Minireview

Neural Mechanisms of Alarm Pheromone Signaling

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Alarm pheromones are important semiochemicals used by many animal species to alert conspecifics or other related species of impending danger. In this review, we describe recent developments in our understanding of the neural mechanisms underlying the ability of fruit flies, zebrafish and mice to mediate the detection of alarm pheromones. Specifically, alarm pheromones are detected in these species through specialized olfactory subsystems that are unique to the chemosensitive receptors, second messenger-signaling and physiology. Thus, the alarm pheromones appear to be detected by signaling mechanisms that are distinct from those seen in the canonical olfactory system.

INTRODUCTION

When animals encounter dangerous stimuli, they often emit an alarm pheromone to alert conspecifics or closely related species of the imminent danger. The response to alarm pheromones is an innate and stereotypical behavior characterized by either an attempt to escape from the threat or attempt to mount a defensive attack. The behavioral response elicited by alarm pheromones is both species- and context-dependent. For example, eusocial insects such as ants and honeybees are more likely to attack the threat (e.g. an intruder in their nest), whereas non-social insects are more likely to escape from the source of alarm pheromone (Verheggen et al., 2010). Moreover, the response of the pea aphid (*Acyrtosiphon pisum*) to a single alarm pheromone varies with the quality of food and the risk for desiccation associated with the amount of environmental humidity the aphid is exposed to (Dill et al., 1990).

Despite the many studies of the ecological, ethological and chemical nature of alarm pheromones that have been carried out, our understanding of the neural mechanisms underlying the pheromone detection through the olfactory system remains limited. These chemicals are known to induce stereotypical alarm behaviors through a defined neural mechanism. The response to alarm pheromone in the wild is often similar to the response seen in laboratory settings. Therefore, a laboratory study of the neural mechanisms responsible for alarm pheromone signaling could improve our understanding of the neural basis of these innate behaviors.

In this review, we will focus on recent advances in our under-

standing of the detection of alarm pheromones through the olfactory system and the mechanisms underlying the process of odor-evoked information in the brain. This review will focus on studies in three genetically tractable organisms: the fruit fly (*Drosophila melanogaster*), zebra fish (*Danio rerio*) and house mouse (*Mus musculus*).

ALARM PHEROMONES IN *DROSOPHILA MELANOGASTER*

Studies of alarm pheromones in insects have revealed a stark difference in the responses of social insects versus nonsocial insects. Social insects- such as ants, bees and wasps- respond with highly developed programmed behaviors that often involve the recruitment of workers/soldiers to participate in the defense of the nest against an intruder (Verheggen et al., 2010). By contrast, nonsocial insects tend to exhibit avoidance behaviors in the response to the potentially harmful stimuli.

In previous studies of *D. melanogaster*, we reported that stressed flies emit an odorant, called *Drosophila* stress odorant (dSO), which elicits avoidance behavior in naïve flies (Suh et al., 2004). When electric shock or mechanical agitation caused the emitter flies to release dSO, this odorant induced robust avoidance behavior in naïve, responder flies. The flies did not exhibit a response to the odor from a tube in which flies merely occupied without producing dSO. These findings indicate that flies must be stressed to emit dSO and that dSO may function as an alarm pheromone.

Gas chromatography-mass spectrometry analysis identified carbon dioxide (CO₂) as one of the molecules emitted by stressed flies. CO₂ alone can act as a repellent on walking flies at a concentration as low as 0.1% (Faucher et al., 2006; Suh et al., 2004; Wasserman et al., 2013). However, the amount of CO₂ emitted by stressed flies is an order of magnitude lower than the concentration required to elicit a strong avoidance response in responder flies (Faucher et al., 2006). Moreover, flies in which the CO₂-sensing olfactory sensory neurons were inactivated failed to avoid CO₂, but still had a robust avoidance response to dSO (Suh et al., 2004). These findings suggest that dSO contains an additional component(s) beside CO₂.

Evidence of the role of CO₂ as an alarm pheromone is weakened by the fact that the increase in CO₂ emission by stressed flies could be a result of the increase in locomotor activity and

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hyperventilation associated with stress, given that each of these reactions increases the metabolic rate and, therefore, the rate of CO₂ production and release. The fact that stressed flies emit large amounts of CO₂ at the moment of agitation, however, suggests that there is a reservoir of CO₂ that is ready to be released into the atmosphere. Indeed, insects have been shown to store and release large amounts of CO₂, while resting (Lighton, 1996). This suggests that CO₂ can act as an alarm signal in conjunction with as yet unidentified components of dSO to generate robust avoidance behavior. The identity of the other components of dSO, however, remains to be determined.

The olfactory system in the fruit fly consists of two anatomical organs: the third segment of the antenna and the maxillary palp. Each of these structures is covered with hundreds of sensory hairs, called *sensilla*, that harbor the dendritic terminals of olfactory sensory neurons (OSNs). Three types of sensilla have been described based on their morphology: basiconic, coelomic, and trichoid. OSNs in the basiconic and trichoid sensilla mainly express the 7-transmembrane odorant receptors (ORs), which are typical olfactory receptors: basiconic OSNs respond primarily to food and environmental odorants, and trichoid OSNs respond to sexual pheromones (Su et al., 2009). Coelomic sensilla mainly express the ionotropic glutamate receptors (IRs), which respond to water-soluble amines and acids (Hansson and Stensmyr, 2011). All of these olfactory receptors are localized to the dendritic terminals of the sensilla, where they interact directly with odorant molecules.

CO₂-sensitive OSNs are located in the ab1c basiconic sensilla (de Bruyne et al., 2001). The ab1c neurons have a unique ring-shaped dendritic morphology (Shanbhag et al., 1999). Unlike other OSNs in the basiconic sensilla, which express OR receptors, ab1c OSNs express the gustatory receptors (GRs), GR21a and GR63a (Jones et al., 2007; Kwon et al., 2007; Scott et al., 2001; Suh et al., 2004). OR (and likely GR) receptors act as ligand-gated ion channels with an unclear role in G-protein-mediated signaling (Sato et al., 2008; Wicher et al., 2008). The detection of CO₂ requires the G-protein G α_q , suggesting that a G-protein-mediated signaling cascade is involved (Yao and Carlson, 2010). Similar to its role in the CO₂-sensing neurons, G α_q also mediates GR-signaling in sugar-sensing taste neurons (Ishimoto et al., 2005; Ueno et al., 2006).

GR21a-expressing sensory neurons appear to be highly selective for molecular CO₂; this is suggested by the fact that they were activated specifically by CO₂ from among a panel of approximately 70 odorants tested thus far (Kwon et al., 2007; Suh et al., 2004; Turner and Ray, 2009). These neurons were not activated by related odorants such as carbonic acid, which is derived from CO₂ when elevated levels of CO₂ lower the pH in the aqueous environment surrounding the OSN dendrites. Furthermore, our recent finding showed that GR21a-positive neurons were activated by carbon disulfide (CS₂), which is structurally similar to CO₂ (Ai and Suh, unpublished data). This suggests that the ligand for GR21a-positive sensory neurons is a carbon molecule with two double bonds onto either oxygen or sulfur.

Another striking feature of ab1c neurons is that they are not subjected to presynaptic inhibition (Root et al., 2008). This is in contrast to other pheromone-sensing glomeruli, which strongly express the gamma-amino butyric acid (GABA) receptor and therefore, are highly sensitive to presynaptic inhibition (Olsen and Wilson, 2008; Root et al., 2008). Presynaptic inhibition serves as a gain-control mechanism in many types of sensory neurons to expand the dynamic range of sensory detection without saturating the response in the postsynaptic neurons

(Olsen et al., 2010). The trade-off for presynaptic inhibition is that weak inputs may not be recognized by sensory neurons because the detection threshold is elevated (Root et al., 2008). In contrast, a lack of presynaptic inhibition results in a lower detection threshold, which would support the need for a sensitive alarm pheromone receptor. Thus, GR21a-positive neurons that are devoid of presynaptic inhibition can allow the detection of even small amounts of an alarm pheromone.

ALARM PHEROMONE IN ZEBRAFISH

In fish, the alarm pheromone is known as Schreckstoff ("fear substance" in German), a term coined by the Austrian biologist Karl Von Frisch who described this phenomenon in 1942 [(Frisch, 1942) cited in (Stensmyr and Maderspacher, 2012)]. Intending to study hearing in fish, Von Frisch marked individual fish used for his experiment with an incision close to the tail that severed the sympathetic nerve. When the marked fish were released into water, he noticed that the other members of the shoal appeared stressed and were reluctant to approach the injured individuals. In later studies, investigators found that these aversive behavioral responses to Schreckstoff were prevalent in many species of fish. Fish in a shoal respond to Schreckstoff with increasingly erratic movements (darting), by descending to the bottom of the tank and by moving closer together to form a tighter shoal (Smith, 1992). An isolated fish would react by increasing its freezing responses (Mathuru et al., 2012). It is worth noting that responses to Schreckstoff that have been seen in tanks in laboratory settings are similar to those seen in natural settings, making a laboratory-based study appropriate for investigating its effects (Brown et al., 2000; Speedie and Gerlai, 2008).

Schreckstoff is composed of oligosaccharides of the glycosaminoglycans (GAGs) chondroitin-4-sulfate, chondroitin-6-sulfate and other low-molecular-weight components (Mathuru et al., 2012). Hypoxanthine-3(N)-oxide (H3NO), which consists of a purine skeleton with a nitric oxide (NO) side group, has been suggested as the active component of the Schreckstoff mix (Brown et al., 2000; Pfeiffer et al., 1985). However, several findings have weakened this proposal. First, synthetic H3NO elicits only a slight increase in darting behavior and does not appear to elicit a tightening of shoal movement or bottom dwelling (Mathuru et al., 2012). Second, studies have shown that it is the NO group of H3NO rather than the purine skeleton that triggers alarm behavior (Brown et al., 2000). Third, H3NO does not activate the same glomeruli as Schreckstoff (see below). These observations suggest that another molecule with an NO group may serve as an active component of Schreckstoff.

Specialized cells in the epidermal layer of the skin known as "club cells" have been suggested as the source of Schreckstoff (Pfeiffer, 1977; Ralphs and Benjamin, 1992). Club cells have no contact with the external water. There is no evidence that Schreckstoff is actively released from them when fish are exposed to hazardous situations or stimuli. Rather, it is released through a passive process after a physical insult or tissue damage, which often occurs as a result of a predatory attack. This is in contrast to the manner in which alarm pheromones are released by other animals, which does not involve tissue damage. Thus, the alarm pheromone in fish is released into the aqueous surrounding by passive diffusion after tissue damage.

There are three types of OSNs in the zebrafish olfactory organ (the olfactory rosette): ciliated OSNs, microvillous OSNs and crypt neurons. Ciliated OSNs express OR-type of olfactory receptors and the OMP-protein, mainly project to the antero-

Table 1. Comparison between the alarm pheromone-sensing olfactory system and the canonical olfactory system

	Fruit fly		Zebrafish		Mouse	
	Alarm	Canonical	Alarm	Canonical	Alarm	Canonical
Anatomical compartment	Antenna	Antenna	Olfactory rosette	Olfactory rosette	Grüneberg ganglion	Main olfactory epithelium
OSN type	OSN	OSN	Crypt OSN	Ciliated OSN	GGNs	OSN
OSN morphology	Bipolar with ring-shaped dendrites	Bipolar with straight dendrites	Globose with an invagination containing microvilli and cilia	Round with a long cilia	Globose ciliated	Bipolar ciliated
Receptor type	Gr	Or	V1R	Or	V2r83 TAAR	Or
G-protein	G α_q	Unclear	G α_i	G α_{OLF}	G α_o G α_i	G α_{OLF}
Second messenger	Unknown	Unknown	cAMP	cAMP	cGMP	cAMP
Presynaptic inhibition	No	Yes	Unknown	Yes	Unknown	Yes

medial glomeruli and respond to bile acid, social pheromones in fish (Yoshihara, 2008). Microvillous OSNs express vomeronasal receptor (V)2r-type of olfactory receptors, project to lateral chain and ventrolateral glomeruli and respond to amino acids and food-related odorants (Yoshihara, 2008). Crypt neurons express V1R-type receptors; the great majority expresses the V1R-type receptor *ora4* (Oka et al., 2012) and projects to the dorsomedial glomeruli (Gayoso et al., 2012). Calcium imaging studies showed that the dorsomedial nuclei in the zebrafish olfactory bulb are activated by Schreckstoff, which indicates that the crypt cells are the OSNs that respond to Schreckstoff (Mathuru et al., 2012). Similar observations have been made in the Crucian carp (*Carassius carassius*), where extracellular recordings from the dorsomedial nucleus showed a increased firing frequency in response to skin extract from conspecifics (Hamdani et al. and Doving, 2003).

Crypt neuron is an atypical type of OSN that is characterized by a globose morphology and the appearance of both microvilli and cilia within a crypt (a small invagination of the plasma membrane) (Hansen and Zeiske, 1998). Crypt neurons are sparsely distributed in the olfactory epithelium (Braubach et al., 2012). Immunohistochemical staining indicated that crypt neurons in the zebrafish express a G α_i -type of G-proteins (Oka et al., 2012). Functional studies demonstrated that approximately 40% of crypt neurons rely on cAMP-activated signaling for the detection of odorants (Vielma et al., 2008).

ALARM PHEROMONES IN MOUSE

Alarm pheromones are known to be released from mice that have been subjected to such stressors as foot-shock (Zalaquett and Thiessen, 1991), CO₂ asphyxiation (Brechtbühl et al., 2008) or injection of the pain-inducing hypertonic solution (Rottman and Snowdon, 1972). Additionally, body odor from the loser in a fight elicits strong avoidance responses in naïve individuals (Carr et al., 1970). None of the alarm pheromones has been yet determined. It has been suggested that the pheromone is a water-soluble molecule, given that it can be collected by bubbling the odor from stressed mice through a solution of artificial cerebrospinal fluid (aCSF) (Brechtbühl et al., 2008). Stressed rats have increased levels of 2-heptanone in their urine, which elicits avoidance behavior in naïve rats (Gutierrez-Garcia et al., 2007). However, the low solubility of 2-heptanone in water and the failure of 2-heptanone to elicit avoidance behavior and stimulate the Grüneberg ganglion (GG) in mice suggest that it is

not likely to be an alarm pheromone in mice (Brechtbühl et al., 2008).

The mouse olfactory system consists of four sub-systems: the main olfactory epithelium, which responds to general odorants; the vomeronasal organ, which responds mainly to pheromones; the septal organ of Maseria; the Grüneberg Ganglion (GG) (Breer et al., 2006; Munger et al., 2009). Calcium imaging showed that odors collected from asphyxiated mice can activate neurons in the GG and that the severing of GG axons eliminates the alarm response without affecting olfactory acuity to general odors. This suggests that the GG is the olfactory subsystem that detects alarm pheromones (Brechtbühl et al., 2008). The GG also contains a temperature sensor that detects a decrease in ambient temperature (Schmid et al., 2010). Given that pups are not adequately prepared for regulating their own body temperature, removing them from their mother or from the nest could be life-threatening (Blumberg et al., 1992). Thus, a temperature sensor on the tip of the nose would be a useful “alarm system” to alert the pups when they no longer get benefited from the warmth of their mother. The combination of the alarm pheromone-sensitivity and thermosensitivity in the GG provides further evidence that these neurons can serve as detectors of alarm responses to specific stimuli (Schmid et al., 2010).

The GG consists of a small cluster of OSNs (~800 cells) located at the tip of the nose close to the opening of the naris (Grüneberg, 1973). The OSNs in the GG, known as Grüneberg ganglion neurons (GGNs), have a globular shape and multiple cilia. These cells are embedded in connective tissue under a keratinized epithelium and have no morphological connection to the nasal lumen. However, permeabilization assays revealed that water-soluble molecules could reach the cilia of the OSNs. In neonates exposed to dimethylpyrazine and related odorants, an increase in c-fos immunoreactivity was observed, which indicates that water-soluble molecules can be detected by GGNs (Brechtbühl et al., 2008; Mamasuew et al., 2011).

The olfactory receptor expressed by the majority of GGNs is the V2r83 receptor (also known as V2R2). A few GGNs express the TAAR-type receptors in a one-receptor: one-neuron manner that is typical for OSNs (Fleischer et al., 2006; 2007). V2r83 is a putative co-receptor expressed in several vomeronasal neurons (Martini et al., 2001). Some of the V2r83-expressing GGNs co-express the membrane-bound guanylate cyclases, pGC-G or pGC-A, which could act as odorant receptors (Fleischer et al., 2009; Liu et al., 2009). Other components

of cGMP-mediated signaling - such as cGMP-dependent phosphodiesterase 2A (PDE2A) and cGMP-activated cyclic nucleotide-gated channel 3 (CNGA3) - are also expressed by the GGNs. The temperature-sensitivity of GGNs is dependent on cGMP signaling (Schmid et al., 2010).

The GGNs innervate an atypical type of glomeruli surrounding the rostral part of the accessory olfactory bulb. These "necklace" glomeruli are also innervated by GC-D-positive neurons, which comprise a small subpopulation of OSNs in the main olfactory epithelium that does not express OR-type olfactory receptors. The GC-D expressing OSNs respond to CO₂, CS₂ and urine-derived semiochemicals (Hu et al., 2007; Leinders-Zufall et al., 2007; Munger et al., 2010). Similar to fruit flies, mice exhibit avoidance behavior to elevated concentrations of CO₂ and do so in a GC-D-dependent manner (Hu et al., 2007). Similar to GGNs, GC-D-expressing OSNs use a similar cGMP-dependent signaling system that depends on CNGA3 channel to function. The similarity in signaling components, types of odorants and the projection patterns of GGNs and GC-Ds suggest that the necklace glomeruli may be an integrative point of the olfactory bulb that is involved in mediating aversive behavioral responses to hazardous stimuli (Fleischer and Breer, 2010).

CONCLUSION

Some of the main features of the olfactory system that are involved in the detection of alarm pheromones are compared to those in the canonical olfactory system (which detects general odors) in Table 1. The canonical olfactory system monitors a multitude of odorants to generate an odor image of the surrounding air, whereas the alarm pheromone-sensing apparatus requires the detection of a specific semiochemical that is tightly coupled with a stereotypical behavioral response. Consistent with this, the olfactory cells of fruit flies, zebrafish and mice appear to transform alarm pheromones into neural signals with unique properties that differ from those of canonical OSNs.

In fruit flies, for example, the OSNs that are activated by CO₂ are not susceptible to presynaptic inhibition and have a unique ring-shaped dendrite of unknown function. In zebrafish, the crypt neurons have a distinct morphology, are sparsely distributed in the olfactory epithelium and use a G α_{OLF} -dependent pathway rather than the canonical G α_{OLF} pathway. In mice, the alarm pheromone-sensing OSNs are located at the tip of the nose, project to a unique set of glomeruli and use a cGMP-dependent signaling system.

It may be challenging to establish unifying principles for the alarm pheromone-detecting OSNs in these disparate model systems as fruit flies, zebrafish and mice; however, it appears that alarm pheromones are detected by specialized subsystems in all three species. It may be interesting to determine whether these differences exist in axonal projections into the brain. In the eye, retinal ganglion cells (RGC) that generate "images" project to the thalamus, whereas a small subpopulation of RGCs responding to looming objects on a collision course project to the superior colliculus (Dean et al., 1989). These ominous objects trigger a defensive posture or avoidance on the part of the object being approached. Thus, specialized subsystems for defensive alarm signals may be a common principle used in multiple sensory systems.

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