Dose-Dependent Nonassociative Olfactory Learning in a Fly

Iole Tomassini Barbarossa, Patrizia Muroni, Maria D. Setzu and Anna Maria Angioy

Department of Experimental Biology, Section of General Physiology, University of Cagliari, Monserrato-Cagliari 09042, Italy

Correspondence to be sent to: Anna Maria Angioy, Department of Experimental Biology, Section of General Physiology, University of Cagliari, Monserrato-Cagliari 09042, Italy. e-mail: amheart@unica.it

Abstract

Olfactory sensory stimulation induces a fast-phase arrest response (FPA-R) of the blowfly heart activity that has been described as a sensitive tool for testing insect reactivity to odor perception. We analyzed FPA-R occurrence to repeated olfactory stimulation with low and high 1-hexanol concentrations that are behaviorally attractant and repellent, respectively, in the blowfly. FPA-R occurrence diminished and ceased with repeated presentations of low and medium odor concentrations, according to dynamics inversely related to odor doses. On the other hand, repeated stimulation with higher odor concentrations induced persistent FPA-Rs. Sensory input amplitude to repeated presentations of singly tested odor concentrations did not change throughout stimulation sessions. A spontaneous restoration of FPA-R to olfactory stimulation was recorded 30 min after cessation of FPA-R to a previous olfactory stimulation session. However, a prompt restoration of FPA-R to olfactory stimulation after cessation of FPA-R was obtained following mechano-taste stimulation of labellar sensilla. Our findings show that the FPA-R habituates to olfactory sensory stimulation with low and medium odor concentrations according to dynamics inversely related to odor intensities. On the other hand, the FPA-R does not habituate to higher odor concentrations. Therefore, flies learn to disregard nonaversive odor information, but they cannot ignore iterative detection of a repellent volatile.

Key words: habituation, heart response, insect, odor information

Introduction

In nature, olfactory-guided behavior of most animals results from repeated odor samplings, such as sniffing in mammals (Freeman 1978) or antennal flicking in arthropods (Mellon 1997). Because of such sampling modality and of a nonuniform odor plume structure (Murlis et al. 1992; Vickers et al. 2001), volatiles are perceived according to a discontinuous pattern at variable concentrations. Stimulus repetition can induce adaptation or activate habituation-learning process at sensory and central nervous system levels, respectively, depending on stimulation intensity and pattern (Nirit and van der Kooy 2000). Habituation is a widespread occurrence through the animal kingdom, representing a simple form of nonassociative learning in which a response diminishes and ceases with repeated presentation of a single sensory cue, in the absence of peripheral adaptation or muscle fatigue.

In Drosophila, the role of specific K⁺ channel subunits in habituation of the giant fiber escape pathway response has been described (Engel and Wu 1998). Cyclic guanosine monophosphate (cGMP) has been demonstrated to play a role in habituation of the proboscis extension response in the honey bee (Müller and Hildebrandt 2002), and cGMP-dependent protein kinase has been shown to affect habituation of the proboscis extension response in Drosophila (Scheiner et al. 2006).

A highly developed sense of smell allows insects to sensitively recognize and specifically discriminate biologically meaningful odorants (Stensmyr et al. 2002) and to perform appropriate behavior to high-quality olfactory information supplied to their brain (Hansson and Christensen 1999; Vickers et al. 2001). Here we asked if concentration-dependent influences of repeated odor presentation on response habituation could be examined on an insect experimental paradigm allowing for recording response decrement simultaneously with sensory input and muscle fatigue.

In several species of adult insects, olfactory sensory stimulation induces the occurrence of a heart response that has been described as an effective indicator of sensory reception (Angioy et al. 1987, 1998, 2003). The heart is a contractile tubular vessel running along the dorsal midline of the insect body (Angioy and Pietra 1995). An arrest of fast-phase heartbeat and a premature setting in of slow-phase heartbeat occur after olfactory sensory stimulation with several kinds of volatiles (Angioy et al. 1987). By performing stimulus application at the beginning of a fast phase during a regular heart cycle, the
immediate phase arrest, and the decrease of its duration (by 70% or more) reliably prove the occurrence of the fast-phase arrest response (FPA-R). Simultaneous recordings of heart activity and olfactory sensory input from the antennae can be monitored on an intact blowfly (Angioy et al. 1987), and fatigue mechanisms are not called into play in the case of heart activity in this insect species (Angioy and Pietra 1995).

We have monitored the FPA-R to olfactory sensory stimulation in blowflies for analyzing dose-dependent influences on the dynamics of FPA-R occurrence decrement to repeated stimulus presentation.

Materials and methods

Insects

Experiments were performed on 3- to 4-day-old adults of *Protophormia terraenovae* blowflies from a colony reared in standard conditions (24 °C, 70–80% relative humidity, 16:8 day/night cycle). Insects were kept without food but were provided with water ad libitum during 24 h prior to performing experiments. Intact blowflies were fixed dorsal side down on a strip of low melting point dental wax; both wings and legs were immobilized. Single specimens were placed on a microscope stage in the visual field of a stereomicroscope (Wild M5 A; Wild Leitz Ltd, Heerbrugg, Switzerland) within a Faraday shield on an antivibration surface. Tests were made in a controlled environment at the same standard conditions used for rearing the insect colony.

Electrophysiological recordings

Standard electrophysiological techniques were used for simultaneously recording heart activity (electrocardiogram [ECG]) and antennal olfactory input (electroantennogram [EAG]) on intact specimens (Angioy et al. 1987, 2003).

Monopolar extracellular ECGs were obtained using a pair of metal electrodes (Ag-AgCl wires, 250-μm diameter) in contact with the insect cuticle by means of a conductive ECG gel. The active and the ground electrodes were positioned on the second abdominal segment and at the base of a foreleg, respectively. Olfactory input recordings were performed from the third antennal subsegment. The active electrode, a saline-filled micropipette (2 μm in diameter at the tip) containing an Ag-AgCl wire, was positioned on an olfactory pit on the posterior antennal surface. The different electrode was the same grounded one used for ECG recording. After amplification (Altec Elettronica s.n.c., Cagliari, Italy), ECG and EAG signals were displayed on the screen of an oscilloscope (Tektronix 5111 A; Tektronix Inc. Beaverton, OR), stored on a modified video recorder (Vetter; A.R. Vetter Co. Inc. Rebesburg, PA), and later analyzed with an integrated system of hardware and software (PowerLab/4S; ADInstruments Ltd, Castle Hill, Australia).

Olfactory stimulation

Olfactory stimulation was performed using a standard olfactometer (Angioy et al. 1987). A main flow of humidified and charcoal-filtered air (2 l/min) was continuously delivered through a glass tube (internal diameter 10 mm), ending 2 cm in front of the blowfly antennae. By means of a polyethylene tube, a 5-ml glass syringe containing a 10 × 1 cm piece of filter paper with (stimulus) or without (control) an odor stimulus was connected to the glass tube. By using a puffing device provided with a timer-controlled opening of a 3-way air valve (Altec Elettronica s.n.c.), a 2-s pulse of a secondary airflow (400 ml/min) was sent through the syringe to the glass tube. For stimulation, a 2-s pulse of clean air (control) or air containing an odor stimulus was added to the main airflow. A glass funnel (internal diameter 5 cm) connected to an air suction system was positioned close to the preparation to take away the odor-carrying air after stimulation. Vapors of 1-hexanol were used as a single olfactory cue. The chemical was diluted in hexane in decadic steps, a 100% and a 0.1% doses corresponding to the pure chemical and a 1000-fold dilution of it respectively, and solutions impregnating the filter paper were singly tested after solvent evaporation.

Experimental procedure

Heart activity was recorded after a 15-min period of recovery from electrode positioning, allowing additional time when spontaneous insect movements affected regular activity pattern (Thon 1982). Continuous ECG recording started 4 heart cycles before the delivery of the main airflow over the fly antennae. This airflow permanently flushed the preparation until the end of tests on each specimen. Because the sensory induced heart response of the blowfly consists of a fast-activity arrest (Thon 1982; Angioy et al. 1987), stimulation was applied 2 s after the recording of a fast-phase beginning in the oscilloscopic screen. Control stimulation was performed at the beginning as well as at the end of an experimental session by delivering a pulse of clean air through a syringe without any odor stimulus. Animals exhibiting an FPA-R to control stimulation were removed from the experimental group. Olfactory stimulation was repeatedly performed at a frequency of 1 stimulation/heart cycle (20–30 s interstimulus interval) by delivering a pulse of air through a syringe containing an odor stimulus.

Dose-related effects of repeated stimulation on FPA-R occurrence and sensory input

Dose-related effects of repeated stimulation were measured by testing each odor dose on a separate group of 22 flies. FPA-R occurrence to successive stimulations with a given odor dose was recorded and then continuing throughout a 10-stimulation series starting from FPA-R cessation. A series of 25 successive odor applications was performed.
when a persistent FPA-R occurred to olfactory stimulation. Threshold and latency of the FPA-R and correspondent EAG amplitude values were measured.

**Effects of a novel stimulation on FPA-R occurrence after FPA-R cessation**

Single specimens of a group of 44 flies were trained with a repeated stimulation with a 0.1% odor dose until FPA-R cessation was detected. Olfactory stimulation continued with 10 successive odor applications in order to verify the cessation persistency. At the end of the latter training, an intense stimulation was performed of mechano-taste trichoidea sensilla that are located on the oral labellar surface of the proboscis (Dethier 1976). By using a micromanipulator (Leica micromanipulator; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany), the smaller end of a glass micropipette containing a 0.5 M sucrose solution was slipped over the tip of 3–4 sensilla that were simultaneously bent for 3–4 s. Mechano-taste stimulation was followed by a last series of 10 odor applications, and FPA-R occurrence and cessation were monitored. In a control fly group ($n = 35$), single specimens were trained with a repeated odor stimulation (a 0.1% odor dose) until FPA-R cessation was detected, thereafter, continuing with a session of 20 successive odor applications.

**Spontaneous restoring of FPA-R after cessation**

A group of 26 specimens were trained to cessation of FPA-R with a first session of olfactory stimulation (a 0.1% odor dose). Flies exhibiting FPA-R to the odor stimulus 30 min later were then trained to cessation of FPA-R with a second session of olfactory stimulation. The dynamics of cessation of FPA-R to the first and the second stimulation sessions were compared with each other.

**Data analysis**

FPA-R threshold was set as the odor dose to which ≥50% of specimens tested showed an FPA-R. Latency of the FPA-R was measured as the time interval between the initiation of the stimulus and the fast-phase arrest, as shown in the ECG. A 2-way analysis of variance (ANOVA; Statistica, StatSoft 7.0) was used to evaluate the dynamics of changes of FPA-R occurrence and latency and EAG variation pattern. A regression line analysis was used to evaluate the significance of FPA-R restoring data (GraphPad Prism 4, software program).

**Results**

Figure 1 is a sample of simultaneous heart activity (ECG) and olfactory input (EAG) recordings on an intact blowfly, before and during a repeated presentation of a low 1-hexanol concentration (0.01% of the pure chemical).

A regular heart activity cycle before stimulation, that is, alternating fast phase and slow phase (line A in the Figure),...
was followed by a series of FPA-Rs (line B in the Figure), that is, a fast-phase arrest and a premature slow-phase beginning, induced by a repeated 2-s odor presentation (line B: bars 1, 2, and 3). The FPA-R was evoked 3 successive times after a progressively increasing latency (from 1 to 3: 0.695, 1.086, and 1.565 s, from stimulation onset to fast-phase arrest in the Figure), and finally it did not occur after a fourth stimulus repetition, EAG amplitude being unchanged (line C in the Figure).

An FPA-R to control stimulation was exhibited by a very limited number of flies (8 out of 140). These specimens were removed from experimental groups. The lowest odor dose inducing an FPA-R in a significant number of flies (more than 50% of group specimens) was a 0.1% concentration of the chemical solution (Figure 2A). Therefore, the latter as well as higher doses corresponded to stimulus intensities above the FPA-R threshold. The number of responding flies decreased with repetition of above-threshold stimulations with low (0.1%) or moderate (1%) odor doses (Figure 2A), as confirmed with a 2-way ANOVA ($F(24, 1000) = 4.905, P < 0.000001$). No significant changes of correspondent EAG amplitudes across the stimulation training were detected (Figure 2C: $F(5, 177) = 0.263, P = 0.93$). FPA-R latency progressively increased throughout stimulation sessions with low (0.1%) and moderate (1%) odor doses (Figure 2B), as confirmed with a 2-way ANOVA ($F(4, 108) = 3.605, P = 0.008$). Correspondent dynamics of decreases in responding fly percentage was inversely related to stimulating odor doses, FPA-R cessation occurring faster (at the 6th trial) and slower (at the 18th trial) with a low (0.1%) and a moderate dose (1%), respectively. Post hoc comparison with Tukey’s test confirmed dose-dependent repeated-stimulation effects on the 2 patterns of responding fly decrement ($P \leq 0.02$).

Differently from the latter, an FPA-R was repeatedly evoked across an even prolonged stimulation series with high (10%) or highest (100%) odor doses in a significant fly percentage (53%) or in most of them (80%), respectively. Despite a decreasing trend of FPA-R occurrence to the high odor dose (10%), post hoc comparison with Tukey’s test indicated that there were not any reliable differences in the number of responding specimens throughout both trainings with high and highest odor doses ($P > 0.05$). No significant changes of correspondent EAG amplitudes across each stimulation training were detected (Figure 2C; $F(22, 334) = 0.54, P = 0.971$). In both cases, the FPA-R latency did not significantly change throughout the training (Figure 2B; $F(27, 322) = 0.331, P = 0.999$). Figure 3 is a sample of FPA-R restoring following the application of a novel stimulus on a fly previously trained to FPA-R cessation with a series of a 0.1% odor dose stimulation. A regular heart activity before stimulation was followed by an FPA-R to 3 successive olfactory stimulations (+, 1–3; line A in the Figure). FPA-R cessation was detected at the fourth stimulus application (−, 4; line A in the Figure) and persisted until a thirteenth one (−, 13; lines B and C in the Figure). Stimulation of mechano-taste labellar sensilla at the successive fast phase induced a prompt FPA-R (+, MT; line D in the Figure). Thereafter, olfactory stimulation during successive heart cycles turned out to be effective again, an FPA-R occurring 4 consecutive times.

![Figure 3](image-url)  
Figure 3 Effects of a novel stimulation on FPA-R occurrence after FPA-R cessation in a Protophormia terraenovae blowfly. Sample of a continuous ECG recording before stimulation (line A), during a first olfactory stimulation session (1–13; lines A, B, and C), during mechano-taste stimulation of labellar sensilla (MT; line D), and during a second olfactory stimulation session (1–5; line D). Bars mark stimulus delivery (a 0.1% dose of 1-hexanol vapors). FPA-R occurrence (+) and absence (−) are shown.
(+, 1–4; line D in the Figure) before ceasing at the fifth stimulation (−, 5).

A restoration of the FPA-R to olfactory stimulation following mechano-taste stimulation of labellar sensilla was obtained in 26 out of 44 flies previously trained with an olfactory stimulation session (10 stimulations with a 0.1% odor dose) after FPA-R cessation (Figure 4, A bar).

These flies displayed equivalent dynamics of FPA-R occurrence decrease to a first ($y = -8.41x + 77.05, R^2 = 0.887, P = 0.0001$) and a second stimulation session performed after the application of the novel stimulus ($y = -6.23x + 53.82, R^2 = 0.630, P = 0.0106$) (parallel lines, 1-way ANOVA [$F(1, 14) = 1.045, P = 0.3239$]; top and bottom lines, respectively; Figure 5). In the control group of flies receiving no other stimuli but a first session of olfactory stimulation (10 applications of a 0.1% odor dose) after FPA-R cessation ($n = 35$), response absence was consistently detected throughout a second session of 10 more olfactory stimulus applications (Figure 4, B bar).

Taken together, these results demonstrate that the FPA-R cessation to a repeated olfactory stimulation and its restoration following a novel stimulation correspond to habituation and dishabituation phenomena.

By testing a group of flies ($n = 26$) with a repeated stimulation (a 0.1% odor dose) before habituation (Figure 6, A bar) and at a 30-min time interval after FPA-R cessation (Figure 6, B bar), a spontaneous recovery from habituation was detected in a significant number of flies ($n = 18$).

A regression line analysis of results showed that this group of flies displayed equivalent FPA-R habituation dynamics to the first ($y = -7.58x + 89.50, R^2 = 0.882; P < 0.0001$) and the second stimulation session ($y = -5.50x + 63.91, R^2 = 0.718; P = 0.0005$) (parallel lines, 1-way ANOVA [$F(1, 20) = 2.227, P = 0.1512$]; top and bottom lines, respectively; Figure 7), the latter spaced by a 30-min time period from the former.

### Discussion

Olfactory sensory stimulation induces the FPA-R that has been described as an effective tool for testing insect responsiveness to odor detection (Angioy et al. 1987, 1998, 2003). Here we show that the FPA-R the blowfly exhibits to

![Figure 4](image-url)  
**Figure 4** Effects of a novel stimulation on FPA-R occurrence in *Protophormia terraenovae* blowflies previously trained to FPA-R cessation with a first session of olfactory stimulation. Bars are percentages of specimens (A, $n = 44$; B, $n = 35$) showing an FPA-R to the first odor application of a stimulation session performed after (A) and in the absence (B, control) of mechano-taste stimulation of labellar sensilla. A 0.1% odor dose was always used as olfactory stimulus.

![Figure 5](image-url)  
**Figure 5** Effects of a novel stimulation on FPA-R occurrence after FPA-R cessation in a group of *Protophormia terraenovae* blowflies. Percentage of specimens ($n = 26$) showing an FPA-R to sessions of olfactory stimulation (a 0.1% odor dose) performed before (open squares, top) and after (filled circles, bottom) mechano-taste stimulation of labellar sensilla. Regression line analysis; parallel lines, $P = 0.3239$ (GraphPad Prism 4, software program).
olfactory stimulation can be regarded as a suitable experimental paradigm for studying nonassociative learning processes such as habituation.

A decrease of FPA-R occurrence and its cessation was obtained by a repeated olfactory sensory stimulation. This phenomenon was not dependent on sensory adaptation because a constant sensory input amplitude was recorded throughout a repeated stimulus application. Besides, it was not caused by heart-fatigue mechanisms that are absent in the case of blowflies (Angioy and Pietra 1995). After cessation, the FPA-R to olfactory stimulation was promptly restored following an intense mechano-taste stimulus applied to the labellar sensory area. Taken together, these findings demonstrate that FPA-R cessation to repeated olfactory stimulation and its restoration following a novel stimulation correspond to habituation and dishabituation phenomena.

Previous studies showed dose-dependent opposite effects of 1-hexanol stimulation on odor-induced blowfly behavior, low and high vapor concentrations causing low-moderate attractancy and repellency, respectively (Dethier and Yost 1952). Our findings showed dose-dependent different effects of 1-hexanol stimulation on habituation in blowflies, the nonassociative learning process occurring faster to a low 1-hexanol dose and slower to a moderate one. Despite a decreasing occurrence trend, formally no evidence for FPA-R habituation to a high odor dose was found. Finally, habituation almost never occurred to the highest odor dose.

Dose-dependent habituation has been described for the chemotaxis response in *C. elegans*, a low odor concentration inducing habituation and a high one causing sensory adaptation (Nirit and van der Kooy 2000). In our experiments, we simultaneously recorded iterative occurrence of FPA-R and constant olfactory input amplitudes to stimulation with 10% and 100% 1-hexanol doses, thus demonstrating that neither habituation nor sensory adaptation to high odor doses occurred. The different patterns adopted for training blowflies and *C. elegans*, that is, a temporally massed odor stimulation (30-s interstimulus interval) and a long-term odor preexposure, respectively, may account for the absence or the occurrence of sensory adaptation to high odor doses detected.
in the 2 research studies. Adoption of a temporally massed stimulation pattern may also account for the short-term duration of memory for habituation measured in the present work, with long-term memories requiring spaced stimulation trainings (Scharf et al. 2002).

In conclusion, the research work we have performed shows that the dynamics of habituation in blowflies is inversely related to the stimulating odor dose, the pure chemical never inducing habituation. Therefore, blowflies can learn to disregard a biologically nonaversive odor information, but they cannot ignore an even iterative detection of a repellent volatile.

Attractive and repulsive olfactory information appear to require 2 opposing modulatory transmitter systems (Schwaerzel et al. 2003; Schroll et al. 2006) and to be processed in different centers (Wang et al. 2003) of the Drosophila brain, to be mediated by separate circuits in C. elegans (Nirit and van der Kooy 2000), and to activate different regions of the human brain (Zald and Pardo 1997; Gottfried et al. 2002). Accordingly, dose-dependent nonassociative learning mechanisms we describe may require different neuronal populations working in separate brain circuits processing segregated olfactory information to high and low concentrations of a single odor compound.

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References


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