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Sensory mechanisms controlling the timing of larval developmental and behavioral transitions require the *Drosophila* DEG/ENaC subunit, Pickpocket1

Joshua A. Ainsley [a], Myung Jun Kim [a,b,1], Lauren J. Wegman [a], Janette M. Pettus [a,2], Wayne A. Johnson [a,b,*]

[a] University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Department of Molecular Physiology and Biophysics, Iowa City, IA 52242, USA
[b] University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Neuroscience Ph.D. Program, Iowa City, IA 52242, USA

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ABSTRACT

Growth of multicellular organisms proceeds through a series of precisely timed developmental events requiring coordination between gene expression, behavioral changes, and environmental conditions. In *Drosophila melanogaster* larvae, the essential midthird instar transition from foraging (feeding) to wandering (non-feeding) behavior occurs prior to pupariation and metamorphosis. The timing of this key transition is coordinated with larval growth and size, but physiological mechanisms regulating this process are poorly understood. Results presented here show that *Drosophila* larvae associate specific environmental conditions, such as temperature, with food in order to enact appropriate foraging strategies. The transition from foraging to wandering behavior is associated with a striking reversal in the behavioral responses to food-associated stimuli that begins early in the third instar, well before food exit. Genetic manipulations disrupting expression of the Degenerin/Epithelial Sodium Channel subunit, Pickpocket1(PPK1) or function of PPK1 peripheral sensory neurons caused defects in the timing of these behavioral transitions. Transient inactivation experiments demonstrated that sensory input from PPK1 neurons is required during a critical period early in the third instar to influence this developmental transition. Results demonstrate a key role for the PPK1 sensory neurons in regulation of important behavioral transitions associated with developmental progression of larvae from foraging to wandering stage.

Introduction

Sensory information from a variety of internal and external stimuli is integrated by the central nervous system to modify both innate and learned behaviors. This sensory input is critical for an animal to produce a relevant response to changes in its environment. As an organism transitions through developmental stages, morphological and physiological changes often require accompanying transitions in behavior that must be temporally coordinated. For example, sexual maturation in many animals is characterized not only by the physical capability for reproduction but also by expression of appropriate behavioral programs (Consoulas et al., 2000; Romeo, 2003; Shirangi and McKeown, 2007). How the timing of these behavioral transitions are coordinated with internal and external factors to ensure normal development is not well understood.

Drosophila melanogaster, like all holometabolous insects, undergo complete metamorphosis to reach adulthood (Riddiford, 1993; Riddiford et al., 2003; Thummel, 2001). Each life cycle phase is characterized by a coordinated program of developmental events and behavioral transitions that have evolved to promote fitness and survival. *Drosophila* larvae develop through three larval instars separated by molting events to allow for continued growth, with a final molt initiating pupation (Riddiford, 1993; Riddiford et al., 2003). Gene transcription cascades involved in regulation of larval molts have been shown to be initiated by transient release of the steroid hormone ecdysone. During most of the larval phase, animals remain immersed within the food source and feed constantly, displaying what is known as foraging behavior (Sokolowski et al., 1983). Since adults do not increase in size after eclosion, growth occurs almost exclusively during the larval instars. During the third and final instar, larvae enter wandering stage, characterized by cessation of eating, purging of the gut, and exiting the food to search for a suitable pupation site. Physiological mechanisms regulating the precise timing of this major developmental and behavioral transition are poorly understood, but undoubtedly require coordination between systemic hormonal signals as well as both internal and external sensory cues.

The third instar behavioral transition is fundamentally a reversal of innate responses to food-associated stimuli from food attraction to

* Corresponding author. University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Department of Molecular Physiology and Biophysics, Iowa City, IA 52242, USA. Fax: +1 319 335 7330.
E-mail address: Wayne-A-Johnson@uiowa.edu (W.A. Johnson).
[1] Present address: University of Minnesota, Department GC, 5-235 Moos Tower, 515 Delaware St SE, Minneapolis, MN 55455, USA.
[2] Present address: University of Iowa, Carver College of Medicine, Department of Internal Medicine, Iowa City, IA 52242, USA.

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food aversion (Wu et al., 2003). Many animal species maintain physical association with a food source by sensing food-associated environmental cues. This has been demonstrated experimentally for the nematode, *Caenorhabditis elegans*, which is capable of responding to food-associated stimuli including odorants, temperature, and oxygen levels (Chalasani et al., 2007; Cheung et al., 2005; Luo et al., 2006; Mohri et al., 2005; Mori, 1999; Mori and Ohshima, 1995). Directed taxis behaviors such as temperature preference have been examined by observing the distribution of animals on a thermal gradient, as well as by direct observation of behavioral responses to changes in temperature (Mori, 1999; Mori and Ohshima, 1995; Zariwala et al., 2003). Individual nematodes will "track" to the specific temperature within a thermal gradient at which it had most recently found food. These food-associated taxis behaviors require the animal to integrate disparate sensory inputs from their current environment for comparison with previously established environmental setpoints associated with a food source.

One foraging strategy utilized to accomplish these taxis behaviors involves switching between two primary modes of locomotion behavior related to speed and turning frequency (Hills et al., 2004; Pierce-Shimomura et al., 1999; Wakabayashi et al., 2004; Zhao et al., 2003). An increase in the number of turns and direction reversals in response to an encounter with a food-associated stimulus, referred to as *area restricted search (ARS) behavior*, serves to keep an animal within a smaller search area. As the amount of time since the last encounter with a food-associated stimulus increases, the turning rate of the organism will decrease, resulting in long, relatively straight locomotion paths (Hills et al., 2004). This *dispersal behavior* serves to increase the probability of finding a food source by expanding the search area. The dynamic balance between straight movements and turns results in efficient exploration of the environment and increased probability of food encounters.

The complete reversal of food attraction to food aversion is a remarkable phenomenon observed in larvae of holometabolous insects, such as *Drosophila*, as they make the developmental transition from foraging to wandering behavior. How these behavioral changes are regulated and coordinated with hormone-induced gene transcription remains an open question. We have previously shown that wild-type wandering stage larvae removed from food and placed on a non-nutritive agarose sheet will initiate ARS behavior, with a high number of stops and turns and a decreased search area (Ainsley et al., 2003). Wandering larvae lacking the degenerin/epithelial sodium channel (DEG/ENaC) subunit, Pickpocket1 (PPK1), will display a low number of stops and turns and an increased search area, indicative of dispersal behavior, when assayed under the same conditions. This is suggestive of a role for PPK1 in the regulation of larval behavioral development. PPK1 is specifically expressed in the class IV multiple dendritic (mdIV) neurons and two bipolar neurons located near the posterior spiracles (Adams et al., 1998; Ainsley et al., 2003). The mdIV neurons have a highly branched dendritic structure that completely tiles the larval body wall and is evolutionarily conserved across species of holometabolous insects (Grueber et al., 2001, 2002).

Results presented here show that wild-type *Drosophila* larvae are able to associate specific environmental conditions with a food source, leading to stereotyped food-seeking behaviors. During the early third instar, as animals prepare to enter the wandering stage, behavioral responses to the same environmental conditions are reversed, resulting in a transition to food-avoiding behaviors. Disruption of PPK1 or PPK1 neuron function caused defects in this major developmental and behavioral transition. These defects are not due to a general delay in development and are specific to a food-associated stimulus. Functional PPK1 neurons are required during a critical period in the early third instar (80–90 h AEL) for the reversal in larval behavior to occur. These results suggest that afferent sensory input from the peripheral sensory neurons expressing PPK1 plays a major role in coordinating the key larval developmental transition from foraging to wandering behavior.

Materials and methods

Drosophila stocks

Flies were raised on standard cornmeal–yeast–agar medium. Stocks and balancer chromosomes not specifically described in the text are as included on FlyBase at http://flybase.bio.indiana.edu. All crosses were performed at 25 °C unless otherwise stated. The w; Df(2L)b88h49/Df(2L)A400 overlapping deficiency stock (Df/Df) was used as a *ppk1* null mutant stock as previously described (Ainsley et al., 2003). Transgenic stocks were generously provided by Dr. Toshi Kitamoto (University of Iowa; *UAS-shi^{ts1}*) and Dr. Cynthia Hughes (The Neurosciences Institute, San Diego, CA; clh24–Gal4). The *ppk1* deficiency chromosome, Df(2L)Exel6035, was maintained over the In(2LR)Gla Bc balancer allowing selection of heterozygous larval progeny by absence of the Bc larval marker.

Immunolabeling and microscopy

Polyclonal PPK1 antiserum was raised in rabbits against a peptide corresponding to the first 17 amino acids at the intracellular amino terminal end of PPK1(MAEIREDEEEKKSGISI). All immunological manipulations including peptide synthesis, conjugation to carrier protein, injections, bleeds and initial affinity-purification were performed commercially according to standard protocols (Biosource). Affinity-purified PPK1 antiserum was stored in small aliquots containing 5% glycerol at −80 °C.

For immunolabeling of mdIV neurons, late third instar stage larvae were dissected in 1× PBS, pH 7.4 by opening lengthwise down the dorsal or ventral surface and pinning flat (inside up) in a custom-made magnetic chamber. After removal of internal organs, the larval body wall was fixed for 10 min in 4% paraformaldehyde in 1× PBS by incubating on a rotating platform at RT. After transfer to a slide well, larval body walls were fixed for an additional 30–40 min in 800 ml 1:1 heptane:4% paraformaldehyde (1× PBS). Fixed larval body walls were then rinsed 3 times for 20 min with PBSS (1× PBS + 0.1% saponin). Fixed tissues were then permeabilized by incubating for at least 1 h in PBSS followed by blocking for 1 h in PBSSB (PBSS + 1% BSA). The anti-PPK1 antibody was incubated with dissected Df(2L)b88h49/Df(2L)A400 third instar larvae lacking PPK1 prior to labeling experiments to reduce nonspecific binding. Tissues were then incubated overnight at 4 °C on a platform shaker with anti-PPK1 primary antibody (1:800 in PBSSB). Primary antibody was removed and tissues rinsed 2× quickly with PBSS at RT followed by 3 rinses with PBSS for 20 min each. After rinses, tissues were blocked for at least 1 h at RT in PBSSBN (PBSSB + 5% Normal Goat Serum). Tissues were then incubated for 3–4 h at RT with the Alexa Fluor 546 secondary antibody diluted in PBSSBN (1:3000; Molecular Probes, Eugene OR), protecting samples from light throughout the incubation. Samples were washed 3 times for 20 min with PBSS at RT followed by one wash for 10 min with 1× PBS. Samples were then slide mounted with Vectashield (Vector Labs). Confocal images were taken using a Zeiss LSM510 confocal microscope and manipulated using Adobe Photoshop.

Initial characterization of the ppk1GAL4 transposon detected expression in a small subset of neurons in the anteromedial upper brain lobes of the larval CNS (Ainsley et al., 2003). After generating a higher quality PPK1 antiserum, we were unable to detect any PPK1 immunoreactivity in the central brain or ventral nerve cord (Fig. S1A–D). Generation and characterization of additional ppk1GAL4 insertions demonstrated that the brain lobe expression was an insertion artifact. All ppk1GAL4 insertions utilized in these studies displayed specific expression in the mdIV and bipolar posterior spiracle neurons with no detectable expression in the CNS.