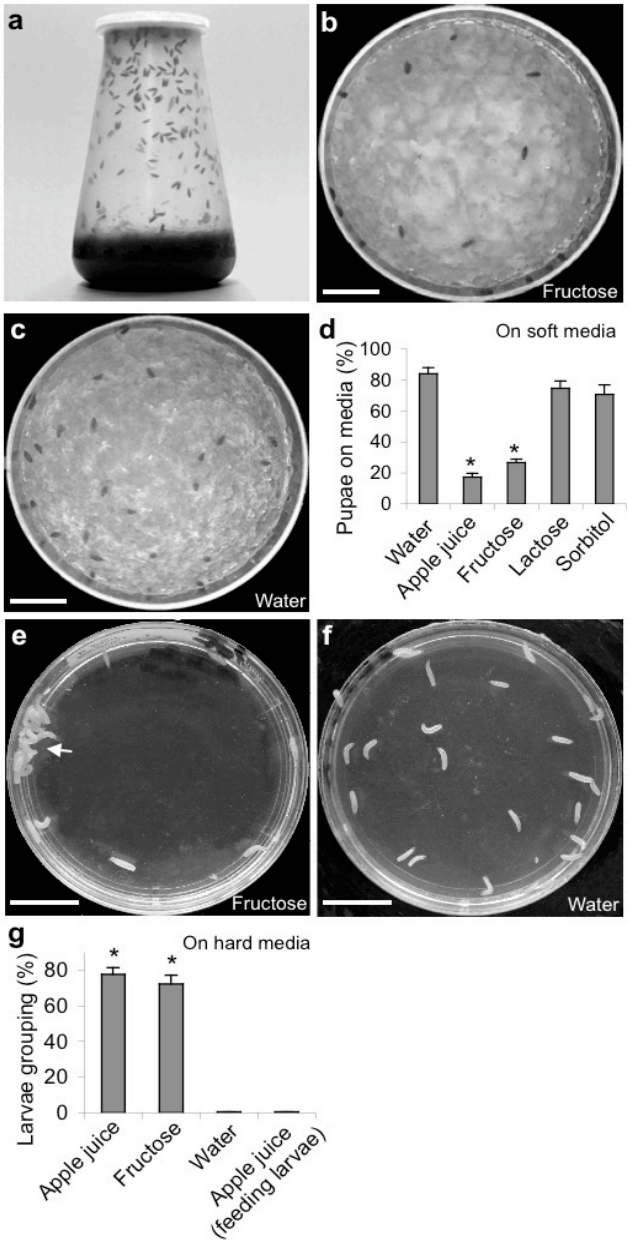
a
b
Fructose
c
Water
d
On soft media
Pupae on media (%)
100
80
60
40
20
0
*
*
Water
Apple juice
Fructose
Lactose
Sorbitol
e
Fructose
f
Water
g
On hard media
Larvae grouping (%)
80
60
40
20
0
*
*
Apple juice
Fructose
Water
Apple juice (feeding larvae)

Figure 2.2. *pain* is involved in larval aversion to fruit juice and fructose

The hypomorphic alleles $pain^1$ and $pain^3$ are resulted from P-element insertions in the 5' region of the *pain* gene. $pain^3$ has been shown to confer a stronger defect in thermal sensation than $pain^1$ (Tracey et al., 2003). (**a**) Larvae with different *pain* mutant alleles showed reduced aversion to apple juice agar medium ($P<0.01$). (**b**) Wild type larvae were aversive to 10% fructose, sucrose and glucose media. The behavioral responses to each sugar were compared between the wild type and each of the pain mutants. Asterisks indicate statistically significant alterations ($P < 0.01$). (**c**) A transgenic construct containing the genomic sequence of the *pain* gene restored the food-averse migration in $pain^3$ larvae. $P <0.001$. (**d** and **e**) $pain^3$ larvae showed no cooperative burrowing throughout the experiment (>1.5 hr). Scale bar, 10 mm. The rescue construct restored the food-conditioned cooperative burrowing in $pain^3$ larvae. (**f**) $pain^3$ and wild type larvae showed comparable motilities on water agarose medium ($P=0.068$).

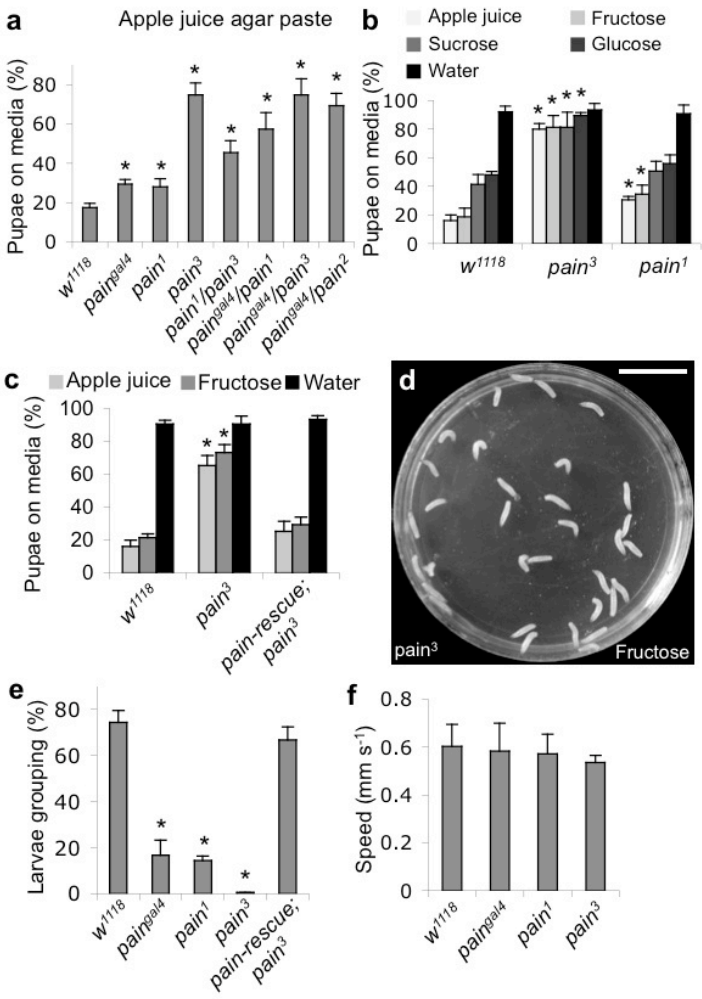
a
Apple juice agar paste
Pupae on media (%)
0
20
40
60
80
w1118
painGal4
pain1
pain3
pain1/pain3
painGal4/pain1
painGal4/pain3
painGal4/pain2
b
Apple juice
Fructose
Sucrose
Glucose
Water
Pupae on media (%)
0
20
40
60
80
100
w1118
pain3
pain1
c
Apple juice
Fructose
Water
Pupae on media (%)
0
20
40
60
80
100
w1118
pain3
pain-rescue; pain3
d
pain3
Fructose
e
Larvae grouping (%)
0
20
40
60
80
w1118
painGal4
pain1
pain3
pain-rescue; pain3
f
Speed (mm s-1)
0
0.2
0.4
0.6
0.8
w1118
painGal4
pain1
pain3

Figure 2.3. Conditional disruption of *pain*-expressing neuronal signaling attenuates larval food aversion

(**a**) A live image of a second-instar larva expressing DsRed driven by *pain-gal4*, which has been shown to recapitulate the endogenous *pain* expression pattern in the peripheral and central nervous system (Tracey et al., 2003). Peripheral *pain*-expressing neurons exist in paired clusters (arrowheads). Scale bar, 200 μm. (**b**) *Gr66a-gal4* has been shown to direct gene expression in larval gustatory neurons of the terminal organs (Scott et al., 2001). UAS-shi^{ts1} encodes a semi-dominant-negative form of dynamin that can block neurotransmitter release at a restrictive temperature (>29 °C). At permissive temperature (23 °C), both experimental larvae (*pain-gal4* X UAS-shi^{ts1}) and control larvae (e.g., *Gr66a-gal4* X UAS-shi^{ts1}) displayed aversive response to apple juice media. At 30 °C, the experimental but not control larvae showed attenuated food aversion. $P<0.001$. (**c**) The transgenic larvae crawled at the speed of about 0.6 mm/sec, which is comparable to those of control larvae (*pain-gal4* alone or *Gr66a-gal4* X UAS-shi^{ts1}).

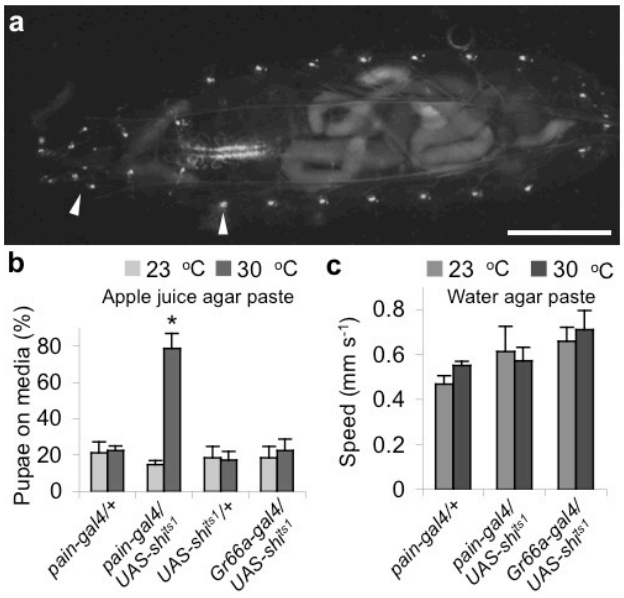
a
b
23 °C
30 °C
Apple juice agar paste
Pupae on media (%)
*
0
20
40
60
80
pain-gal4/+
pain-gal4/ UAS-shits1
UAS-shits1/+
Gr66a-gal4/ UAS-shits1
c
23 °C
30 °C
Water agar paste
Speed (mm s-1)
0
0.2
0.4
0.6
0.8
pain-gal4/+
pain-gal4/ UAS-shits1
Gr66a-gal4/ UAS-shits1

Figure 2.4. Larvae expressing a mammalian vanilloid receptor display capsaicin-averse behaviors

UAS-*VR1E600K* encodes a variant of the mammalian capsaicin receptor VR1. (**a** and **b**) Experimental larvae expressing UAS-*VR1E600K* driven by *pain-gal4* migrated away from agar paste containing 25 μM capsaicin. Control larvae (e.g., UAS-*VR1E600K* alone) mostly remained on the capsaicin medium. Scale bars, 10 mm. (**c**) Quantification of avoidance response of transgenic larvae in media containing capsaicin, apple juice or water only. $P<0.001$. (**d**) In a two-choice assay, *pain-gal4* x UAS-*VR1E600K* feeding larvae (74h AEL) showed no preference for capsaicin-free media. $n > 90$, $P<0.001$.

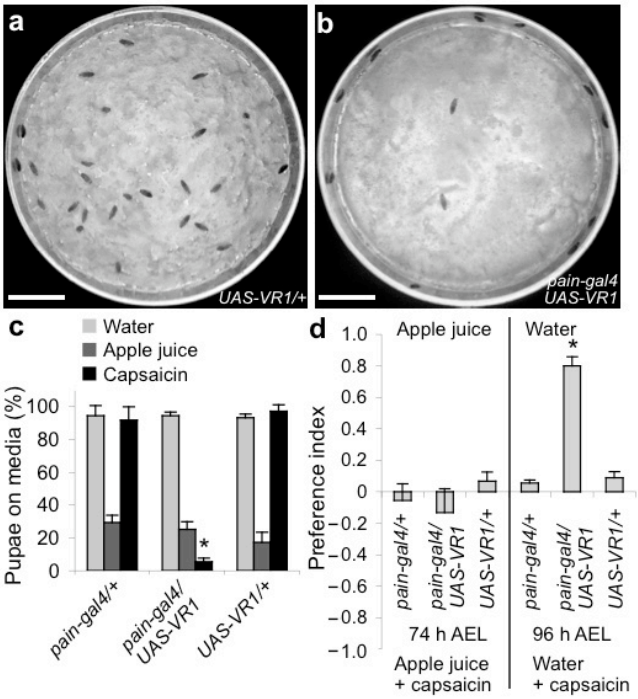
a
UAS-VR1/+
b
pain-gal4
UAS-VR1
c
Water
Apple juice
Capsaicin
Pupae on media (%)
100
80
60
40
20
0
*
pain-gal4/+
pain-gal4/
UAS-VR1
UAS-VR1/+
d
Apple juice
Water
Preference index
1.0
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1.0
*
pain-gal4/+
pain-gal4/
UAS-VR1
UAS-VR1/+
74 h AEL
96 h AEL
Apple juice
+ capsaicin
Water
+ capsaicin

Figure 2.5. The ventral PAIN neurons of the three thoracic segments (T1 to T3) project to the thoracic ganglia and denticle belts

The nervous tissues of *pain-gal4*; UAS-*mCD8-GFP* larvae (96 h AEL, n = 7) were immunostained with anti-GFP antibodies. (**a**) The GFP positive ventral neuron clusters (arrowheads) project to the thoracic ganglia. Scale bar: 50 μm. (**b-d**) Magnified views of neuron clusters in each of the thoracic segments showing their projections to the area near the bristles of the ventral denticle belt (open arrowheads). Scale bar: 20 μm.

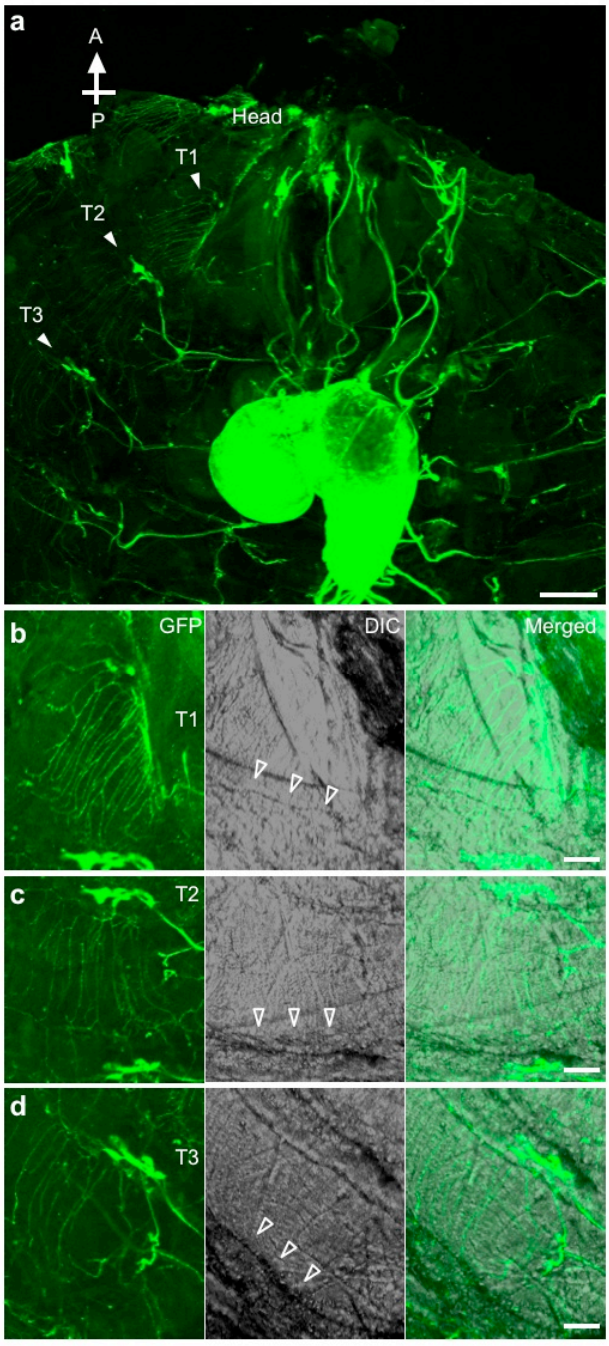
a
A
P
Head
T1
T2
T3
b
GFP
DIC
Merged
T1
c
T2
d
T3

Figure 2.6. Imaging and SOARS analysis of excitation of thoracic PAIN neurons by fructose with the cameleon Ca^{2+} indicator

Stimulation paradigm: the tissue was initially perfused with HL6-Lac solution for up to 10 min before imaging. During imaging, solutions were changed every 120 seconds, alternating between HL6-Lac and HL6-Fru. (**a)** A composite fluorescent and transmitted light image of *pain*-expressing neurons from the ventral left cluster (below the Keilin's organ, see supplementary Figure S1) in the third thoracic segment of the control larva (*pain*^{gal4}; UAS-*YC 2.1*). CFP fluorescence is shown in green, and the numbers indicate neurons. Anterior to the left. (**b**) An eigenimage of the above tissue generated from the SOARS analysis of the cameleon YFP/CFP FRET data. This eigenimage facilitates the identification of pixels that display spatially correlated, temporally anti-correlated fluorescence changes selectively responding to fructose stimulation (see Methods). Light (dark) pixels indicate regions where the calcium concentration increased (decreased) in response to fructose. The circled regions containing light pixels correspond to neuronal cell bodies, which display statistically significant periodic responses to fructose ($p < 10^{-5}$). (**c)** The projection (time-course) of the weighted mask in the data sets shows periodic anti-correlated changes of the CFP and YFP signals. (**d**) The dynamic change of fructose concentration was monitored by flowing 0.0005% fluorescein through the perfusion chamber (blue trace). The black trace indicates the solution switching profile. Note the time lag between on and off switches and the corresponding changes in fluorescein concentration due to connective tubing between the pump and perfusion chamber. Two complete cycles of solution alternation are shown here. (**e**) The periodic change in CFP/YFP ratio in individual neurons

responding to the fluctuation in fructose concentration. The strongest response was observed in neuron 4. $P < 10^{-5}$. In these traces a decrease (increase) in the ratio corresponds with increase (decrease) of calcium concentration. (**f-h**) Imaging and data analysis of the same set of thoracic *pain*-expressing neurons from *pain* mutants ($pain^{gal4}$ / $pain^{3}$; UAS-*YC 2.1*) are performed using the same procedures described above. No spatially correlated, temporally anti-correlated signals were detected. At least 6 tissues were imaged. Scale bars: 20 μm.

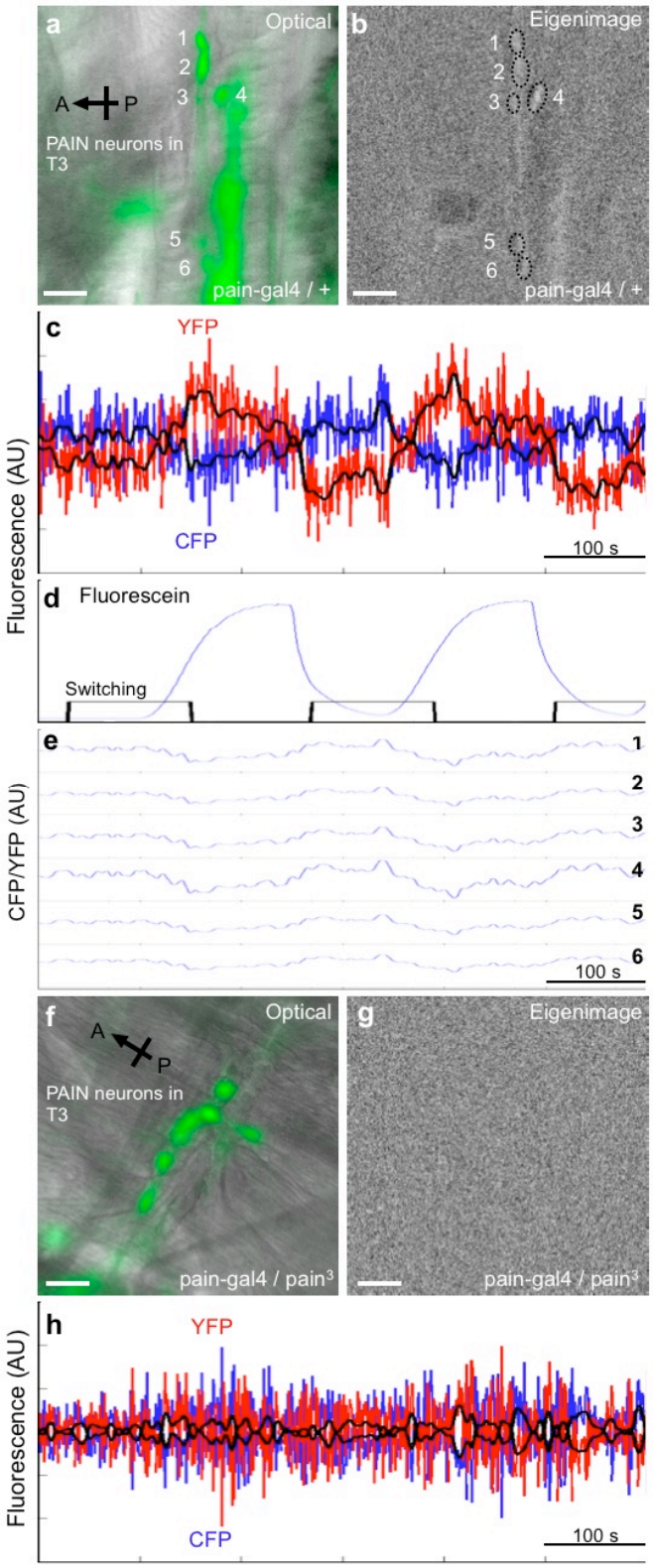
a
Optical
1
2
3
4
A
P
PAIN neurons in
T3
5
6
pain-gal4 / +
b
Eigenimage
1
2
3
4
5
6
pain-gal4 / +
c
YFP
CFP
100 s
Fluorescence (AU)
d
Fluorescein
Switching
e
CFP/YFP (AU)
1
2
3
4
5
6
100 s
f
Optical
A
P
PAIN neurons in
T3
pain-gal4 / pain3
g
Eigenimage
pain-gal4 / pain3
h
YFP
CFP
100 s
Fluorescence (AU)

Figure 2.7. The responses of larval sensory neurons to sugar stimulation

(**a-c**) The test of the response of thoracic PAIN neurons to 10% lactose was performed as follows. Briefly, the tissue was initially perfused with HL6-Lac solution for up to 10 min before imaging. During imaging, solutions were changed every 120 seconds, alternating between same HL6-Lac from two separate reservoirs. (**a**) A composite fluorescent and transmitted light image of *pain*-expressing ventral sensory neurons in third thoracic segment of *pain-gal4*; *UAS-YC 2.1* larvae. Anterior to the right. (**b**) An eigenimage of the same tissue generated from the SOARS analysis of the YFP/ CFP fluorescence data. (**c**) The projection (time-course) of the weighted mask in the data sets showing no significant anti-correlated changes of the CFP and YFP signals. (**d-f**) The response of larval chordotonal neurons to fructose stimulation. Solutions were changed in the same fashion, alternating between HL6-Lac and HL6-Fru. (**d**) A composite fluorescent and transmitted light image of *pain*-expressing chordotonal neurons in first abdominal segment of *pain-gal4*; *UAS-YC 2.1* larvae, which are located near the thoracic PAIN neurons imaged in Figure 2.5. Anterior to the left. (**e**) An eigenimage of the same tissue generated from the SOARS analysis. (**f**) The projection (time-course) showing no significant anti-correlated changes of the CFP and YFP signals. (**g**) The dynamic change of fructose concentration was monitored by flowing 0.0005% fluorescein through the perfusion chamber (blue trace). The black trace indicates the solution switching profile. Note the time lag between on and off switches and the corresponding changes in fluorescein concentration. Two complete cycles of solution alteration are shown here. Scale bars: 20 μm.

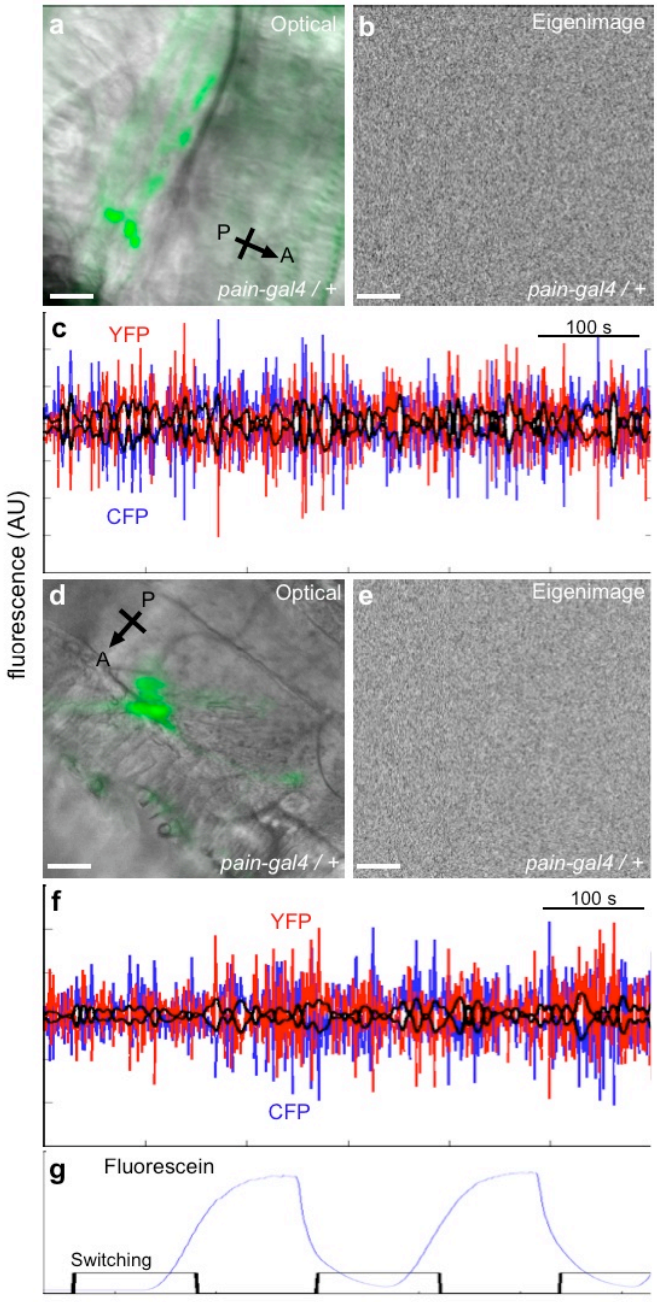
a
Optical
P
A
pain-gal4 / +
b
Eigenimage
pain-gal4 / +
c
YFP
100 s
CFP
fluorescence (AU)
d
Optical
P
A
pain-gal4 / +
e
Eigenimage
pain-gal4 / +
f
100 s
YFP
CFP
g
Fluorescein
Switching

Figure 2.8. Ablation of fructose-responsive PAIN neurons in the thoracic segments disrupts larval food aversion

(**a)** A live image of the anterior of a third-instar larva (74 h AEL) expressing UAS-*YC 2.1* driven by *pain-gal4.* Six clusters of fructose-responsive *pain*-expressing neurons located on the ventral side of three thoracic segments (T_1 to T_3) are shown (boxed). Scale bar, 50 μm. (**b-d)** The magnified images of PAIN neurons in the boxed areas from **a**. There are 6 neurons per cluster in T_1 and seven in T_2 or T_3. Scale bars, 10 μm. (**e**) Each experimental and the mock group include at least 18 larvae. Compared to the mock group, ablation of all six neuron clusters or two T_2 clusters caused significant reduction in food aversion ($n = 19$, $P < 0.01$). Larvae from other experimental groups (e.g., those ablated of one T_2 and one T_3 cluster) showed no significant behavioral changes ($n = 18$, $P > 0.08$).

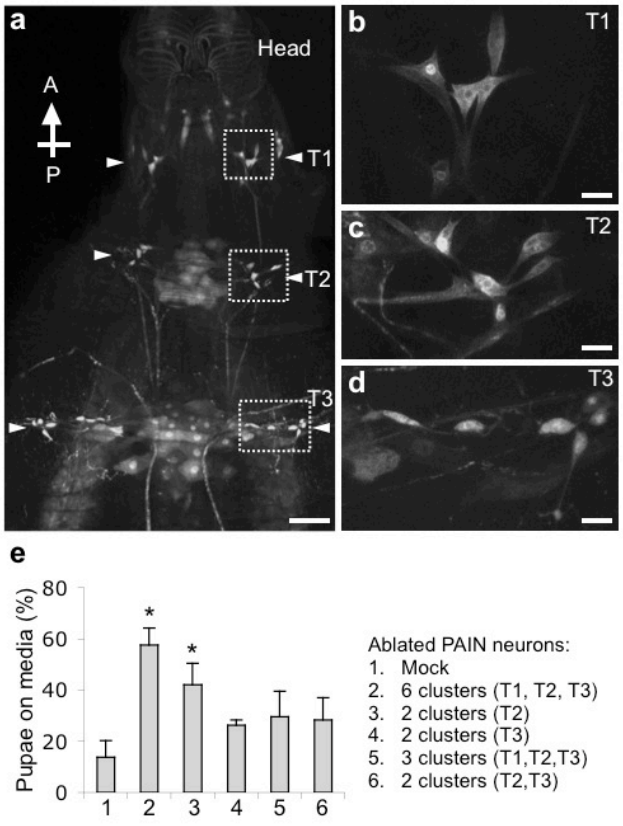
a
Head
A
P
T1
T2
T3
b
T1
c
T2
d
T3
e
Pupae on media (%)
80
60
40
20
0
1
2
3
4
5
6
*
*
Ablated PAIN neurons:
1. Mock
2. 6 clusters (T1, T2, T3)
3. 2 clusters (T2)
4. 2 clusters (T3)
5. 3 clusters (T1,T2,T3)
6. 2 clusters (T2,T3)

Figure 2.9. Localization of NPFR1-positive PAIN neurons

The nervous tissues of *pain-gal4* X UAS-DsRed larvae (96 h AEL, n = 15) were immunostained with affinity-purified anti-NPFR1 peptide and anti-DsRed antibodies, and imaged using a confocal microscope. (**a**) The anti-NPFR1 antibodies selectively stained a small set of *pain*-expressing neurons in three thoracic segments (arrowheads), which are absent in larvae expressing an attenuated diphtheria toxin driven by *npfr1-gal4* (also see Figure S3 in supplemental data). Scale bar, 100 μm. The neurons in the boxed regions are shown below. (**b** and **c**) The magnified views of NPFR1-positive PAIN neurons in the second (T2) and third thoracic (T3) segment, respectively. Scale bars, 30 μm. (**d** and **e**) The new anti-NPFR1 peptide antibodies showed an immunofluorescence staining pattern in CNS similar to those previously published (Wu et al., 2003). A partial stack of confocal images is shown, which include six NPFR1-positive neurons at the dorsomedial surface of the ventral nerve cord (see arrowheads in **e** Merged) and neuropils of the central brain lobes. NPFR1 expression pattern in the CNS does not appear to overlap with that of DsRed directed by *pain-gal4* (see magnified views in **e**). Scale bars, 50 μm.

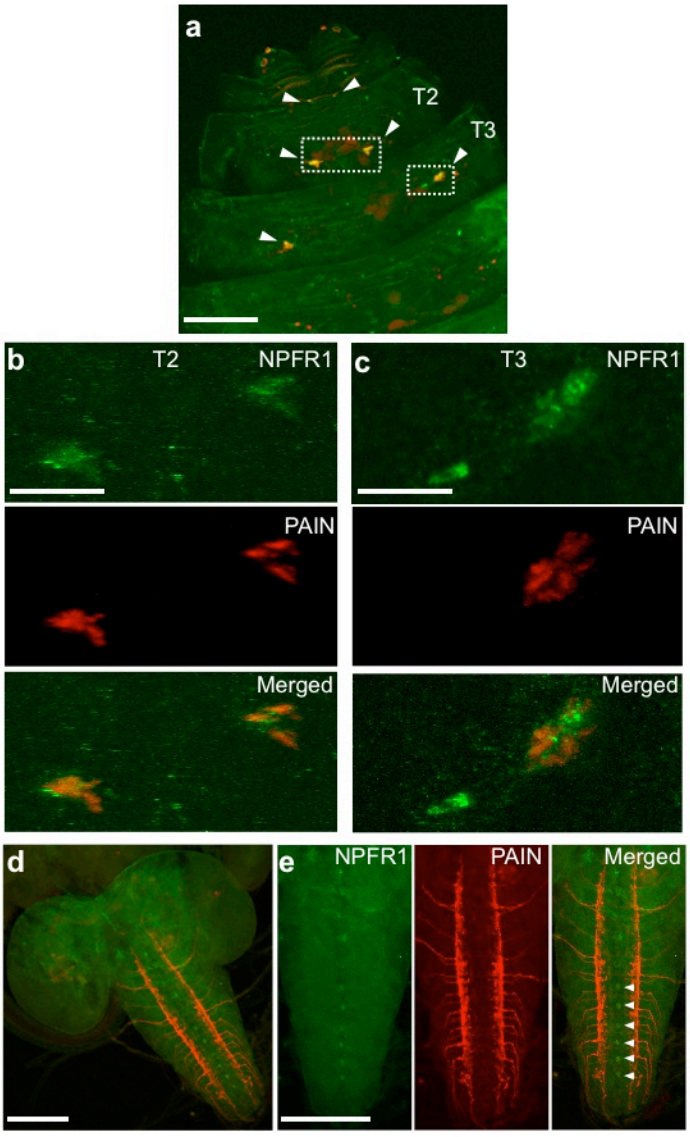
a
T2
T3
b
T2
NPFR1
PAIN
Merged
c
T3
NPFR1
PAIN
Merged
d
e
NPFR1
PAIN
Merged

Figure 2.10. The affinity purified anti-NPFR1 antibody selectively stains cells near Keilin's Organs in larval ventral epidermis

UAS-*DTI* encodes an attenuated diphtheria toxin (Han et al., 2000). (**a**) The NPFR1 immunoreactivity was detected in the ventral epidermis of control larvae (UAS-*DTI* alone; 96 h AEL; n=12.). Arrowheads indicate the NPFR1 positive cells near the Keilin's Organ in the three thoracic segments. (**b**) *npfr1-gal4* X UAS-*DTI* larvae show no specific NPFR1 staining. The dorsal and terminal organs are auto fluorescent. n=9. Scale bars, 100 μm. (**c**-**e**) High-resolution view of NPFR1-positive cells in the three thoracic segments (T1 T2 and T3), respectively. These cells are located below the cuticle near the Keilin's Organs (**c** to **e**, DIC channels). Scale bars, 20μm.

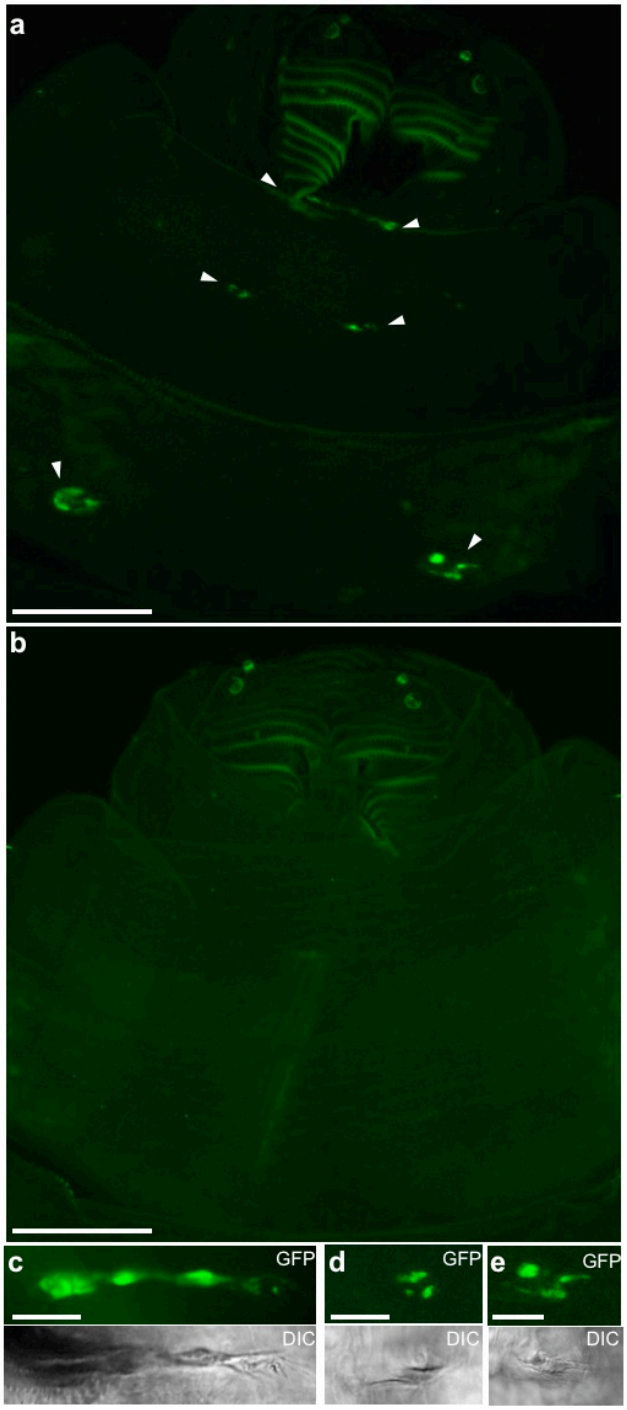
a
b
c
GFP
DIC
d
GFP
DIC
e
GFP
DIC

Table 2.1. Summary of the calcium imaging data.

Line	Age	Sitmulation paradigm	Neuron cluster examined	Anticorrelated signal	Number of tissues
pain^{gal4}/ + ; UAS-*YC 2.1*/ +	96h AEL	Lactose-fructose, alternate, 3 repeats	Ventral sensory neurons in T2 and T3 segments	Yes $p < 1X10^{-5}$	8
pain^{gal4}/ + ; UAS-*YC 2.1*/ +	74h AEL	Lactose-fructose, alternate, 3 repeats	Ventral sensory neurons in T2 and T3 segments	No	9
pain^{gal4}/ *pain*^{3} ; UAS-*YC 2.1*/ +	96h AEL	Lactose-fructose, alternate, 3 repeats	Ventral sensory neurons in T2 and T3 segments	No	8
pain^{gal4}/ + ; UAS-*YC 2.1*/ +	96h AEL	Lactose-lactose, alternate, 3 repeats	Ventral sensory neurons in T2 and T3 segments	No	8
pain^{gal4}/ + ; UAS-*YC 2.1*/ +	96h AEL	Lactose-fructose, alternate, 3 repeats	Chordotonal neurons in A1 segment	No	7

CHAPTER 3

DROSOPHILA NEUROPEPTIDE Y-LIKE PATHWAY DEFINES A CONSERVED ANTINOCICEPTIVE MECHANISM THA SUPPRESSES TRP CHANNEL-MEDIATED STRESSFUL STIMULATION[1]

[1] *Xu, J., *Li, M., & Shen, P., submitted to *Nature Neuroscience*, 04 / 2009

*Co-first authors

ABSTRACT

Pain threshold and tolerance vary greatly among individuals. Recent studies suggest that individuals with haplotypes associated with low neuropeptide Y (NPY) expression display diminished resiliency to pain and stress. *Drosophila* neuropeptide F (NPF, the sole member of the NPY family) also promotes stress resiliency and suppresses avoidance behavior of fly larvae. However, signaling mechanisms underlying antinociceptive activity of NPY family peptides remain largely uncharacterized. Here we show that NPF, like mammalian opioid peptides and endocannabinoids, suppresses peripheral noxious stimulation through its G-protein coupled receptor NPFR1. NPF/NPFR1 signaling negatively modulates different subtypes of fly and mammalian TRP-family ion channels expressed in larval sensory neurons. In human cells, NPFR1 attenuates TRPV1-mediated Ca^{2+} influx and its enhancement by cAMP analogs. Similarly, the NPF/NPFR1 pathway also blocks sensitization of larval aversive response by cAMP-dependent protein kinase (PKA). Our findings suggest that a common signaling mechanism underlies endogenous antinociceptive activities of insects and humans.

INTRODUCTION

The nociceptive system, which senses and processes noxious stimuli, is essential for self-preservation of animals. Malfunction of the nociceptive system could have seriously adverse consequences. Neuropathic pain, a common chronic disorder that remains difficult to treat, is attributed to lesions or dysfunction of the nervous system (Niederberger et al., 2008). It is increasingly recognized that genetic factors may significantly impact the likelihood of development of chronic pain and the effectiveness of pharmacotherapy (Oertel and Lotsch, 2008). For example, erythermalgia, a pain disorder characterized by severe burning sensation in

the extremities, is resulted from a gain of function in *SCN9A*, which encodes the alpha subunit of voltage gated sodium channels Nav1.7 (Cox et al., 2006). NPY family peptides, which are widely conserved among metazoans, have also been implicated in stress and pain response (Bannon et al., 2000; Thorsell and Heilig, 2002; Thorsell et al., 2000; Wu et al., 2005a; Wu et al., 2005b). Recent studies suggest that individuals with haplotypes of low neuropeptide Y (NPY) expression levels are more prone to develop pain and stress disorders (Zhou et al., 2008).

The transient receptor potential (TRP) family cation channel proteins are evolutionarily conserved sensors of diverse stressful stimuli (Montell, 2005a; Montell et al., 2002; Moran et al., 2004; Ramsey et al., 2006). Mammalian TRPV1, a well-characterized polymodal channel protein, responds to noxious heat, protons and capsaicin, and plays a prominent role in nociceptive sensation (Caterina and Julius, 2001; Caterina et al., 1997). Sensory neurons are one of the major sites of TRPV1 expression. Endogenous pain suppressors such as opioid peptides and endocannabinoids attenuate TRPV1-mediated external noxious stimulation through their G-protein coupled receptors expressed in peripheral nociceptors (Diaz-Laviada and Ruiz-Llorente, 2005; Endres-Becker et al., 2007; Mukhopadhyay et al., 2002). On the other hand, TRPV1 activity can also be sensitized by diverse signaling molecules and kinase-mediated pathways, many of which are responsible for inflammatory and neuropathic pain (Hucho et al., 2005; Mostany et al., 2008; Zhu et al., 2007).

The *Drosophila* TRPA channel protein PAINLESS (PAIN) is required for aversive responses to thermal, mechanical and chemical stressors (Al-Anzi et al., 2006; Tracey et al., 2003). *Drosophila* larvae cease feeding before metamorphosis, and display a PAIN-dependent behavioral switch from sugar attraction to aversion (Xu et al., 2008). These sugar-averse behaviors are beneficial to the survival of postfeeding larvae into the adulthood. For example,

they prevent immobile pupae from microbial killing and drowning by liquid food by driving postfeeding larvae out of the feeding habitat (e.g., fallen overripe fruits) to search for safer pupation sites such as food-free soil or the surface of fly bottles (Ashburner, 1989; Chiang, 1950). The PAIN-mediated neuronal pathway for sugar avoidance must be suppressed in younger feeding larvae that live mostly inside sugar-rich food proper. We have shown that fly NPY-like brain peptide NPF suppresses PAIN-mediated sugar aversion throughout early larval development; Loss of NPF signaling in feeding larvae is sufficient to trigger precocious sugar-averse behaviors (Wu et al., 2003). Therefore, NPF regulation of TRP channel-mediated food aversion in *Drosophila* larvae appears to be a useful model for elucidation of signaling mechanisms that regulate an animal's sensation and processing of external stressful stimuli.

In this work, we investigate how the fly NPY-like system suppresses TRP channel-mediated response to peripheral stressful stimuli. We show that NPF and its G-protein coupled receptor NPFR1 negatively regulate activities of different subtypes of fly and mammalian TRP channels expressed in larval sensory neurons. We also found that larval response to aversive cues is sensitized by increased activity of cAMP-dependent protein kinase (PKA) in PAIN neurons, and the NPF/NPFR1 pathway blocks PKA sensitization of PAIN neurons. Like mammalian opioid peptides and endocannabinoids, NPF suppresses TRPV1-mediated Ca^{2+} influx in NPFR1-expressing human embryonic kidney (HEK) 293 cells. Our findings suggest that endogenous antinociceptive activities of invertebrates and mammals utilize a conserved signaling mechanism.

RESULTS

NPFR1-mediated suppression of peripheral aversive stimulation

The G-protein coupled NPF receptor NPFR1 is selectively expressed in a subset of *pain*-expressing, sugar-responsive neurons in the thoracic body wall (Xu et al., 2008, also see Figure 3.1a). We decided to test how NPFR1 overexpression, driven by *pain-gal4*, may affect the sugar-averse behaviors of postfeeding larvae (96 h *a*fter *e*gg *l*aying, AEL). In the apple juice medium, a majority of control larvae (e.g., UAS-*npfr1*^{cDNA} alone) migrated out of the soft food medium and pupated at food-free sites. In contrast, most of the experimental larvae (*pain-gal4/*UAS-*npfr1*^{cDNA}) showed attenuated aversion to apple juice (Figure 3.1b, c). Moreover, postfeeding larvae expressing NPF (*pain-gal4/*UAS-*npf*) also showed attenuated food aversion. Thus, increased NPF or NPFR1 activity dominantly suppresses larval food-averse migration

When exposed to hard sugar-containing media, postfeeding but not younger feeding larvae rapidly swarm towards each other and form stable aggregates (Wu et al., 2003). This instinctive cooperative behavior may enable larvae to quickly burrow through sugar-containing media (e.g. fruit juice-stained top soil underneath fallen fruits) for pupation (Alyokhin et al., 2001; Thomas, 1995). We also found that *pain-gal4/*UAS-*npfr1*^{cDNA} postfeeding larvae failed to display aggregation and burrowing on the 10% fructose agar medium (Figure 3.2b, c). The opposite phenotype was also observed with younger feeding larvae (74 h AEL) expressing double-stranded *npfr1*RNA (*pain-gal4/*UAS-*npfr1*^{dsRNA}) using a sugar-stimulated grouping assay. Like NPF signaling-deficient larvae (Wu et al. 2003), *pain-gal4/*UAS-*npfr1*^{dsRNA} larvae precociously displayed a sugar-induced grouping behavior normally associated with older, postfeeding larvae (Figure 3.2d, e). In combination, these findings provide functional evidence

that NPF directly modulates peripheral sensory neurons through its G-protein coupled receptor NPFR1.

NPFR1 Suppression of TRPA channel-mediated Ca^{2+} influx

To investigate how NPFR1 might affect the activity of PAIN neurons, we imaged neuronal excitation with a Ca^{2+} indicator, yellow cameleon 2.1 (YC2.1; (Miyawaki et al., 1999). The data were subsequently processed using the SOARS algorithm, which is designed to extract the anti-correlated changes between yellow and cyan fluorescence levels in response to fructose stimulation (Broder et al., 2007). We found that similar to the situation in *pain*^{3} larvae (96h AEL), fructose failed to trigger excitation of the thoracic PAIN neurons in *pain-gal4*/UAS-*npfr1*^{cDNA} larvae (96h AEL; Figure 3.3) (Xu et al., 2008). Thus, NPFR1 appears to suppress larval PAIN neuronal activity by blocking the opening of the TRPA channel.

NPFR1 Suppression of TRPA channel-mediated nociception

In *pain-gal4*/UAS-*npfr1*^{cDNA} larvae, NPFR1 is ectopically expressed in the entire set of PAIN neurons including those that are responsive to noxious heat (Tracey et al., 2003). We therefore tested how NPFR1 might affect noxious heat response by those larvae. We found that *pain-gal4* X UAS-*npfr1*^{cDNA} larvae showed significantly delayed aversive response to the touch of a 40 °C probe (Figure 3.4a, (Tracey et al., 2003). In addition, the PAIN-mediated response to benzyl-isothiocyanate, the pungent ingredient of horseradish, was also abolished by NPFR1 overexpression in adult PAIN neurons (Figure 3.4b**)** (Al-Anzi et al., 2006). These results demonstrate that NPF and NPFR1 define a fly antinociceptive system capable of suppressing peripheral stressful stimulation mediated by nociceptive TRPA channels.

Suppression of mammalian TRPV1-mediated avoidance by NPFR1

Wild type *Drosophila* larvae display neither attractive nor aversive response to capsaicin, the spicy substance from hot chili peppers. However, expression of a rat capsaicin receptor TRPV1 in PAIN neurons of postfeeding larvae is sufficient to trigger larval aversion to capsaicin (Xu et al., 2008). This finding provides an opportunity to test whether the NPF system is capable of suppressing a mammalian nociceptive TRP channel of a different subtype in *Drosophila* sensory neurons. We found that postfeeding larvae co-expressing NPFR1 and TRPV1, driven by *pain-gal4*, failed to display capsaicin-averse behaviors (Figure 3.5). Consistent with this finding, younger feeding larvae expressing rat TRPV1 are also insensitive to capsaicin (Xu et al., 2008). These results suggest that the NPF/NPFR1 pathway suppresses different subtypes of TRP channels, and its antinociceptive activity is mediated by a signaling mechanism conserved between flies and mammals.

NPFR1 suppression of TRP channels in human cells

HEK 293 cells have been widely used for studying the suppression of TRPV1 activity by mammalian opioid receptors (Diaz-Laviada and Ruiz-Llorente, 2005; Vetter et al., 2008). To provide direct evidence that NPF/NPFR1 signaling suppresses TRP channels through a conserved antinociceptive mechanism, we tested whether NPFR1 signaling is sufficient to suppress TRPV1 in human cells. Both *npfr1* and rat *TRPV1* cDNAs were co-expressed in HEK 293 cells, and capsaicin-induced Ca^{2+} influx was imaged using Fluo-4 and Fura-red fluorescent dyes (Figure 3.6). We found that HEK293 cells, co-transfected with NPFR1 and rat TRPV1 cDNAs, displayed significantly reduced TRPV1-mediated Ca^{2+} influx relative to control groups during a 300-sec test period (Figure 3.6a-f). For example, HEK293 cells transfected with

TRPV1 cDNA alone showed significant Ca^{2+} influx in response to capsaicin within 30 seconds, and TRPV1 channels remained active during the entire test period. Experimental cells co-transfected with both NPFR1 and TRPV1 cDNAs, in the presence of NPF, showed drastically attenuated and delayed responses to capsaicin. During the initial 200-second period, cells showed low levels of calcium-dependent fluorescence, and a subset of cells displayed an increase of intracellular Ca^{2+} between 200-300 seconds (Figure 3.6e, f). We have observed that addition of NPF to cells transfected with TRPV1 cDNA alone caused a mild reduction in TRPV1 activity (Figure 3.6f). Since HEK293 cells express endogenous NPY receptor subtypes (Y2 and Y5, unpublished data), this mild inhibitory effect of NPF might be due to its cross-activation of an endogenous NPY receptor. Our results demonstrate again that NPFR1 suppression of nociceptive TRPV1activity is mediated by a signaling mechanism conserved between flies and humans.

Modulation of TRPV1 by cyclic nucleotides and NPF

It has been shown that the cAMP / PKA pathway potentiates TRPV1 activity in HEK 293 cells and nociceptive sensory neurons, and may be acutely involved in inflammatory and neuropathic pain (Bhave et al., 2002). We also observed that bath application of a cAMP analog (8-Br-cAMP) to HEK 293 cells significantly increased TRPV1-mediated Ca^{2+} influx (Figure 3.7a). Using this sensitized *in vitro* model, We found that NPFR1 was capable of attenuating the potentiation of TRPV1 by 8-Br-cAMP, providing further evidence that fly NPFR1 functions well in heterologous mammal cells. In addition, 8-Br-cGMP, a cGMP analog, slightly reduced TRPV1 activity, and NPFR1 suppression of TRPV1 was significantly enhanced in the presence of 8-Br-cGMP (Figure 3.7b). Pharmacological evidence suggests that NPFR1 is coupled with

$G_{i/o}$ protein (Garczynski et al., 2002). Therefore, it is possible that the antinociceptive NPFR1 may involve downregulation of intracellular cAMP though inhibition of adenylyl cyclase.

NPF/NPFR1 suppression of PKA-induced hypersensitivity

The *in vitro* finding has led us to wonder if PKA has a sensitizing effect on PAIN-mediated larval sugar aversion. Indeed, we found that postfeeding larvae expressing a constitutively active form of PKA (UAS-*PKAc*), driven by *pain-gal4*, showed aversive response to agar media regardless of the presence or absence of sugar (Figure 3.7c,d). For example, a majority of *pain-gal4 /* UAS-*PKAc* larvae migrated out of the sugar-free soft medium, while control larvae (e.g., UAS-*PKAc* alone) pupated mostly on the medium. The *pain-gal4/* UAS-*PKAc* larvae also displayed sugar-independent grouping behavior (data not shown). These results indicate that the increased activity of the cAMP / PKA pathway causes sensitized behavioral response to media. Mammalian PKA has been shown to sensitize TRPV1 channels and acutely mediates hyperexcitation of nociceptive sensory neurons (Hu et al., 2001; Song et al., 2006). It is possible that fly PKA may have similar excitatory effects in PAIN neurons.

We further tested whether the NPF pathway is able to suppress sugar aversion of PKA-sensitized larvae. Postfeeding larvae expressing UAS-*PKAc* and UAS-*npf*, directed by *pain-gal4*, were placed onto apple juice and sugar-free soft agar media. We found that most of the larvae pupated on both media (Figure 3.7c, d). Moreover, larvae coexpressing UAS-*PKAc* and UAS-$npfr1^{cDNA}$ also displayed similar phenotype (Figure 3.7d). Consistent with these observations, younger feeding larvae that overexpress PKAc (*pain-gal4/* UAS-*PKAc*; 74hAEL) did not show any precocious food-averse behaviors (data not shown). These results indicate that the NPF pathway remains fully capable of suppressing sensitization of PAIN neurons by

exuberant PKA activity. These results indicate that the NPF pathway has a dominant suppressive effect on the sensitization of PAIN neurons by exuberant PKA activity. It is important to note that constitutively active PKAc is insensitive to the reduction of intracellular cAMP (Kiger et al., 1999). Therefore, the inhibitory effect of the $G_{i/o}$-protein coupled NPF receptor on PAIN neurons may involve additional signaling mechanism(s) independent of PKA.

DISCUSSION

Nociception of invertebrates and mammals is mediated by conserved signaling mechanisms involving TRP family channels (Montell, 2005a; Montell et al., 2002; Moran et al., 2004; Ramsey et al., 2006). In this study, we provide the first evidence that the antinociceptive functions of invertebrates and mammals are also mediated by a conserved mechanism that suppresses TRP family channels. Given the implicated role of human NPY in stress and pain resiliency (Bannon et al., 2000; Thorsell and Heilig, 2002; Thorsell et al., 2000; Zhou et al., 2008), our findings suggest that the *Drosophila* NPY-like system can be a useful model for the identification and characterization of genetic factors that influence pain threshold and tolerance as well as genetic predispositions to pain disorders.

The G-protein coupled receptors of mammalian opioid peptides and endocannabinoids are expressed in peripheral nociceptors, and mediate suppression of peripheral noxious stimulation (Endres-Becker et al., 2007; Pertwee, 2001). In this study, we demonstrate that the *Drosophila* NPY-like system suppresses peripheral stressful stimulation in feeding larvae. Several lines of evidence suggest that NPF directly acts on sensory neurons expressing TRPA channel protein PAIN (Xu et al., 2008). First, the G-protein coupled NPF receptor NPFR1 is expressed selectively in a subset of PAIN sensory neurons responsive to aversive sugar

stimulation. Second, laser ablation of NPFR1-expressing PAIN neurons abolished larval aversion to sugar. Finally, overexpression of NPFR1 in PAIN neurons blocks sugar-stimulated TRPA channel activity. The mammalian NPY receptor Y1 is also expressed in the primary nociceptive neurons of dorsal root ganglia (DRG) and trigeminal ganglia (Brumovsky et al., 2007). Y1 has been reported to inhibit capsaicin-stimulated release of calcitonin gene-related peptide (Gibbs and Hargreaves, 2008). Together, these findings suggest that NPY family peptides are involved in the modulation of the sensation of external stressful stimuli in flies and possibly mammals.

TRPV1 appears to be one of the primary targets of endogenous antinociceptive activities in mammals (Gunthorpe and Chizh, 2009). TRPV1 and the receptors of opioid peptides, endocannabinoids and NPY colocalize in different nociceptors (Endres-Becker et al., 2007; Gibbs and Hargreaves, 2008; Pertwee, 2001). It has also been shown that both opioid and cannabinoid receptors suppress TRPV1-medited Ca^{2+} influx in primary sensory neurons or in HEK 293 cells (Endres-Becker et al., 2007; Vetter et al., 2008). Now we have obtained evidence that activation of NPFR1 also suppresses TRP channel activities in larval sensory neurons and heterologous HEK293 cells. These findings suggest that a conserved signaling mechanism may underlie the suppression of peripheral stressful stimulation by invertebrate and mammalian antinociceptive activities. It remains largely unclear how NPFR1 and mammalian opioid and cannabinoid receptors negatively regulate TRPV1 activity. Since all of these receptors are coupled with $G_{i/o}$ (Demuth and Molleman, 2006; Diaz-Laviada and Ruiz-Llorente, 2005; Garczynski et al., 2002; Mukhopadhyay et al., 2002; Pertwee, 2001), their antinociceptive activities may be mediated by a common mechanism involving downregulation of adenylate cyclase and intracellular cAMP.

Expression of mammalian TRPV1 causes the transgenic larvae to display migratory and grouping behaviors in response to aversive capsaicin stimulation. However, increased PKA activity in the PAIN neurons of postfeeding larvae is sufficient to elicit such behaviors without the need of any aversive chemicals in the medium. Thus, higher PKA activity appears to sensitize larval nociceptive sensory neurons. Consistent with this notion, PKA has been shown to potentiate TRP channel activity through direct phosphorylation of the N-terminal domain (Bhave et al., 2002). PKA activity can also reverse the desensitized TRP channels. In the DRG sensory neurons of rats with spinal nerve ligation, PKA activity is acutely required for the peripheral neuropathic pain (Hu et al., 2001). Future experiments will determine whether the PKA-elicited independence of aversive stimulation is resulted from its sensitization of an endogenous TRP channel (e.g., PAIN).

It remains to be determined how NPFR1 signaling dominantly suppresses the migratory and grouping behaviors in wild type and PKA-overexpressing larvae. One simple explanation is that NPFR1 signaling may lead to a large reduction of the intracellular cAMP level, thereby downregulating the activity of PKA. However, we consider this explanation is not completely satisfactory because the transgenic UAS-PKAc construct encodes a constitutively active form of PKA whose activity is cAMP-independent. Therefore, our finding strongly argues for the existence of an additional PKA-independent mechanism(s) by which NPFR1 suppresses TRP channel in PKA-overexpressing PAIN neurons.

NPY family peptides have been shown to promote diverse stress-resistant behaviors (Bannon et al., 2000; Thorsell and Heilig, 2002; Thorsell et al., 2000). Overexpression of NPFR1 in flies and administration of NPY in mice rendered animals to be more willing to work for food and become more resilient to aversive taste and deleteriously cold temperature (Flood

and Morley, 1991; Jewett et al., 1995; Lingo et al., 2007; Wu et al., 2005a; Wu et al., 2005b). Our finding of suppression of different TRP family channels by a single receptor NPFR1 provides a molecular explanation of how a NPY family peptide could mediate resiliency to diverse gustatory, thermal and mechanical stressors. We previously showed that in food-deprived larvae, reduced fly insulin signaling triggers NPFR1-mdiated stressor-resistant foraging activities (Wu et al., 2005a; Wu et al., 2005b). Our findings also raise the possibility that TRP family channels may be indirectly regulated by insulin signaling.

METHODS

Flies, media and larval growth

Conditions for rearing adult flies and egg collection were described previously (Shen and Cai, 2001; Wen et al., 2005). The larvae were raised at 25 °C with exposure to natural lighting. Synchronized eggs were collected within a 2 h interval, and late second instars were transferred to a fresh apple-juice plate with yeast paste (< 80 larvae per plate). The *pain-gal4*, UAS-$TRPV1^{E600K}$, UAS-$npfr1^{cDNA}$, UAS-$npfr1^{dsRNA}$, UAS-*npf* and UAS-*PKAc* lines are in the *w* background (Kiger et al., 1999; Marella et al., 2006; Tracey et al., 2003; Wen et al., 2005; Wu et al., 2003).

Behavioral assays

The migration assay on soft agar media was performed as described previously (Xu et al., 2008). Twenty-five postfeeding larvae (96h AEL) in one plate were allowed to move freely on the medium, and those that crawled onto the plastic surface became less mobile, and eventually formed pupae there. The percentage of pupae on agar media was scored after 24 hours. The

clumping assay was also described before (Wu et al., 2003). Briefly, 45 mm petri dishes containing solid fructose agar (3% agar in a 10% fructose solution) were coated with a thin layer of yeast paste (0.5 g yeast powder in 10% fructose solution). Thirty larvae per plate were allowed to browse for 30 min, and those in clumps were scored immediately. The social burrowing assay was performed on solid agar media containing apple juice or 10% fructose, as described previously (Wu et al., 2003). All assays, unless stated otherwise, were performed at room temperature in the dark. At least three separate trials were performed per assay.

The thermonociception assay was performed according to previously published procedure with modifications (Tracey et al., 2003). The temperature of the electric heating probe was set at 40 °C using a variable transformer (Model 72-110, Tenma), and monitored by a digital thermometer (Model 52 II, Fluke). At least 150 larvae (96h AEL) were individually tested for each line.

The two-choice preference test was based on a published procedure with some modifications (Al-Anzi et al., 2006). 2-day-old males withheld from food for 24 hr were presented with a 48-well plate containing two colored food media in the alternating well rows. They contain 1% agar /10% fructose with 0.4 mM benzyl-isothiocyanate (BITC) in 75% ethanol or ethanol only. In each assay, 90–100 flies per line were tested in the dark. A preference index was defined as the fraction of larvae choosing the BITC medium, minus the fraction of larvae choosing the BITC-free medium. A preference index close to +1 indicates that the larvae are attracted to BITC, whereas −1 indicates strong rejection. At least three trials were done for each line.

Immunohistochemistry

The larval epidermis was filleted from the dorsal side. Tissues were fixed for 35 min with 4% paraformaldehyde, washed in PBS with 0.4% TritonX-100, and permeablized with Proteinase K digestion followed by post-fixation. Tissues blocked in 10% BSA were then incubated overnight at 4˚C with affinity-purified rabbit anti-NPFR1 antibodies (1:100). The NPFR1 peptide antibodies were raised and purified using two peptide antigens (CMTGHHEGGLRSAIT and SSNSVRYLDDRHPLC). Alexa 488-conjugated anti-rabbit IgG secondary antibody was diluted to 1:2,000. At least 15 epidermal tissues were examined.

***In vivo* calcium imaging**

Detailed procedures for calcium imaging of sensory neurons and SOARS (Statistical Optimization for the Analysis of Ratiometric Signals) analysis were described previously (Xu et al., 2008). The SOARS method extracts the anti-correlated change of Fluo-4 and Fura-Red signals in a cell (represented by a cluster of ~100 pixels) that respond to stimulations in a common dynamic pattern. At least 6 epidermal tissues were imaged for each group. To quantify the significance of anti-correlated FRET response to fructose stimulation, we calculated p-values for a periodic (sinusoidal) response at the stimulus frequency using multitaper harmonic analysis, a common method for the detection of sinusoids in noisy data (Thomson, 1982).

Transfection and Calcium imaging of HEK 293 cells

HEK293 cells were maintained under standard conditions (37 °C, 5% CO2) in Dulbecco's Modified Eagle's Medium (Mediatech) supplemented with 10% fetal bovine serum, penicillin and streptomycin. pcDNA3.1D directional expression system (Invitrogen) was used to

clone and express rat TRPV1 and fly NPFR1 cDNA in HEK293 cells. TRPV1 cDNA was PCR amplified from $TRPV1^{E600K}$ sequence (Marella et al., 2006), using the following primers: 5' CACCATGGAACAACGGGCTAGCTTA 3' and 5' TTCTTTCTCCCCTGGGACCATGGA 3'. NPFR1 cDNA was PCR amplified from an NPFR1 cDNA plasmid (Garczynski et al., 2002), using two primers: 5' CACCATGATAATCAGCATGAATCAGA 3' and 5' TTACCGCGGCATCAGCTTGGT 3'. HEK293 cells were cultured on 8-well polyornithine-coated chambered coverglasses (Nunc) for 24 hrs before transfection. A suspension of 200 μl of water containing 0.4 μg plasmid DNA and 0.8 μl of Lipofectamine 2000 (Invitrogen) was used for transfection. The amounts of TRPV1 and NPFR1 cDNA used were 20 ng and 200 ng, respectively. pcDNA3.1 vector DNA was supplemented, when necessary, to ensure the equal amount of total DNA per transfection.

Calcium imaging was performed between 36-42 hours post transfection at 23 °C. Cells were loaded with 1 μM Fluo-4 and 2 μM Fura-red (Invitrogen) for 90 min in Hanks' balanced salt solution (HBSS; Gibco), washed once with HBSS, and imaged using a Zeiss Axiovert 200M scope equipped with a Zeiss LSM 510 Meta laser scanning module. Dyes were excited at 488nm with argon laser. Emission fluorescence was filtered by a BP505-530 and an LP585 filter. Images were collected for 300 s upon capsaicin stimulation at 1 frame s^{-1}, and analyzed with the SOARS analysis. NPF was synthesized by Quality Controlled Biochemicals. NPF and cyclic nucleotide analogs (Sigma) were pre-incubated at 23 °C with cells for 20-25 min before adding capsaicin.

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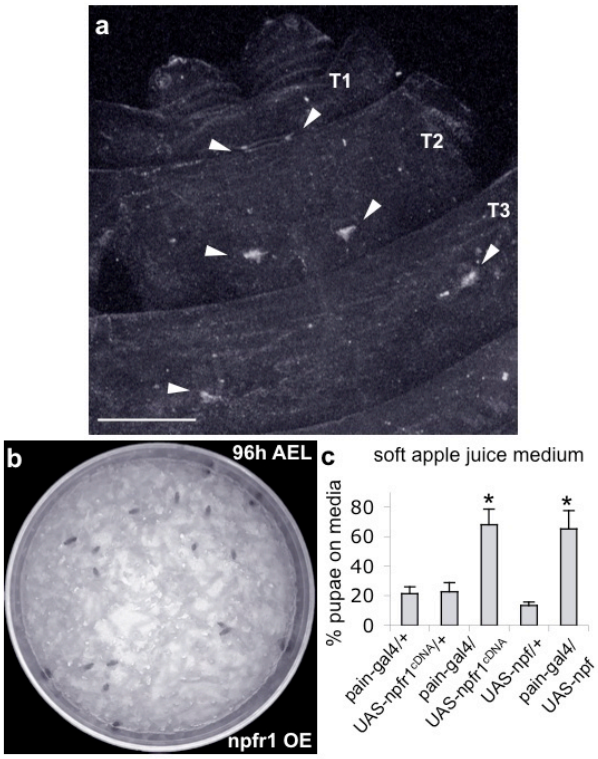
Figure 3.1. Suppression of PAIN neuron-mediated food-averse migration by the NPF/NPFR1 pathway

(**a**) An confocal image of sugar-responsive thoracic PAIN neurons expressing NPFR1. NPFR1 immnunoreactivity is detected in small set of *pain*-expressing neurons in three thoracic segments using affinity-purified anti-NPFR1 peptide antibodies (n = 15; Xu et al., 2008). Scale bar, 100 μm. (**b**) UAS-*npfr1*^{cDNA} and UAS-*npf* contain an *npfr1* and *npf* coding sequence, respectively. Most postfeeding larvae overexpressing NPFR1 in PAIN neurons pupated on apple juice agar paste, while control larvae migrated out of the food medium to pupate on food-free surface. (**c**) Quantification of the larval migratory activities. $P<0.001$. In all figures, unless otherwise stated, error bars represent standard deviations. At least 3 separate trials were performed for each experiment. Asterisks indicate statistically significant differences from paired controls using ANOVA followed by Student-Newman-Keuls analysis.


a
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pain-gal4/+
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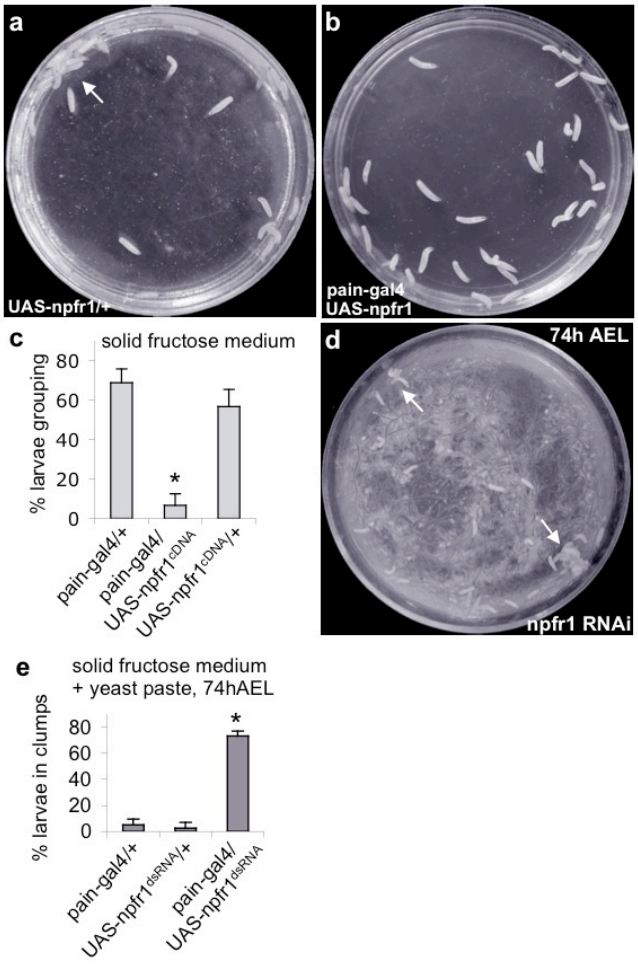
pain-gal4/
UAS-npfr1cDNA
UAS-npf/+
pain-gal4/
UAS-npf

Figure 3.2. Regulation of sugar-stimulated social response by increased or decreased NPFR1 signaling

(**a**) Postfeeding control larvae (e.g., UAS-*npfr1cDNA*/+, 96h AEL) normally show a sequential display of dispersing, clumping and cooperative burrowing on the solid 10% fructose agar medium within a 30-min period. The arrow indicates a cooperative burrowing site. Pictures were taken 30 min after the larvae were transferred onto the medium. (**b**) Postfeeding larvae over-expressing NPFR1 in PAIN neurons showed no clumping or burrowing activity on 10% fructose agar medium even after 1.5 hr. (**c**) Quantification of the larval clumping activities. $P<0.001$. (**d**) UAS-$npfr1^{dsRNA}$ encodes an *npfr1* double-stranded RNA. Young control larvae (74h AEL) disperse randomly on solid fructose agar coated with a thin layer of 10% fructose yeast paste. But at least 70% of younger experimental larvae (*pain-gal4*/UAS-$npfr1^{dsRNA}$, 74h AEL) behaved like older postfeeding larvae, displaying stable aggregation at the edge of the plate (arrows; (Wu et al., 2003). (**e**) Quantification of the larval clumping activities. $P<0.001$.


a
UAS-npfr1/+
b
pain-gal4
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solid fructose medium
% larvae grouping
80
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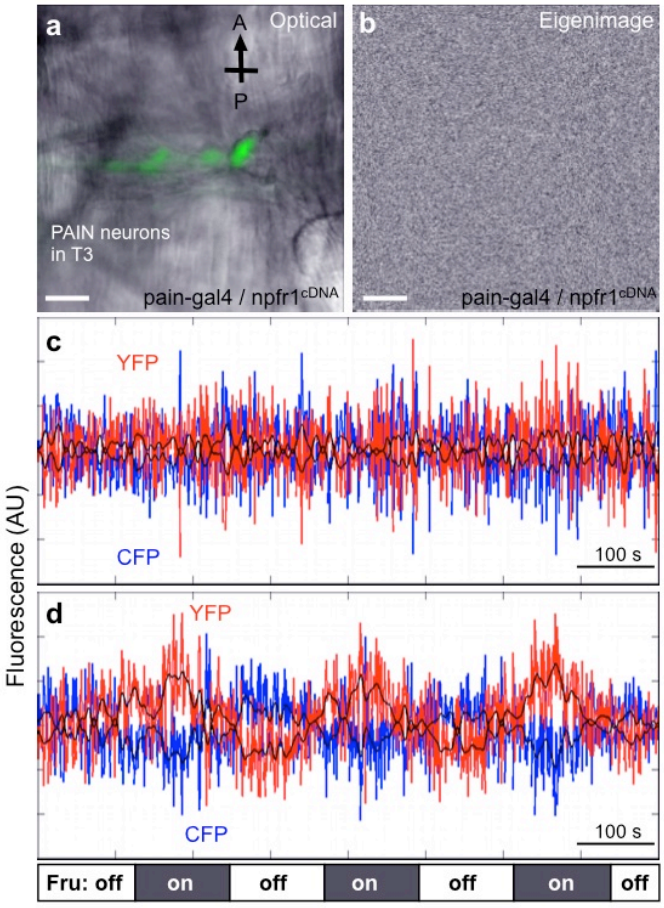
*
pain-gal4/+
pain-gal4/
UAS-npfr1cDNA
UAS-npfr1cDNA/+
d
74h AEL
npfr1 RNAi
e
solid fructose medium
+ yeast paste, 74hAEL
% larvae in clumps
80
60
40
20
0
*
pain-gal4/+
UAS-npfr1dsRNA/+
pain-gal4/
UAS-npfr1dsRNA

Figure 3.3. Imaging and SOARS analysis of excitation of thoracic PAIN neurons by fructose with the cameleon Ca^{2+} indicator

Stimulation paradigm: the tissue was initially perfused with HL6-Lactose solution for up to 10 min before imaging. During imaging, solutions were changed every 120 seconds, alternating between HL6-Lactose and HL6-Fructose. At least 6 tissues per group were imaged. Scale bars: 20 μm. (**a**) A composite fluorescent and transmitted light image of *pain*-expressing neurons from the ventral left cluster in the third thoracic segment of the NPFR1-overexpressing larva (*pain-gal4*, UAS-$npfr1^{cDNA}$; UAS-*YC 2.1*). CFP fluorescence is shown in green. (**b**) An eigenimage of the above tissue generated from the SOARS analysis of the cameleon YFP/CFP FRET data. This eigenimage facilitates the identification of pixels that may display spatially correlated, temporally anti-correlated fluorescence changes selectively responding to fructose stimulation (Xu et al., 2008). No spatial-correlated pixels were detected. (**c**) The time-course of the weighted mask in the data sets shows no periodic anti-correlated changes of the CFP and YFP signals (blue and red traces, respectively). (**d**) Imaging and data analysis of the same set of thoracic *pain*-expressing neurons from control larvae (*pain-gal4*; UAS-*YC 2.1*) that display periodic anti-correlated changes of the CFP and YFP signals under the same conditions as described above. The fructose stimulation paradigm is indicated at the bottom. Three complete cycles of solution alternation are shown here.


a
A
P
Optical
PAIN neurons
in T3
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pain-gal4 / npfr1cDNA
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Fluorescence (AU)
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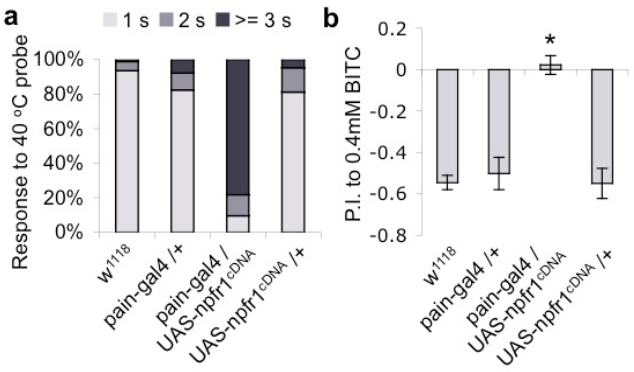
on
off

Figure 3.4. NPFR1 suppresses PAIN-mediated thermal nociception in larvae and chemical nociception in adults

(**a**) NPFR1 suppresses PAIN-mediated thermal nociception in larvae. Most wild type larvae display a stereotypical rolling behavior within 1 sec when touched by a 40 °C probe to the lateral body wall (Tracey et al., 2003). In contrast, the majority of NPFR1 Over-expressers respond after 3 sec. $n > 100$ for each line tested. (**b**) NPFR1 suppresses PAIN neuron-mediated chemical nociception in adults. 2-day old control flies mostly avoided 0.4 mM BITC. However the NPFR1 over-expressing flies showed no preference to either of the two types of food. $n > 250$ for each line tested.


a
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Response to 40 °C probe
100%
80%
60%
40%
20%
0%
w1118
pain-gal4 /+
pain-gal4 / UAS-npfr1cDNA
UAS-npfr1cDNA /+
b
P.I. to 0.4mM BITC
0.2
0
-0.2
-0.4
-0.6
-0.8
*
w1118
pain-gal4 /+
pain-gal4 / UAS-npfr1cDNA
UAS-npfr1cDNA /+

Figure 3.5. NPFR1 suppresses larval avoidance to capsaicin induced by ectopically expressed mammalian TRPV1

UAS-*TRPV1*^{E600K} encodes a variant of the mammalian vanilloid receptor TRPV1. (**a**) Control larvae expressing UAS-*TRPV1*^{E600K} driven by *pain-gal4* migrate away from agar paste containing 400 nM capsaicin. (**b**) Experimental larvae expressing both NPFR1 and TRPV1^{E600K} in PAIN cells mostly remained on the capsaicin medium. (**c**) Quantification of avoidance response of transgenic larvae in media containing capsaicin. $P<0.001$. (**d**) Quantification of capsaicin-induced larval aggregation on the sugar-free capsaicin medium. Larvae expressing TRPV1 alone (*pain-gal4/* UAS-*TRPV1*^{E600K}) but not those co-expressing TRPV1 and NPFR1 (*pain-gal4/* UAS-*TRPV1*^{E600K}/UAS-*npfr1*^{cDNA}) showed capsaicin-elicited aggregation. $P<0.001$.

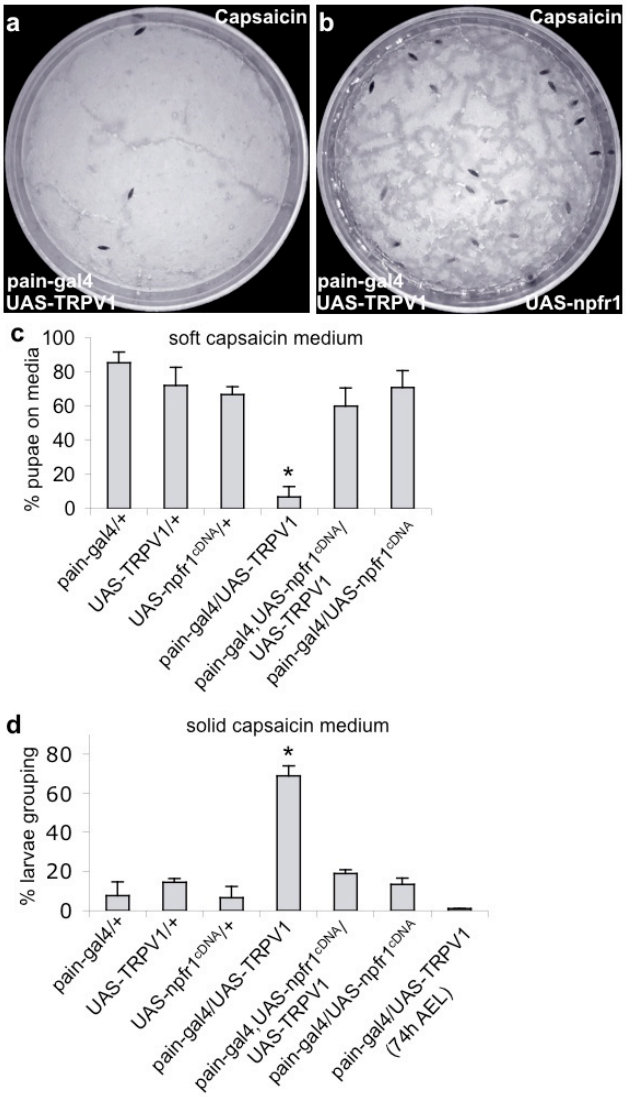

a
Capsaicin
pain-gal4
UAS-TRPV1
b
Capsaicin
pain-gal4
UAS-TRPV1
UAS-npfr1
c
soft capsaicin medium
% pupae on media
100
80
60
40
20
0
*
pain-gal4/+
UAS-TRPV1/+
UAS-npfr1cDNA/+
pain-gal4/UAS-TRPV1
pain-gal4,UAS-npfr1cDNA/
UAS-TRPV1
pain-gal4/UAS-npfr1cDNA
d
solid capsaicin medium
% larvae grouping
80
60
40
20
0
*
pain-gal4/+
UAS-TRPV1/+
UAS-npfr1cDNA/+
pain-gal4/UAS-TRPV1
pain-gal4,UAS-npfr1cDNA/
UAS-TRPV1
pain-gal4/UAS-npfr1cDNA
pain-gal4/UAS-TRPV1
(74h AEL)

Figure 3.6. NPFR1 suppresses Ca^{2+} influx mediated by rat TRPV1 in human cells

Ca^{2+} imaging and SOARS analysis of Human Embryonic Kidney (HEK) 293 cells expressing the TRPV1 channel. HEK 293 cells were loaded with the Fluo-4 and Fura-red fluorescent Ca^{2+} indicators, stimulated by 400 nM capsaicin (CAP) and imaged for 300s. Eigenimages highlight the clusters of pixels showing statistically significant anticorrelated changes in Fluo-4 and Fura-red fluorescence intensities. (**a**) An eigenimage of HEK cells transfected with empty pcDNA3.1 vectors; (**b**, **c**) cells transfected with TRPV1 cDNA and stimulated by CAP in the absence or presence of 1mM NPF. (**d**, **e**) cells co-transfected with TRPV1 and NPFR1 cDNAs and stimulated by CAP in the absence or presence of NPF. (**f**) SOARS analysis of changes in the ratio between Fluo-4 to Fura-red fluorescence levels during the entire 300-sec recording period. Each trace is generated from at least 3 independent experiments.

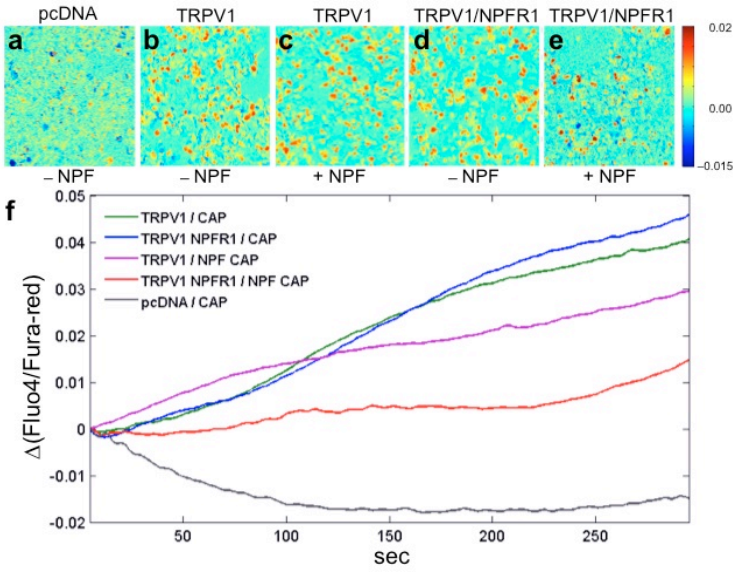
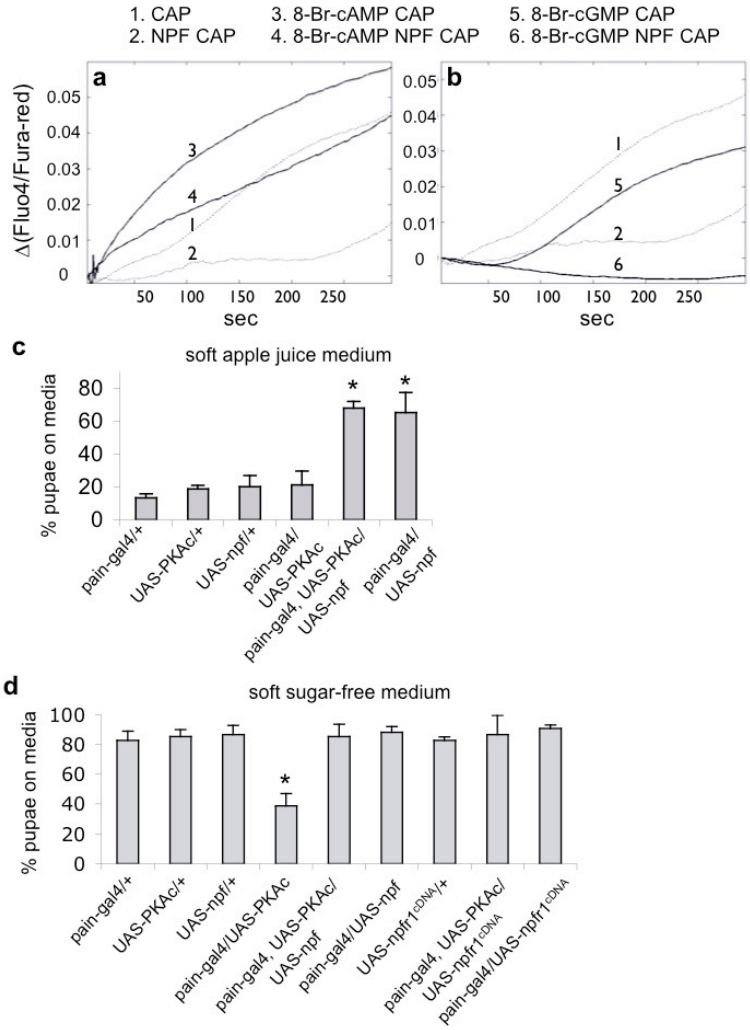

pcDNA
TRPV1
TRPV1
TRPV1/NPFR1
TRPV1/NPFR1
a
b
c
d
e
0.02
0.00
-0.015
– NPF
– NPF
+ NPF
– NPF
+ NPF
f
TRPV1 / CAP
TRPV1 NPFR1 / CAP
TRPV1 / NPF CAP
TRPV1 NPFR1 / NPF CAP
pcDNA / CAP
Δ(Fluo4/Fura-red)
0.05
0.04
0.03
0.02
0.01
0
-0.01
-0.02
50
100
150
200
250
sec

Figure 3.7. Effects of cyclic nucleotides, PKA and NPFR1 on TRP channel activities

(**a**) TRPV1 is sensitized by 8-Br-cAMP and slightly inhibited by 8-Br-cGMP (see line 1, 3 and 5). NPFR1 significantly attenuated sensitization of TRPV1 by 8-Br-cAMP (compare line 3 and 4). NPFR1 suppression of TRPV1 is enhanced in the presence of 8-Br-cGMP (compare line 5 and 6). (**b**) Quantification of avoidance response of transgenic larvae in media containing apple juice. Larvae co-expressing PKAc and NPF in PAIN neurons showed attenuated food-averse migration. Most pupated on the medium. $P<0.001$. (**c**) Quantification of avoidance response of transgenic larvae in sugar-free media. Control larvae that express UAS-*PKAc* alone pupated mostly outside of the agar medium. Larvae co-expressing PKAc and NPF or NPFR1 in PAIN neurons displayed attenuated food-averse migration. $P<0.001$.


1. CAP
2. NPF CAP
3. 8-Br-cAMP CAP
4. 8-Br-cAMP NPF CAP
5. 8-Br-cGMP CAP
6. 8-Br-cGMP NPF CAP
a
b
Δ(Fluo4/Fura-red)
sec
c
soft apple juice medium
% pupae on media
pain-gal4/+
UAS-PKAc/+
UAS-npf/+
pain-gal4/ UAS-PKAc
pain-gal4, UAS-PKAc/ UAS-npf
pain-gal4/ UAS-npf
d
soft sugar-free medium
% pupae on media
pain-gal4/+
UAS-PKAc/+
UAS-npf/+
pain-gal4/UAS-PKAc
pain-gal4, UAS-PKAc/ UAS-npf
pain-gal4/UAS-npf
UAS-npfr1cDNA/+
pain-gal4, UAS-PKAc/ UAS-npfr1cDNA
pain-gal4/UAS-npfr1cDNA

CHAPTER 4

GENETIC DISSECTION OF MOLECULAR AND NEURONAL PATHWAYS MEDIATING STRESS-INDUCED SOCIAL BEHAVIORS IN *DROSOPHILA* LARVAE[1]

[1] Xu, J., Li, M., & Shen, P., 04 / 2009

ABSTRACT

Animals display social aggregations induced by environmental stressors, but underlying neural mechanisms remain unclear. *Drosophila* postfeeding larvae show food-averse social burrowing through food proper and migrate to food-free surface for pupation. This social burrowing facilitates food penetration more efficiently and therefore is beneficial to larvae. Here we use a simple behavior paradigm to dissect the neuronal pathways regulating larval social burrowing. We show that the social burrowing is comprised of three genetically separable behaviors, seeking, clumping and burrowing. The cAMP-dependent Protein Kinase A (PKA) activity in a subset of peripheral neurons expressing *painless* (*pain*), a *Drosophila* Transient Receptor Potential (TRP) channel, is essential for the onset of seeking, clumping and burrowing behaviors. We also found that the PKA in *atonal*-expressing neurons and a subset of Va neurons differentially regulate clumping but not seeking or burrowing activity. Our results reveal a complex signaling network controlling social burrowing in postfeeding larvae.

INTRODUCTION

Across many species, animals display social interactions and aggregations when presented with environmental stressors including noxious chemicals, predators and food scarcity (Wilson, 1975). For instance, *C. elegans* feed socially in the environment containing noxious chemicals (de Bono et al., 2002), and certain African tadpole aggregate in large clumps in the presence of fish predators (Glos, 2007). Social interactions can profoundly affect individual wellbeing, population dynamics and biodiversity (Shorrocks, 1995). However, little is known about how genetic and neural factors regulate the complex social behaviors.

The genetically tractable *D. melanogaster* larva provides an excellent opportunity to investigate the genetic and neural basis underlying the regulation of social interactions (Wu et al., 2003; Xu et al., 2008). Third-instar *Drosophila* larvae display two opposing food responses: younger larvae live mostly inside aqueous food media such as overripe fruits; in contrast, older postfeeding larvae avoid food media and display migration (also known as wandering) towards food-free sites such as soils for pupation. In the process of migration, postfeeding larvae display a social response to aversive food stimuli; these larvae instinctively seek each other and subsequently form large clumps on solid apple juice-agar media and burrow cooperatively through the food proper. This food-averse social burrowing allows larvae to penetrate food layer at a faster rate (Wu et al., 2003), and is likely beneficial to the survival of pupae by minimizing their exposure to harmful microorganisms and drowning in the feeding habitat such as rotten fruits (Ashburner, 1989; Chiang, 1950).

Our previous studies revealed that the onset of social burrowing activity is dependent on the presence of sugar stimulation. The sensation of aversive sugar cues is mediated by peripheral sensory neurons expressing *pain*, a *Drosophila* TRPA channel. The *pain* mutant larvae, whose ventral thoracic sensory neurons are defective in fructose-induced excitation, showed abolished social burrowing on solid apple juice-containing agar medium (Xu et al., 2008). These results suggest that the *pain*-expressing peripheral sensory neurons are a part of the neuronal pathway that mediates the social burrowing behavior.

PKA is an important regulator of neural activity. Pharmacological studies revealed that locally administered PKA activators can potentiate the nociception by TRPA1 channel (Wang et al., 2008). On nociceptive terminals, PKA phosphorylation of TRPV1 results in the sensitization to many different stimuli, contributing to the development of hyperalgesia (Jeske et al., 2008).

Presynaptic PKA phosphorylation of SNAP 25 regulates the size of releasable vesicle pool (Nagy et al., 2004), and therefore can facilitate the release of neurotransmitter in neurons and form the basis for a form of long-term potentiation (LTP) (Weisskopf et al., 1994). We have found that upregulation of PKA activity in *pain*-expressing neurons has a sensitizing effect on sugar-induced migratory behavior (Chapter 3). Taken together, these results suggest that PKA may also play a prominent role in regulating sugar-averse social burrowing behavior.

In this report, we used the *Drosophila* larva model to dissect the molecular and neuronal pathways mediating social burrowing. We have shown that PAIN-mediated sensation of aversive sugar stimuli is essential for the onset of social burrowing, and that PKA activities in *pain*-expressing neurons are critical. We have also identified two sets of CNS neurons important for controlling clumping activity. The PKA activities in those neurons differentially regulate clumping, but not seeking or burrowing activities. Our results reveal a complex neuronal signaling network that controls social burrowing in postfeeding larvae.

RESULTS

Characterization of cooperative burrowing activity in postfeeding larvae

Drosophila larvae display social burrowing upon exiting feeding phase and entering wandering phase (ca. 96 h *a*fter *e*gg *l*aying, AEL). When newly-emerged wild type postfeeding larvae are transferred on to solid apple juice-containing agar medium, they move across the medium and actively seek each other, and subsequently form clumps before burrowing cooperatively through the agar layer. The percentage of larvae grouping into clumps is used to quantify the social burrowing activity (Wu et al., 2003).

We found that the initiation of the social burrowing is dependent on the presence of aversive food cues. When transferred on to apple juice-containing medium, a majority of the wild type larvae remained on the medium and actively seek each other. In contrast, the same larvae did not display seeking behavior when transferred on to sugar-free agar medium. Instead, a significant portion of larvae wandered out of the medium within 30 min (Figure 4.1a). Consistent with this result, *pain*^{3} mutant larvae, which are defective in sensing noxious sugar stimulation, do not display active seeking behavior (Xu et al., 2008). These results suggest that a chemosensory pathway, possibly mediated by PAIN, is required for the onset of seeking and the subsequent clumping and burrowing behaviors. We also found that fructose, an abundant substance in fruit juice, can potently elicit social burrowing in wild-type larvae (Figure 4.1b). The cooperative burrowing behavior was not observed when larvae are transferred onto soft apple-juice containing agar paste (Figure 4.1b). The larvae instead dispersed evenly across the medium. This suggests that the behavior is dependent on texture / surface property, and it may depend on mechanical stimulation mediated by mechanosensory mechanisms.

To provide further evidence that PAIN-mediated chemosensory pathway is essential for the initiation of social burrowing behavior, we tried to activate peripheral *pain*-expressing sensory neurons by expressing a mammalian TRP channel protein TRPV1^{E600K} that responds to capsaicin (Caterina et al., 1997; Marella et al., 2006; Tobin et al., 2002). The control postfeeding larvae (e.g., TRPV1^{E600K} alone) randomly browsed on the solid agar medium containing 50 μM capsaicin and showed no social burrowing activity throughout the experiment (> 1 h) (Figure 4.1d). However, a majority of the larvae expressing TRPV1^{E600K} (*pain-gal4* X UAS-TRPV1^{E600K}) formed clumps on solid capsaicin-agar medium within 30 min (Figure 4.1c and d). Meanwhile, the same experimental larvae did not show clumping activity on soft capsaicin agar

paste (Figure 4.1d), suggesting a separate sensory modality, possibly of mechanical origin, is required for initiating cooperative burrowing behavior. Taken together, these data strongly argue that the peripheral PAIN neuron-mediated sugar-sensing pathway is essential for inducing social burrowing behavior. They also support the notion that the neural circuits regulating sugar-averse behaviors are hardwired (Xu et al., 2008).

PKA activity in PAIN neurons regulates cooperative burrowing activity in postfeeding larvae

To explore cellular mechanisms that underlie PAIN neuron-mediated induction of social burrowing, we investigated a number of evolutionarily conserved intracellular signaling pathways in the nervous system for their potential roles in larval social burrowing. For the analysis of PKA signaling, we downregulated PKA activity in PAIN neurons by using *pain-gal4* to direct the expression of a dominant-negative form of the PKA inhibitory subunit (UAS-PKA^{inh}, Li et al., 1995). Interestingly, we found that social burrowing was not observed with the *pain-gal4* X UAS- PKA^{inh} larvae. Instead, they browsed randomly on the surface of the apple juice-containing medium (Figure 4.2a, b). In contrast, the controls (e.g., UAS-PKA^{inh} alone) showed normal seeking, clumping and burrowing activities (Figure 4.2b). These results indicate that the PKA activity in PAIN neurons is critical for the onset of sugar-elicited social burrowing.

We further investigated how upregulation of PKA activity in PAIN neurons might affect the social burrowing behavior. In this case, *pain-gal4* was used to express a UAS-*PKAc* transgene encoding a constitutively active catalytic subunit of PKA (UAS-*PKAc*, Kiger et al., 1999). As expected, the *pain-gal4* X UAS-*PKAc* larvae showed social burrowing activity on apple juice-containing medium (data not shown). Strikingly, the same larvae also displayed the

same phenotype even on solid sugar-free medium, while other controls (e.g., w x UAS-*PKAc*) showed no seeking, clumping or burrowing activities (Figure 4.2c, d). Therefore, enhanced PKA signaling in PAIN neurons is capable of substituting the sugar stimulation to induce social burrowing. Note that the same larvae with high PKA activity in PAIN neurons did not show social burrowing activity on soft sugar-free medium (Figure 4.2d). Again, this evidence points to the existence of a separate sensory modality that is required for the initiation of the behavior. Taken together, our findings indicate that PKA is a key regulator in the PAIN neuronal pathway that controls the induction of postfeeding social burrowing by aversive chemosensory cues.

PKA activity in *ato*-expressing neurons regulates clumping activity in postfeeding larvae

To identify other neuronal pathways involved in social burrowing behavior, we tried to manipulate PKA levels in various sets of neurons. We selected a panel of neuronal drivers to direct the expression of UAS-PKA^{inh} or UAS-PKAc, and examined the phenotypes of resulting larvae. We found that a subset of central neurons expressing a preneural gene *atonal* (*ato*) are important for larval clumping activity. Downregulation of PKA activity in *ato*-expressing neurons abolished clumping activity (Figure 4.3b). A majority of control larvae (e.g., UAS-PKA^{inh} alone) formed clumps within 30 min on apple juice-containing medium. However, the experimental larvae (*ato*-gal4 X UAS-PKA^{inh}) wandered around and did not show clumping activity throughout the experiment. Meanwhile, their aversive response to sugar stimulation remained normal. Most of them moved out of soft apple juice agar paste, and pupated outside (Figure 4.3c). *ato* is expressed in a cluster of ~15 neurons in each brain lobe and around 10 neurons in the ventral ganglion (Figure 4.3a). No *ato* expression in the PNS was detected (data not shown). These data suggest that at least a subset of *ato*-expressing central neurons is

important for clumping activity, and that PKA levels in those neurons are critical for the onset of clumping activity.

A genetic mutation disrupts larval clumping but not seeking and burrowing behaviors

We have identified a mutant line whose larvae were deficient in clumping but proficient in burrowing. As a results, these larvae burrowed solitarily on the solid medium (Figure 4.4a). Interestingly, such larvae tend to burrow in adjacent sites, suggesting their seeking behavior likely remains normal (Figure 4.4a). The mutant harbors a single copy of P-element containing a UAS-*PKAc* transgene, which was mapped to the chromosome site 49A7 (denoted as UAS-$PKAc^{49A7}$, Figure 4.5a). The transgene insertion does not appear to be inside coding sequence of local genes. To see whether the insertion is responsible for the loss of clumping activity, we remobilized the insertion using transposase. The newly generated UAS-*PKAc* larvae showed completely restored social burrowing (Figure 4.4b), indicating that the P element insertion is responsible for the dominant suppression of the clumping activity of postfeeding larvae.

To test whether the loss of clumping activity is associated with the *PKAc* coding sequence, we replaced the UAS-$PKAc^{49A7}$ transgene with the sequence of a GFP-tagged atrial natriuretic factor fusion (ANF-GFP) through homology-dependent P-element replacement (Rao et al., 2001; Sepp and Auld, 1999). The resulting transgene was named as UAS-$ANF\text{-}GFP^{49A7}$. Sequencing analysis showed that the UAS-*ANF-GFP* precisely replaced the UAS-$PKAc^{49A7}$ (Figure 4.5b). Unlike UAS-$PKAc^{49A7}$ larvae, UAS-$ANF\text{-}GFP^{49A7}$ larvae showed normal social burrowing on the solid apple-juice medium (Figure 4.4b, c), indicating that the loss of clumping activity is likely due to leaky expression of UAS-*PKAc* transgene.

We attempted to visualize the leaky expression pattern of *PKAc* by staining for GFP in UAS-*ANF-GFP*^{49A7} larvae. The GFP immunoreactivity was detected in ten cells in larval central nervous system: two in each brain lobe and six in the ventromedial surface of the abdominal ganglia (Figure 4.6a). No immunofluorescence staining was detected in other larval tissues including the gut, salivary and ring glands, imaginal discs or peripheral nervous system. Interestingly, the six neurons in the ventral ganglion appear to be peptidergic Va neurons, as indicated by their immunoreactvity to perivisceroki nin II (PVK II) antibody (Figure 4.6b, d) (Santos et al., 2006). The neurons in the brain lobe, however do not appear to be overlapping with Va neurons (Figure 4.6b, c). Taken together, these findings suggest that some or all of the ten neurons, possibly Va neurons, may be a part of a neuronal circuit that selectively mediates larval clumping behavior, and the ectopic expression of UAS-*PKAc* in those neurons may interfere the functioning of this circuit.

DISCUSSION

In this study, we have shown that the social burrowing of postfeeding larvae is mediated by a complex neuronal signaling network comprised of *pain*-expressing peripheral sensory neurons, *ato*-expressing central neurons and possibly Va neurons. We have provided evidence that the social burrowing can be broken down into three sequential behavior components: seeking, clumping and burrowing. These behavior components are genetically separable, as indicated by the UAS-*PKAc*^{49A7} insertional mutant, which showed abolished clumping but not seeking and burrowing behaviors. The wild-type larvae do not show clumping activity on soft apple juice-containing paste, suggesting that the surface property / texture may be important for eliciting clumping. In the natural environment, postfeeding larvae burrow out of fruit proper and

move to the fruit juice-stained soil, which they burrow into and pupate (Thomas, 1995). These observations raise the possibility that a mechanosensory modality maybe involved and that the sensation of solid surface may work in conjunction with sensation of aversive sugar cues to send signal to the CNS to initiate clumping and subsequent burrowing activity.

We have shown that PKA activities in *pain*-expressing neurons regulate sugar-averse migration (Xu et al., 2008), as well as social burrowing. Remarkably, postfeeding larvae expressing constitutively active PKA in PAIN neurons displayed social burrowing in the absence of sugar stimulation. One possible explanation for this effect is that the upregulated PKA activity in PAIN neurons may trigger the sensation of sugar stimuli by constitutively activating the nociceptive signaling pathway. Consistent with this notion, elevating PKA activity through pharmacological measures in nociceptors can result in hyperalgesia (Aley and Levine, 1999). PKA may sensitize *pain*-expressing neurons in different ways. One possibility is that PKA directly phosphorylate PAIN channel to increase channel activity, similar to what has been shown in the case of TRPV1 (Bhave et al., 2002). The other possibility is that PKA may interact with exocytosis mechanisms to increase the release of neurotransmitters (Nagy et al., 2004; Thakur et al., 2004). Further work is needed to reveal the exact mechanisms.

Our data suggests that downregulation of PKA activity in *ato*-expressing neurons suppresses clumping activity on solid apple juice-containing agar medium. However the same larvae do not appear to have a defect sensing aversive sugar stimulation. Therefore, it appears that either *ato*-expressing neurons are involved in mediating other peripheral sensory modalities (possibly of mechanical origin) required for clumping, or they are a part of a central neural circuit that regulates clumping. *ato* is a preneural gene important for the development of chordotonal neurons, which are considered to be stretch sensors (Kernan, 2007; Okabe and

Okano, 1997). It is therefore possible that downregulation of PKA in *ato*-exopressing neurons affects early development of mechanosensors, which in turn affects the onset of clumping activity at postfeeding stage. However, the *ato-gal4* X UAS-PKA^{inh} larva display normal pausing response when a light touch is applied to the nose, suggesting that mechanosensation is likely intact (unpublished data). Interestingly, in third-instar larvae, *ato* is expressed mostly in the mushroom bodies in the CNS (Figure 4.3a). No expression is detected in chordotonal neurons. Thus, it is unlikely that the loss of clumping is due to the inhibition of peripheral mechanosensors. Mushroom bodies are processing centers for many important behaviors including learning and memory. It is likely that low PKA activities in a subset of mushroom body neurons can affect complex behaviors such as social burrowing.

Our mutant analysis also led to the discovery of ten CNS neurons important for clumping activity. We also demonstrated that 6 of them are Va neurons (Figure 4.6b). However, it not clear whether the Va neurons or the non-VA neurons or all of them are involved in regulating clumping behavior. Previous studies indicated that Va neurons are peptidergic neurons and they secret a neuropeptide Capability (CAPA). It was shown that CAPA peptide and its receptors are involved in acceleration of pupariation in larvae (Zdarek et al., 2004). It is possible that changes of PKA levels in Va neurons can affect CAPA production and thus affecting pupariation-related biological process. However, substantial work is needed to find out the underlying mechanisms.

METHODS

Flies, media and larvae growth

Conditions for rearing adult flies and egg collection were described previously (Roberts, 1986; Shen and Cai, 2001). Synchronized eggs were collected within a 2 h interval, and late

second instars were transferred to a fresh apple-juice plate with yeast paste (< 80 per plate). The larvae were raised at 25 °C with the exposure to natural lighting. The UAS-*PKAc*, UAS-PKA^{inh}, UAS-$TRPV1^{E600K}$, UAS-*YC2.1* and UAS-*ANF-GFP*, *ato-gal4* and *painless-gal4* are in *w* background (Hassan et al., 2000; Husain and Ewer, 2004; Kiger et al., 1999; Kiger and O'Shea, 2001; Li et al., 1995; Liu et al., 2003; Scott et al., 2001; Tracey et al., 2003).

Behavioral Assays

The social burrowing assay was performed on solid 1.6 % agar media containing apple juice, 10% fructose or 50 μM capsaicin as described previously (Wu et al., 2003). 30 newly-emerged postfeeding larvae was washed thoroughly and then transferred onto the medium. After 30 min the plate was photographed and the number of larvae in the clumps was scored immediately.

The sugar-averse migratory assay on soft agar media was performed as described previously (Xu et al., 2008). 25 postfeeding larvae (96h AEL) in one plate were allowed to move freely on the medium. After 30 min, the numbers of larvae in clumps was recorded. The larvae were given additional time to form pupae and the percentage of pupae on agar media was scored after 24 hrs.

All assays were performed at room temperature in the dark. At least three separate trials were performed per assay. Statistical analysis of the data was performed using one-way ANOVA, followed by Student-Newman-Keuls analysis.

Molecular biology and immunocytochemistry

We follow the standard procedures for cloning, genomic PCR and the southern blot analysis (Sambrook et al., 1989). The immunostaining of tissues was carried out according to a previously published procedure (Brown et al., 1999; Shen and Cai, 2001). Primary antibody dilutions: rabbit anti-GFP, 1:1000; rabbit anti-PVK II, 1:3000. Alexa 488-conjugated anti-rabbit IgG and Alexa 568-conjugated anti-mouse IgG secondary antibodies (Molecular Probes, OR) were diluted 2000-fold. At least 8 tissues were examined for each line.

ACKNOWLEDGEMENTS

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Figure 4.1. Characterization of cooperative burrowing activity in postfeeding larvae

Unless stated otherwise, newly-emerged postfeeding larvae (96h AEL) were used in all assays. (**a**) 30 wild-type w^{1118} larvae were transferred onto the solid agar plate with or without apple juice, and allowed to browse freely. A majority of larvae stayed on apple juice-containing medium. In contrast, a significant portion of larvae moved out of sugar-free medium. At least three trials were performed for every line. $P<0.001$. (**b**) Quantification of clumping activity of different transgenic larvae on different types of media. The larvae showed no clumping activity on solid sugar-free agar or any soft agar paste. $P<0.001$. (**c**) *pain-gal4* directs transgene expression in a subset of CNS and PNS neurons (Tracey et al., 2003). UAS-TRPV1 encodes a variant (E600K) of rat TRPV1. The larvae expressing TRPV1 in PAIN neurons display strong social burrowing activity on solid agar plate containing 50 μM capsaicin. Pictures were taken at 30 min. The arrows indicate social burrowing sites. (**d**) Quantification of larval clumping activity on capsaicin-containing media. $P<0.001$.

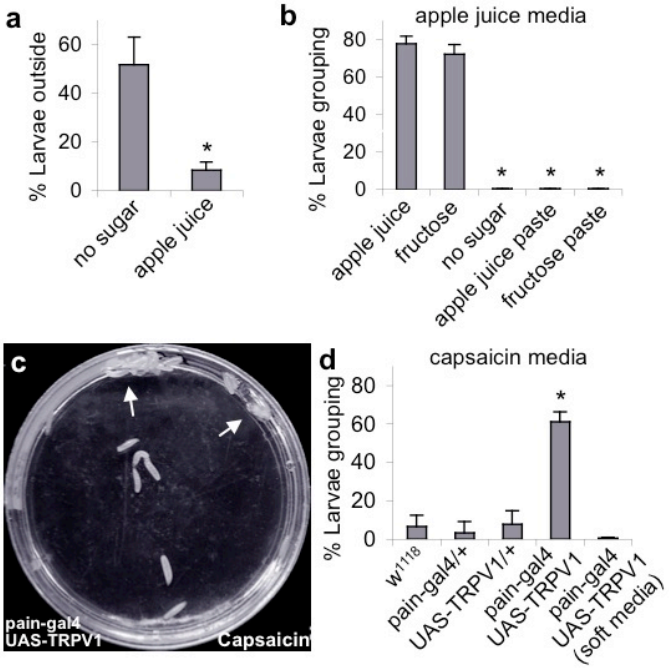

a
% Larvae outside
60
40
20
0
*
no sugar
apple juice
b
apple juice media
% Larvae grouping
80
60
40
20
0
*
*
*
apple juice
fructose
no sugar
apple juice paste
fructose paste
c
pain-gal4
UAS-TRPV1
Capsaicin
d
capsaicin media
% Larvae grouping
80
60
40
20
0
*
w1118
pain-gal4/+
UAS-TRPV1/+
pain-gal4
UAS-TRPV1
pain-gal4
UAS-TRPV1
(soft media)

Figure 4.2. PKA activity in *pain*-expressing neurons regulates clumping activity in postfeeding larvae

UAS-PKA^{inh} encodes a dominant negative form of PKA. UAS-*PKAc* encodes a constitutively active form of PKA. (**a**) Larvae with PKA activity downregulated in PAIN neurons (*pain-gal4* X UAS-PKA^{inh}) showed no clumping activity on solid apple juice-containing medium. Pictures were taken at 30 min. (**b**) Quantification of larval clumping activity on the solid apple juice-containing agar plate. $P<0.001$. (**c**) Larvae with PKA activity up regulated in PAIN neurons (*pain-gal4* X UAS-*PKAc*) showed strong clumping activity on solid sugar-free medium. The arrows indicate social burrowing sites. (**d**) Quantification of larval clumping activity on solid and soft sugar-free media. $P<0.001$

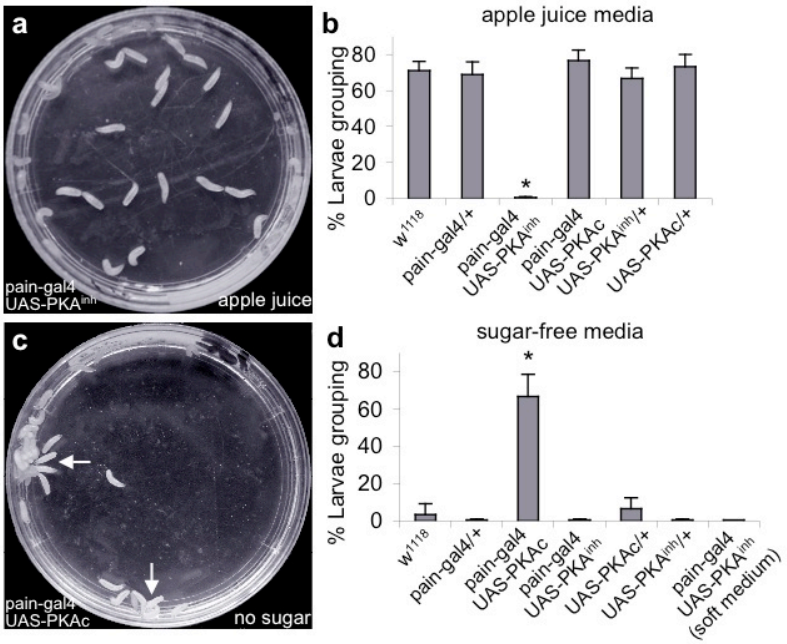

a
pain-gal4
UAS-PKA^inh
apple juice
b
apple juice media
% Larvae grouping
0
20
40
60
80
*
w^1118
pain-gal4/+
pain-gal4 UAS-PKA^inh
pain-gal4 UAS-PKAc
UAS-PKA^inh/+
UAS-PKAc/+
c
pain-gal4
UAS-PKAc
no sugar
d
sugar-free media
% Larvae grouping
0
20
40
60
80
*
w^1118
pain-gal4/+
pain-gal4 UAS-PKAc
pain-gal4 UAS-PKA^inh
UAS-PKAc/+
UAS-PKA^inh/+
pain-gal4 UAS-PKA^inh (soft medium)

Figure 4.3. PKA activity in *ato*-expressing neurons mediates larval clumping activity

(a) *ato*-gal4 directs fluorescent reporter UAS-YC2.1 expression a subset of CNS neurons. The fluorescence was detected in ~15 neurons in each brain lobe (arrowheads) and ~10 neurons in ventral ganglion (arrows). (**b**) Quantification of larval clumping activity on solid apple juice-containing agar medium (n=30 for each of three trials, $P<0.001$). (**c**) Quantification of sugar-averse migratory response by percentage of pupae formed on the surface of soft apple juice-agar paste (n=25 for each of three trials, $P<0.001$).

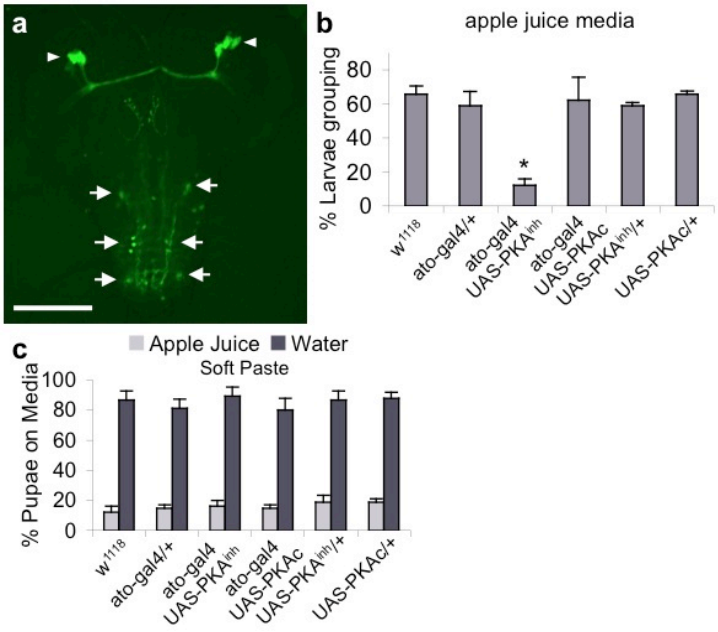

a
b
apple juice media
% Larvae grouping
80
60
40
20
0
*
w1118
ato-gal4/+
ato-gal4 UAS-PKAinh
ato-gal4 UAS-PKAc
ato-gal4 UAS-PKAinh/+
UAS-PKAc/+
c
Apple Juice
Water
Soft Paste
% Pupae on Media
100
80
60
40
20
0
w1118
ato-gal4/+
ato-gal4 UAS-PKAinh
ato-gal4 UAS-PKAc
ato-gal4 UAS-PKAinh/+
UAS-PKAc/+

Figure 4.4. A genetic mutation disrupts larval clumping but not seeking and burrowing activities

(**a**) Larvae (UAS-*PKAc*^{49A7}) harboring a P-element insert containing the UAS-*PKAc* sequence are deficient in grouping behavior, and burrowed solitarily on solid apple-juice agar plate. Arrows indicate individual larvae digging in an upside-down position. The insertion is mapped to the chromosomal 2R, site 49A7. (**b**) Quantification of the social burrowing activities of mutants and larvae with restored behavioral phenotypes. Larvae (UAS-*PKAc*^{49A7} and w^{1118} X UAS-*PKAc*^{49A7}, homozygous and heterozygous for UAS-*PKAc*^{49A7}, respectively) showed no group burrowing. A new UAS-*PKAc* line was obtained through precise excision of UAS-*PKA*^{c49A7} followed by its reinsertion elsewhere in the genome. The resulting larvae (designated as UAS-*PKAc* Excised) showed normal clumping behavior. UAS-*ANF-GFP*^{49A7} was generated by replacing UAS-*PKAc*^{49A7} with a UAS-ANF-GFP by homology-dependent precise gene conversion (Sepp and Auld, 1999). $P<0.001$. (**c**) UAS-*ANF-GFP*^{49A7}larvae displayed restored clumping behavior. The arrowhead indicates the social burrowing site.

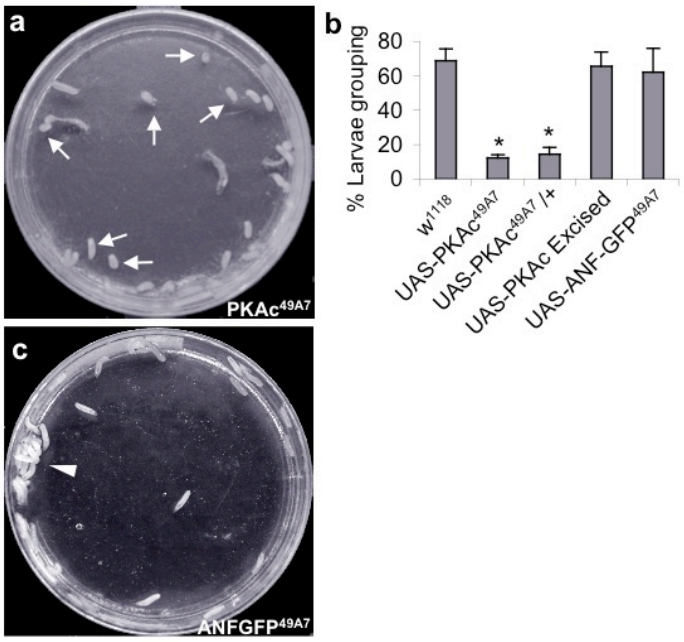

a
PKAc49A7
b
% Larvae grouping
80
60
40
20
0
*
*
w1118
UAS-PKAc49A7
UAS-PKAc49A7 /+
UAS-PKAc Excised
UAS-ANF-GFP49A7
c
ANFGFP49A7

Figure 4.5. The insertion site of the UAS-*PKAc* transgene

(**a**) The P-element insertion harboring UAS-*PKAc* was mapped to the chromosome 2R site 49A7. (**b**) The UAS-*PKAc* insertion was precisely replaced by UAS-*ANF-GFP* by homology-dependent gene replacement and was verified by sequencing the genomic PCR product (Sepp and Auld, 1999). The primers used for genomic PCR are 5'-GCAGGATAAGTATCGTGCAGA-3' and 5'-CCTGGTACTTCAAATACCCTTGG-3'. The sequences around insertion site and neighboring genes are shown. The triangle inside the inserted fragment indicates the direction of the insertion. The southern blot analysis indicated that genome of UAS-*PKAc*^{49A7} and UAS-*ANF-GFP*^{49A7} flies contains a single copy of the corresponding transgene (data not shown). Insertion not drawn to scale.

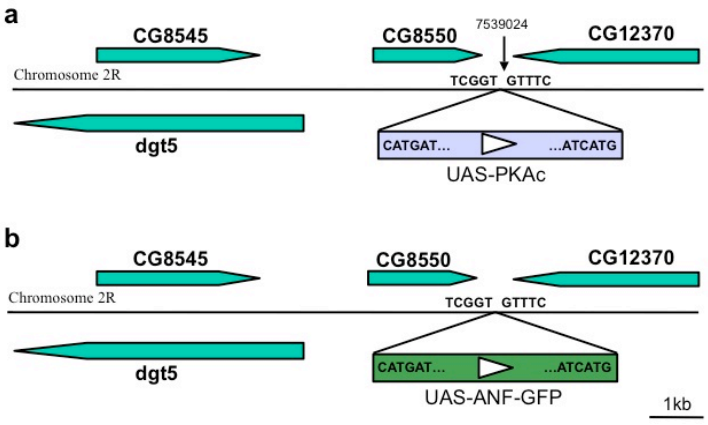
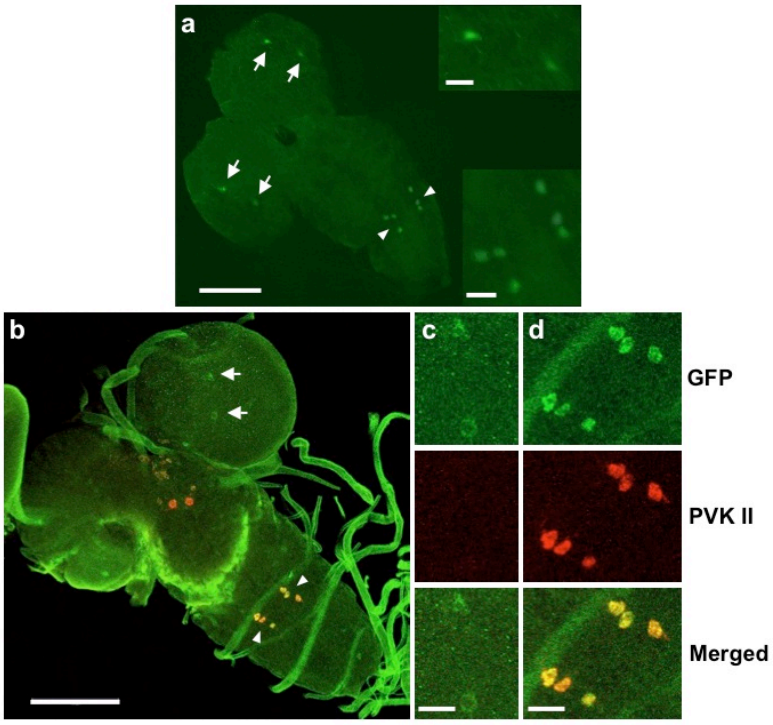

a
CG8545
CG8550
7539024
CG12370
Chromosome 2R
TCGGT GTTTC
dgt5
CATGAT...
...ATCATG
UAS-PKAc
b
CG8545
CG8550
CG12370
Chromosome 2R
TCGGT GTTTC
dgt5
CATGAT...
...ATCATG
UAS-ANF-GFP
1kb

Figure 4.6. The pattern of Leaky PKAc expression

(**a**) Immunofluorescence staining of the CNS of UAS-*ANF-GFP*^{49A7} larvae with anti-GFP antibodies revealed immunoreactivity in four neurons in brain lobes (arrows) and six ventromedial neurons in the abdominal ganglia (arrowheads). Insets show magnified view of the neurons. At least 20 CNS tissues were examined. Scale bar: 100 μm. Inset scale bar: 20 μm. (**b**) The six ventral neurons are Va neurons (arrows). Green channel: anti-GFP staining. Red channel: anti-PVK II (Va neuronal marker) staining. The neurons in brain lobes do not appear to overlap with Va neurons (arrowheads). Scale bar 100 μm. (**c**-**d**) magnified view of neurons indicated in **b**. Scale bar, 20 μm.


a
b
c
d
GFP
PVK II
Merged

CHAPTER 5

CONCLUSIONS AND DISCUSSION

As a part of body's defense system, the nociceptive system is vital to the survival and the wellbeing of the animal. It is also involved in various pathological processes such as inflammation and cancer. Further understanding of nociception and its regulation is of wide interest. In the past, pharmacological studies have provided great insights into how noxious stimuli are sensed and transmitted at tissue and neuronal levels. Recently work in mouse genetics also provided valuable understanding of sensation and modulation of noxious signals by generating an array of knockout transgenic mice. Nonetheless, investigating the basic molecular and cellular mechanism remains difficult in mammalian systems, partly due to the complex techniques and long duration of generating transgenic animals, as well as the existence of compensatory network in mammalian nervous systems.

Drosophila has been proven to be a versatile and powerful model system to uncover molecular mechanisms of sensory biology. Thus far, Tracey and colleagues established a larva model for thermal nociception, and successfully identified a novel TRP channel PAIN required for sensation of noxious heat in larvae (Tracey et al., 2003). Al-Anzi and colleagues established a chemonociception in adult flies, and showed that PAIN is required for sensation of pungent chemicals such as wasabi (Al-Anzi et al., 2006). However, there are few models for sensation of stressful / aversive stimuli that are more physiologically relevant. My work provided the first molecular evidence for the function of nociceptive system under normal physiological conditions. I have shown that fructose, an abundant chemical in larval feeding habitat, can

potently elicit avoidance behavior once the larvae exiting feeding phase, and the sensation of aversive food chemicals is mediated by the conserved TRPA channel PAIN. It has been shown that PAIN is essential for sensation of high temperature (> 42 °C) and noxious chemicals (isothiocyanates) in larvae and adults, respectively (Al-Anzi et al., 2006; Tracey et al., 2003). However, whether PAIN is directly gated by those stimuli was not clear until recently. Tominaga group showed that PAIN can be directly activated by noxious heat but not by isothiocyanates (Sokabe et al., 2008). This result suggests that either PAIN is not the direct receptor for isothiocyanates, or it needs co-factors to be activated. While we demonstrated that PAIN is essential for fructose-stimulated neuronal excitation, our unpublished data showed that PAIN is probably not gated by fructose, since the HEK cells transfected with *pain* cDNA showed no calcium influx upon fructose stimulation, while they display PAIN-dependent calcium influx upon heat stimulation. The actual function of PAIN in the sugar-responsive thoracic neurons is not yet clear. Our preliminary data shows that in *pain* mutant background, the *pain*-expressing neurons in terminal organs (presumably gustatory neurons) display transient instead of sustained increase of intracellular calcium upon continuous fructose stimulation. This piece of evidence has led us to speculate that PAIN has a similar function as the store-operated calcium channels. The calcium increase inside the neurons resulting from activation of sensory receptors may cause PAIN to open and allow more calcium to enter the cell, resulting in an "amplified", more substantial signal. Our finding is consistent with the notion that although most TRP channels do not have the usual properties of store-operated channels, they may nonetheless participate in this process (Flockerzi, 2007).

We have also identified for the first time a set of fructose-responsive neurons in larval PNS outside the terminal organs (the larval taste center). Activation of those neurons can

effectively elicit aversive behaviors, indicating that the avoidance circuit is hardwired. Taken together, these evidences support the notion that neural circuits which convey the attractiveness of the food are separate from the ones that convey the aversiveness of the food. In the feeding phase, the gustatory neurons in the terminal organs are responsible for transmitting food signals to CNS, which in turn directs the larva to stay and feed inside the food. Upon exiting the feeding phase, the CNS switches to the avoidance circuit, comprised of *pain*-expressing thoracic neurons, thus promotes the onset of food-averse behaviors to facilitate postfeeding migration towards food-free pupation sites. Combining all of the above discoveries, we have shown a novel function of nociceptive system under physiological conditions: facilitating larval development and promoting survival of the animal.

Nociception of invertebrates and mammals are mediated by conserved signaling mechanisms involving TRP family channels (Montell, 2005a; Montell et al., 2002; Moran et al., 2004; Ramsey et al., 2006). My work has provided the first evidence that the antinociceptive functions of invertebrates and mammals are also mediated by a conserved mechanism that suppresses TRP family channels. We demonstrated that the *Drosophila* NPY-like system suppresses peripheral stressful stimulation in feeding larvae. I have provided several lines of evidence suggesting that NPF directly acts on the sensory neurons expressing TRPA channel protein PAIN. The G-protein coupled NPF receptor NPFR1 is expressed selectively in a subset of PAIN sensory neurons responsive to aversive sugar stimulation. Overexpression of NPFR1 in PAIN neurons blocks fructose-stimulated TRPA channel activity. Moreover, we have shown that *Drosophila* shares a highly conserved anti-nociceptive pathway with mammals. Our evidence indicates that activation of NPFR1 also suppresses mammalian TRPV1 channel activities in heterologous HEK293 cells. Furthermore, the suppression of NPFR1 can be partially reversed

by a cAMP analogue. Finally, increased PKA activity in the PAIN neurons of postfeeding larvae is sufficient to elicit such aversive behaviors without the need of any aversive chemicals in the medium. Thus, high PKA activity appears to sensitize larval nociceptive sensory neurons. Consistent with this notion, PKA has been shown to potentiate TRP channel activity through direct phosphorylation the N-terminal domain (Bhave et al., 2002). PKA activity can also reverse the desensitized TRP channels (Mohapatra and Nau, 2003). These findings suggest that a conserved signaling mechanism may underlie the suppression of peripheral stressful stimulation by invertebrate and mammalian antinociceptive pathways, and the antinociceptive activities of NPY / NPF receptors may be mediated at least partly by a common mechanism involving downregulation intracellular cAMP and thus PKA activities.

Animals display social aggregations when presented with environmental stressors such as noxious chemicals and predators (Wilson, 1975). The neural and molecular mechanisms remain underexplored. Using the social burrowing of postfeeding larvae as a model, we genetically dissected the neuronal pathways mediating the complex social behaviors. We have again established that PKA is an important regulator of neural functions, and depends on the cellular contexts, it may have different effects on the final behavioral outcomes. My work has also led to the discovery of two sets of central neurons, *ato*-expressing neurons and Va neurons, which differentially regulate clumping activity in larvae. Although detailed mechanisms remain to be determined, our data suggests that regulating clumping activity might be the novel functions of *ato*-expressing neurons and Va neurons, in addition to their roles in chordotonal organ development and flow-rate control in Malphigian tubules (*Drosophila* kidney), respectively (Davies et al., 1995; Okabe and Okano, 1997).

My work also contributed to the technical aspects of *Drosophila* neuroscience. The common hurdle of *in vivo* calcium imaging is the very low signal to noise ratio. This is especially problematic with genetically-encoded calcium indicator, since they mostly have very low dynamic range, typically < 10% ΔF/F. As a result, despite their advantages such as non-invasive nature and genetically controllable localizations, their applications are rather limited. Here we have developed a high-sensitivity calcium imaging protocol to extract the signal of calcium level changes in a noisy background. The statistical algorithm has greatly increased the ability to detect minute signals among noises that will otherwise be ignored by the conventional Region of Interest (ROI) method. It has greatly expanded the usage of genetically-encodes calcium indicators and allowed non-invasive imaging of live neuronal activity in awake animals. I also pioneered the surgical ablation of specific neurons by nitrogen laser in *Drosophila*. Previously it was shown that, in *C. elegans*, focusing controlled laser beam of proper energy on the nucleus of neurons could ablate them without affecting the survival of neighboring cells. Therefore it is considered as one of the cleanest way of analyzing the functional importance of given neurons, since it does not have the inherent issues such as the side effects on animal physiology resulting from expressing molecules by transgenic methods. We have demonstrated for the first time in *Drosophila* larvae that this method is feasible and effective. This ablation protocol that we developed will be tremendously beneficial to our future work on functional dissection of neural circuits, as well as other researches of *Drosophila* neurobiology field.

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