The Journal of Experimental Biology 213, 4163-4173

doi:10.1242/jeb.046474

Presynaptic Ca^{2+} stores contribute to odor-induced responses in *Drosophila* olfactory receptor neurons

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Accepted 31 August 2010

SUMMARY

In both vertebrates and invertebrates, olfactory receptor neurons (ORNs) respond to several odors. They also adapt to stimulus variations, and this is considered to be a simple form of non-associative learning and neuronal plasticity. Different mechanisms have been described to support neuronal and/or synaptic plasticity. For example in vertebrates, presynaptic Ca^{2+} stores relying on either the ryanodine receptor (RyR) or the inositol (1,4,5)-trisphosphate receptor (InsP_3R) have been reported to participate in synaptic transmission, in hippocampal pyramidal neurons, and in basket cell–Purkinje cell synapses. However, in invertebrates, especially in sensory neurons such as ORNs, similar mechanisms have not yet been detected. In this study, using *Drosophila* and taking advantage of an *in vivo* bioluminescence Ca^{2+}-imaging technique in combination with genetic and pharmacological tools, first we show that the GFP–aequorin Ca^{2+} sensor is sensitive enough to detect odor-induced responses of various durations. Second, we show that for a relatively long (5 s) odor application, odor-induced Ca^{2+} responses occurring in the axon terminals of ORNs involve intracellular Ca^{2+} stores. This response is decreased by specifically targeting InsP_3R or RyR by RNAi, or application of the specific blockers thapsigargin or ryanodine, suggesting that Ca^{2+} stores serve to amplify the presynaptic signal. Furthermore, we show that disrupting the intracellular Ca^{2+} stores in the ORNs has functional consequences since InsP_3R- or RyR-RNAi expressing flies were defective in olfactory behavior. Altogether, our results indicate that for long odor applications in *Drosophila*, the olfactory response depends on intracellular Ca^{2+} stores within the axon terminals of the ORNs.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/24/4163/DC1

Key words: olfactory receptor neuron, aequorin bioluminescence, ryanodine receptor, InsP_3 receptor, Ca^{2+} store.

INTRODUCTION

Calcium plays central roles in activity-dependent modification of synaptic plasticity. In mammals, Ca^{2+} release from intracellular stores mediated by either the inositol (1,4,5)-trisphosphate receptor (InsP_3R) or ryanodine receptor (RyR) Ca^{2+}-release channels, has been implicated in several cellular processes, such as postsynaptic signaling, presynaptic control of neurotransmitter release and long-term synaptic plasticity (Berridge, 1998; Fitzjohn and Collingridge, 2002). Indeed, a Ca^{2+} increase within the presynaptic terminals during repetitive action potentials causes use-dependent enhancement of transmitter release (Katz and Miledi, 1968; Zucker and Regehr, 2002), and, in the hippocampus, particularly in the CA3–CA3 pyramidal neuron synapses, presynaptic Ca^{2+} stores are required for long-term depression (LTD) (Emptage et al., 2001; Unni et al., 2004; Berridge et al., 2003). Although it has been reported in invertebrates that almost all sensory systems (including olfactory) adapt to stimuli variations and display synaptic plasticity, similarly to mammals, the cellular and molecular mechanisms, and more particularly, the involvement of the internal Ca^{2+} stores remain relatively unexplored.

In *Drosophila*, olfactory receptor neurons (ORNs), with their cell bodies and dendrites located in the antennae, detect odors and transmit olfactory information to the antennal lobes. The antennal lobes comprise 43 glomeruli and contain about 100 interneurons. Each glomerulus receives about 20 axons from the ORNs expressing the same receptor gene (Stocker, 1994; Vosshall and Stocker, 2007). The axon terminals of the ORNs make synaptic contact with dendrites of a few uniglomerular projection neurons (PNs), which propagate olfactory information to higher brain centers, such as the mushroom bodies. They also make contact with local interneurons (Stocker, 1994; Wilson and Laurent, 2005). It is well documented that sensory neurons, and particularly the olfactory sensory neurons, adapt to variable ambient conditions, as for instance, the strength of the stimulus (concentration or duration of odor application) (Getchell and Shepherd, 1978a; Getchell and Shepherd, 1978b; Firestein et al., 1993), a physiological mechanism considered as a simple form of non-associative learning and neuronal plasticity. Particularly in mammals, cAMP (Reed, 1992; Kurahashi and Menini, 1997), cGMP (Zufall and Leinders-Zufall, 1997) and inositol (1,4,5)-trisphosphate receptor (InsP_3R) pathways (Breer et al., 1990) have been shown to play a role in olfactory signal transduction and adaptation. However, their precise mechanisms, at the cellular level, remain incompletely understood. In *Drosophila*, the use of genetic and behavioral approaches have shown that mutations in InsP_3R affect olfactory response and adaptation to the stimuli (Deshpande et al., 2000). The InsP_3R-mutated flies are defective in olfactory adaptation, as determined both behaviorally and physiologically (as electroantennograms). However, as these studies were based on the use of mutations, which affect all cells of the organism that express the given gene, the precise neurons in which the olfactory defects occurs and the specific role of the InsP_3R pathways in the ORNs still require clarification.

In this study, we have used the binary P[GAL4] expression system in *Drosophila* [with the P[Or83b-GAL4] line (Wang et al., 2003)]

to specifically target the expression of the GFP–aequorin (GA) bioluminescence probe to the ORNs. In addition, genetic [targeting different RNA interference (RNAi) constructs] and pharmacological approaches were used to perturb specific pathways. Then, taking advantage of an *in vivo* bioluminescence imaging technique (Martin et al., 2007), which allowed us to monitor the neuronal Ca^{2+} activity continuously over a long time range without autofluorescence, phototoxicity or photobleaching, we showed that the odor-induced Ca^{2+} response occurring at ORN axon terminals depends on the duration of the stimulus. Moreover, following a relatively long stimulus such as 5 s, the response was sustained by the intracellular Ca^{2+} stores, through RyR and InsP_3R signaling pathways. Finally, to demonstrate the functional consequences of disrupting the intracellular Ca^{2+} stores in the ORNs, we show that InsP_3R- or RyR-RNAi-expressing flies are defective in olfactory behavior.

MATERIALS AND METHODS

Fly strains

Drosophila melanogaster Meigen were maintained on standard medium at room temperature (24°C). P[UAS–GFP–aequorin] (GA) transgenic flies previously developed by Martin et al. (Martin et al., 2007) were used in conjunction with the P[GAL4]Or83b line, to target GA to the ORNs. P[GAL4]Or83b (obtained from the Bloomington Stock Center) is expressed in a large population (approximately 80%) of sensory neurons (J. W. Wang et al., 2003). Since both P[GAL4]Or83b and P[UAS–GFP–aequorin] are inserted on the third chromosome, they have been recombined on the same chromosome, allowing further genetic crosses directly with the two different RNAi lines. Progeny of flies containing both the P[GAL4]Or83b driver and the P[UAS–GFP–aequorin] transgene (Or83b,GA/Canton-S) as transheterozygotes, were used for all imaging experiments. To precisely knock-down the two different investigated genes specifically in the ORNs, we used specific RNAi (P[UAS–InsP_3R-RNAi], P[UAS–RyR-RNAi]; courtesy of R. Ueda, Japan).

Brain preparation

Female flies at 4-days old were used for *in vivo* brain imaging. The flies were briefly cold (ice) anesthetized, inserted in a truncated 1 ml commercial pipette tip until the head protruded and finally fixed in place with dental glue (Protemp III, ESPE, Seefeld, Germany). The assembly was then placed in an acrylic block and secured with Parafilm. A drop of Ringer's solution (Martin et al., 2007) was deposited on the head, and a tiny window in the head capsule was cut out to expose the ORNs. Care was taken to not damage the antennae. Exposed brains were then incubated in fly Ringer's solution containing 5 μmol l^{-1} native coelenterazine (Uptima, Interchim, Montluçon, France) for 2 h, before experiments.

In vivo brain imaging

Odor-evoked bioluminescence signals in the sensory neurons of *Drosophila* were monitored with an electron multiplier CCD camera (EM-CCD, Andor, iXon, Belfast, Ireland; cooled to –80°C) fitted onto a microscope (Nikon, Eclipse-E800). The setup was housed inside a tight dark box (Science Wares, Inc., Falmouth, MA, USA). Using a 20× air objective lens (N.A. 0.75; Plan Apochromat, Nikon France S.A., Champigny-sur-Marne, France) the field of view was 400×400 μm (512×512 pixels). To improve the signal-to-noise ratio, data were acquired with a 2 s integration time, and 2×2 binning was used (1 pixel=1.56 μm×1.56 μm). To acquire and store data, each detected photon was assigned *x* and *y*-coordinates and a time point. Response of individual flies to three different odors: spearmint, citronella and octanol (3-octanol) were recorded. The laboratory-made odor-delivery apparatus consisted of five identical channels, one of which was devoted to control air (without odor). From the air pump and a moistening bottle, each channel included a 50 ml flask with, on either side, a solenoid activated pinch valve (Sirai S-104) isolating those not in use; all connecting tubes were made of silicone. Air was flowing continuously (500 ml min^{-1}) through the control channel except when a logic command issued by the imaging software switched the flow for the pre-determined odor test times (1, 3 or 5 s) to one of the test (odor) channels. Test flasks contained 50 μl of undiluted pure odor (all from Sigma-Aldrich, Saint-Quentin Fallavier, France), deposed on a piece of filter paper. Finally the air stream was delivered to the fly's antennae through a small glass tube placed a few millimeters away.

Pharmacology

Thapsigargin and ryanodine were used to interfere with calcium-induced calcium release (CICR). At a concentration of 100 μmol l^{-1}, ryanodine is known to block the calcium channel associated with the so-called ryanodine receptor of the ER, whereas thapsigargin is a potent inhibitor of the endoplasmic/sarcoplasmic reticulum Ca^{2+}-ATPase pump (Thastrup et al., 1990). Ryanodine (Latoxan, Valence, France) was prepared as a 10 mmol l^{-1} stock solution and diluted to 100 μmol l^{-1} in fly Ringer's solution. Thapsigargin (Sigma) was dissolved in fly Ringer's to a final concentration of 5 μmol l^{-1}. Prior to drug application, the flies were stimulated once with each odor to verify their responsiveness. The flies' brains were then incubated with either drug for 15 min. Thereafter, the response of individual flies to odor stimulation was recorded.

Olfactory T-maze test

Olfactory responses at the behavioral level were determined using an olfactory T-maze test, slightly modified from that of Störtkuhl et al. (Störtkuhl et al., 1999). The maze was fitted with two side arms (chambers) and one sliding central chamber. Odorant-containing airstreams were drawn, from a 50 ml bottle containing 5 μl of undiluted odorant placed on a filter paper, through one side arm. Similarly, the other side arm was supplied with air only. Air was continuously drawn through the system by a pump at a rate of 1 l min^{-1}. Ten flies, starved beforehand for 6 h, were placed in the central chamber, in an upper position. Then, the central chamber was slid down into the bottom position, from which flies, given 15 s at most, could choose between the two side arms. For the pre-exposure condition, the flies were pre-exposed to the odorant for 5 min in the top chamber and then moved down, *via* the central chamber, to choose between the control (air) and the odor-containing airstreams. In both cases the total number of flies in each side chamber was then counted. The response index (RI) was calculated by subtracting the number of flies in an odorant-containing arm from the number of flies in the control arm and dividing by the total number of flies. The RI value ranged between 1 and –1. In this test, if all flies were repelled by an odorant, the RI would equal –1.0, whereas if attracted, the RI would equal 1.0. RI equals 0 if the flies were indifferent to the odorant (randomly distributed).

Quantitative and statistical analysis

Imaging data were analyzed using the Photon Viewer (1.0) software (Science Wares) written in LabView 7.1 (National Instruments, Nanterre, France). Odor-evoked bioluminescence signals (raw data) are presented as the total amount of emitted photons, or as photons s^{-1} for the amplitude (within the drawn ROI). Image recordings were obtained from 5–10 flies for each genotype. For the olfactory T-

maze tests, a total of 10 groups of 10 flies (100 flies) for each genotype were analyzed and then averaged. All statistics were done using the Statistica (7.1) software (StatSoft, Inc., Maison-Alfort, France). One-way analysis of variance was used to test the total amount of emitted photons and the duration between the control (Or83b,GA/CS) and the experimental groups (Or83b,GA/InsP_3R-RNAi, Or83b,GA/RyR-RNAi, and pharmacologically treated). Additionally, one-way ANOVA, followed by a Newman–Keuls comparison test was used to test significance differences in the olfactory responses of control (both Or83b,GA/CS and RNAi/CS) and experimental flies (that expressed a RNAi) in the T-maze choice test.

Deconvolution analysis

Peak light intensity (photons s^{-1}) and quantity of light (total number of photons) detected during a response were determined after the background value (mean of the 10–30 data points preceding a response) was subtracted from the raw data points. The latter was computed as the integral over time of the light intensity. The presence of a 'shoulder' on the decay of light responses to 5 s applications of odors suggested they were made of two components, slightly out of phase. Decomposition (or deconvolution) of the light responses (light intensity *versus* time) into two components was performed using Origin 7 statistical software (Origin Lab Corp., Northampton, MA, USA). Initially these two components were separated by fitting a simple exponential decay function [$y=A\cdot\exp(-t/\tau)$, where A is the initial value of the function, t is time and τ is a time constant] to the few data points collected between the peak value and the 'shoulder' (visible on the 5 s application records) forcing the exponential function to return to zero level, giving an estimate of the decay of the first component. The second component was then computed by subtracting the first one from the raw data points. This approach was, however, partially arbitrary and tedious, thus it was assumed that the light responses due to the changes in $[Ca^{2+}]_i$ resulted from influxes and thus can be adequately described by the equations derived by Hodgkin and Huxley (Hodgkin and Huxley, 1952) to describe the rise and decay of the Na^+ and K^+ conductance in a nerve. Each component could then be described by a function comprising four elements: (1) an exponential rise (time constant τ_1); (2) an exponential decay (τ_2) modulated respectively by, (3) a coefficient (power function) specific of the channels, and (4) a maximum value (product function), for a total of eight parameters for the two components. Then, a ninth parameter was introduced as the time interval between the two components. These nine parameters and the combined functions were introduced into the fitter wizard of Origin 7 with some initial values: for example: τ_{1-1}=1 s, τ_{1-2}=3 s, max_1=2000, $coefficient_1$=2 and τ_{1-2}=4 s, τ_{1-2}=8 s, max_2=300, $coefficient_2$=2, delay=6. Generally the regression converged spontaneously, although in some cases it was necessary to freeze temporarily one parameter or another to reach convergence. The correlation coefficient was usually close to 0.99.

RESULTS

Odor-induced Ca^{2+} response in ORNs monitored by *in vivo* bioluminescence imaging

GFP–aequorin (GA) is a Ca^{2+}-sensitive bioluminescent photoprotein that can be utilized, in continuous recordings, for long-term imaging with an excellent signal-to-noise ratio (Martin et al., 2007; Baubet et al., 2000; Rogers et al., 2008). We have developed an *in vivo* preparation that allows the detection of odor-evoked changes in bioluminescence intensity in the sensory neuron terminals of a living fly at the level of the antennal lobes (Fig. 1). The fluorescent properties of the GFP moiety of the chimeric GA protein, makes it possible to check whether the targeted expression of GA (Or83b,UAS-GA/CS) has been successful and reveals the anatomical organization of the ORN terminals (Fig. 1Ai,Aii). Delivery of an odor, spearmint (Sp), citronella (Ci) or octanol (Oct), for 5 s to the antennae induced a considerable increase in the bioluminescence intensity at ORN synaptic terminals in the antennal lobe, reflecting activity-dependent increases in the cytosolic Ca^{2+} concentration (Fig. 1Bi,Ci,Di). Odorant-evoked bioluminescent transients occur without any appreciable delay (with our time resolution) after starting odor presentation (Fig. 1Bii,Cii,Dii). Higher resolution shows that the Ca^{2+} response is composed of two main components: a fast rising phase, and the decay phase which is made up of a slower rising phase with a slower decay. Three parameters were routinely measured from the responses: the amplitude (in fact, the amplitude of the first component), the total amount of emitted photons, and the total duration of the response (Fig. 2). Our results indicate that the value of each parameter varied with the odor: for example, for spearmint, the average number of detected photons was 15183 (±3435; Fig. 2B), for citronella, 7851 (±1154; Fig. 2E), whereas it was 27156 (±7066) for octanol (Fig. 2H). The total response duration was also odor specific (77±9.2 s) on average for spearmint (Fig. 2C), much shorter for citronella (42±4.2 s; Fig. 2F), whereas much longer for octanol (109±14.3 s; Fig. 2I). However, the amplitudes of the responses were in the same range: about 600 photons s^{-1} at the peak, for all three odors (Fig. 2A,D,G). A deconvolution analysis showed that the main contribution to the response in terms of the total amount of emitted photons was provided by the first phase (see supplementary material Fig. S1A–C), thus the main source of difference between the responses to the three odors comes from the second component, since the first phase is quite similar for each odor and lasts about 20 s.

The Ca^{2+} response correlates with odor duration

To explore the variability of the odor-induced response in the ORNs, distinct groups of flies were exposed to each odorant for 1, 3 or 5 s. As found in former studies based on the use of various fluorescent probes, such as pH-sensitive green fluorescent protein (synapto-pHluorin) (Ng et al., 2002; Shang et al., 2007) or Ca^{2+}-sensitive fluorescent probes, such as G-CaMP (J. W. Wang et al., 2003; Root et al., 2008; Ignell et al., 2009), we observe that a brief application (1 s) of an odorant induces a small response at the ORN axon terminals (Fig. 2). Compared with 1 s, the 3 s application causes a slightly stronger response for all three measured parameters: amplitude, total photons and duration, especially for spearmint. However, 5 s exposure causes a much larger response again for the three measured parameters but with a marked lengthening of the responses and a clear shoulder, which in fact was just discernable at 3 s for spearmint and octanol (Fig. 2; particularly visible in some individual responses). Altogether, these results demonstrate that the magnitude of the Ca^{2+} response of the ORNs varies with odor duration. Therefore, although the responses to relatively short duration odor stimulus (<1 s) have been well documented (Wilson and Laurent, 2005; J. W. Wang et al., 2003; Ng et al., 2002; Shang et al., 2007; Root et al., 2008; Ignell et al., 2009), responses to longer applications, such as 5 s, have not yet been precisely described, which, as shown here, induce a second slow phase. Thus, with the aim of characterizing this second component, we adopted this condition (5 s) as our experimental standard condition to dissect further the cellular and molecular mechanisms supporting it.

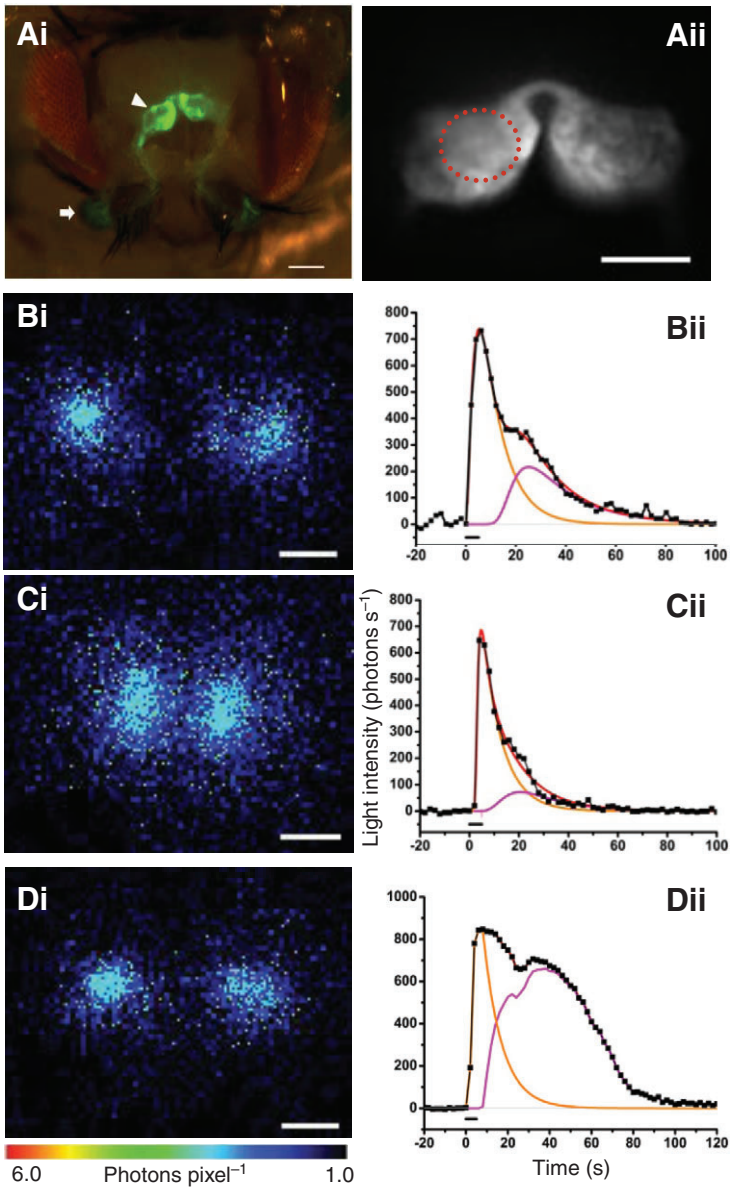

Fig. 1. Odorant-evoked Ca^{2+} responses in the ORNs of a live fly. (Ai) Combined fluorescence and dim-light image showing the distribution of the ORNs over the surface of the antennae (arrow) and their synaptic terminals (arrowhead) in the antennal lobes of a control fly (Or83b,GA/CS; taken with a Leica MZ FLIII binocular microscope; scale bar, 100 μm). (Aii) Fluorescence image of the antennal lobes taken at the beginning of the experiment, used here as a reference image. The red-dashed circle represents the ROI (region of Interest) from which the light emission was quantified (Scale bar, 50 μm). (Bi,Ci,Di) Representative Ca^{2+}-induced bioluminescence images (3 s accumulated light) evoked by a single 5 s application of spearmint (Bi), citronella (Ci) or octanol (Di) shows glomerulus-specific responses. (Bii,Cii,Dii) Raw data of the variation in light intensity (photons s^{-1}) as a function of time of a Ca^{2+}-induced response within the ROI, evoked by a single 5 s application (black bar) of spearmint (Bii), citronella (Cii) or octanol (Dii). Notice the 'shoulder' on the decay of the overall responses (black dotted line), indicating that they result from the summation of two independent components. Indeed, deconvolution of the Ca^{2+} response using statistical analysis shows the two phases. The sum of the two phases (red line) is superimposed on the raw data points (black dotted line). First phase, orange line; second phase, magenta line. Notice that Dii is not exactly at the same scale (*x* and *y* axes) as 1Bii,Cii.

Odor-induced Ca^{2+} activity in the axon terminals occurs through voltage-gated calcium channels

Odor-induced responses in the ORNs are translated as spikes that propagate down to the axon terminals, leading to the opening of the voltage-gated calcium channels (VGCC), responsible for transmitter release. OR83b-GAL4 drives GA expression throughout the ORNs, where it diffuses along the axon down to the terminals, where it can be detected in the antennal lobes (Fig. 1). To verify that the GA-monitored Ca^{2+}-induced activity following odor application primarily results from the activation of the ORNs by odors, we used tetrodotoxin (TTX), the well known sodium-channel blocker, which blocks spike propagation (Su and O'Dowd, 2003). TTX application completely abolished the odor-induced Ca^{2+} responses, indicating that the recorded bioluminescence within the axon terminals directly resulted from the spike propagation within the ORNs (data not shown). To dissect further the different neuronal events leading to Ca^{2+} entry in the axon terminal, we used the VGCC blockers, nifedipine and verapamil (Su and O'Dowd, 2003). Prior to applying either drug, flies were challenged once with the odor, to check their olfactory integrity and provide a control record. Both nifedipine and verapamil application for 15 min completely abolished the Ca^{2+}-induced bioluminescence in the antennal lobes for the three odors, indicating that the presynaptic Ca^{2+} entry was necessary to trigger both phases of the odor-induced Ca^{2+} response (Fig. 3).

InsP_3R and RyR are required for triggering the delayed second slow rising phase

InsP_3R and RyR are the two major channel–receptor complexes controlling the release of Ca^{2+} from intracellular stores (Berridge et al., 2003). In mammals, InsP_3 has been reported as a second messenger during olfactory transduction (Breer et al., 1990), whereas, in *Drosophila,* mutation in the InsP_3R gene (*itpr*) generates defects in olfactory adaptation (Desphande et al., 2000). Moreover, in the hippocampal CA3 pyramidal neurons of mammals, the intracellular Ca^{2+} stores, which are controlled by InsP_3R and/or RyR at the presynaptic terminal, are implicated in neurotransmitter release as well as in synaptic plasticity (Unni et al., 2004; Collin et al., 2005). Thus, to assess whether InsP_3R and/or RyR signaling pathways are implicated in the olfactory response, flies with disturbed InsP_3R or RyR were analyzed.

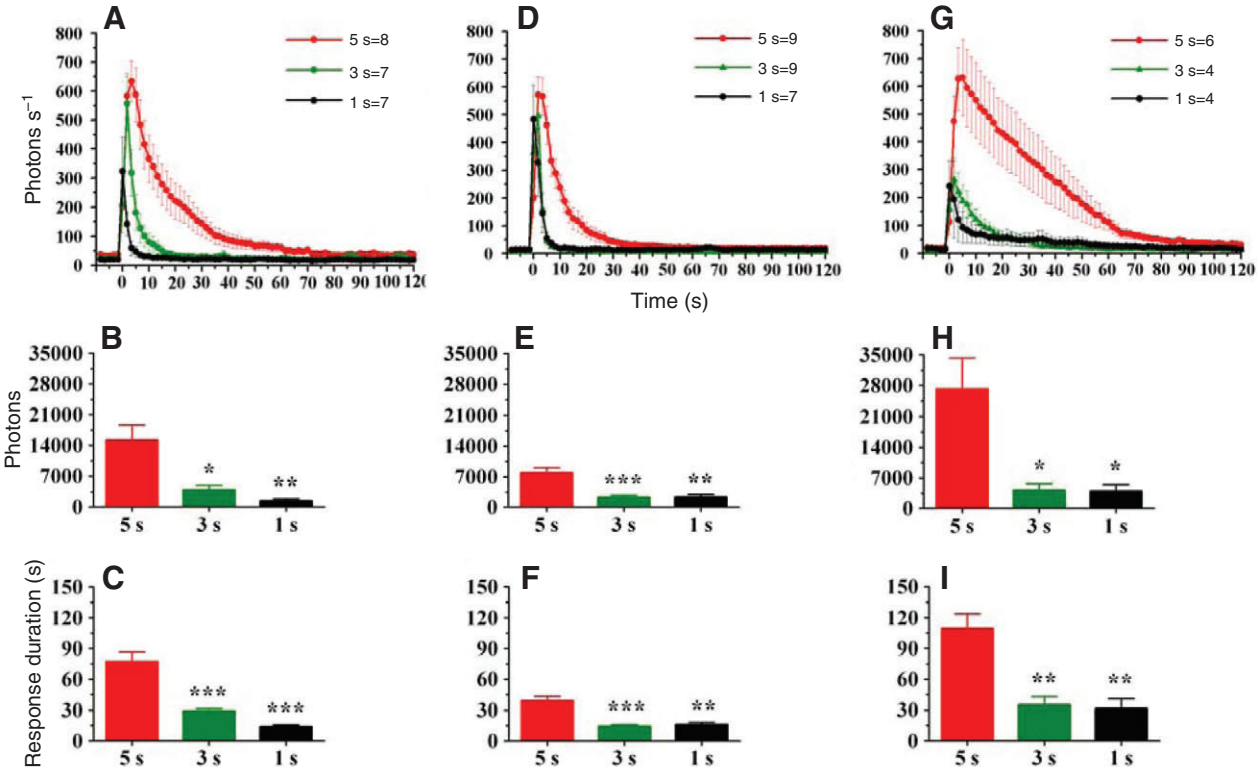

Fig. 2. Odor-induced Ca^{2+} response within the ORNs is correlated with the duration of the stimulus. Ca^{2+}-induced bioluminescence (within the ROI) evoked by 1, 3 and 5 s applications of spearmint (A,B,C), citronella (D,E,F) or octanol (G,H,I). (A,D,G) Mean amplitude (photons s^{-1}) of 4–9 responses (from different flies) over time. Note the shoulder in the 5 s applications but not in the shorter ones. (B,E,H) Mean number of total photons for the three pulse lengths. (C,F,I) Mean response duration (red, 5 s; green, 3 s; black, 1 s). *N*=4–9 flies for each condition. Values are means ± s.e.m. Statistics: A,D,G, one-way ANOVA, followed by a Newman–Keuls comparison test for spearmint (A): 1 s *versus* 5 s: $P<0.05$; 1 s *versus* 3 s: NS; 3 s *versus* 5 s: $P<0.05$. For citronella (D): 1 s *versus* 5 s: $P<0.05$; 1 s *versus* 3 s: NS; 3 s *versus* 5 s: $P<0.05$. For octanol (G): 1 s *versus* 5 s: $P<0.05$; 1 s *versus* 3 s: NS; 3 s *versus* 5 s: $P<0.05$. For B,C,E,F,H,I: Two-tailed *t*-test: *$P<0.05$; **$P<0.001$; ***$P<0.0001$.

Thapsigargin is an inhibitor of the Ca^{2+}-ATPase of the endoplasmic reticulum (ER) that depletes the intracellular Ca^{2+} stores (Thastrup et al., 1990; Rosay et al., 2001). Thapsigargin application for 15 min (Fig. 4) clearly decreased the total number of photons (Sp: 5813±1380.8, $P<0.05$; Ci: 3197±494.9, $P<0.05$; Oct: 4732±3013.7; $P<0.05$), as well as the duration of the response (Sp: 28±5.4 s, $P<0.0001$; Ci: 22±2.0 s, $P<0.001$; Oct: 25±3.0 s, $P<0.001$). The amplitude of the first component of the response was also affected, as compared with control flies, for spearmint ($P<0.05$) and octanol ($P<0.05$; Fig. 4A,G), whereas it was not affected for citronella (Fig. 4D). These results suggest that, although thapsigargin slightly affects the amplitude of the first component (for two of the three tested odors), more importantly, it affects the second component of the response (for the three tested odors). This lead us to suggest that the first component does not necessarily involve Ca^{2+} release from internal stores (e.g. for citronella). To strengthen this conclusion we targeted a specific RNAi to impair the expression of InsP_3R specifically in the ORNs. Flies simultaneously expressing GA and an interfering RNA directed against the InsP_3R (Or83b,GA/InsP_3R-RNAi; Fig. 4) presented an important decrease in light emission during olfactory responses (Sp: 4671±1451.5, $P<0.05$; Ci: 3673±531.7, $P<0.05$; Oct: 7453±2833.1, $P<0.05$; Fig. 4B,E,H). However, again, the amplitude of the first component was only slightly decreased for spearmint ($P<0.05$) and octanol ($P<0.05$), and not at all for citronella, whereas the duration of the responses were significantly decreased in the three cases (Sp: 37±7.5 s, $P<0.001$; Ci: 28±3.8 s ; $P<0.05$; Oct: 39 ±6.6, $P<0.001$).

RyRs have been reported to be present in the antennal lobes (Vázquez-Martínez et al., 2003), but without a precise determination of the neurons that express it. Using a similar strategy to that used for InsP_3R, the contribution of the RyR to the Ca^{2+} responses was evaluated. From 100 µmol l^{-1} and above, ryanodine blocks the channel associated with the RyR (Berridge et al., 2003). Incubations for 15 min decreased the responses (Sp: 5443±1391.6, $P<0.05$; Ci: 3652±686.5, $P<0.05$; Oct: 5466±798.5, $P<0.05$; Fig. 5B,E,H). The amplitude of the first component was affected for spearmint ($P<0.05$) and octanol ($P<0.05$) but not for citronella (Fig. 5A,D,G), whereas the duration significantly decreased for all three odors (Sp: 46±6.0 s, $P<0.05$; Ci: 26±5.1 s, $P<0.05$; Oct: 25±6.7 s; $P<0.0001$; Fig. 5C,F,I). Thus, these results indicate that RyRs are mainly involved in the second component of the response (although an effect on the first component has also been observed for spearmint and octanol). To corroborate the pharmacological approach, a specific RNAi was targeted to the ORNs. Flies simultaneously expressing GA and an interfering RNA directed against the RyR (Or83b,GA/RyR-RNAi) showed a decrease in light emission in response to olfactory stimuli (Sp: 5094±956.8, $P<0.05$; Ci: 3416±894.1, $P<0.001$; Oct: 3566±978.5, $P<0.001$) (Fig. 5B,E,H). The duration of the response was also significantly reduced for all three odors (Sp: 44±5.7 s; $P<0.001$; Ci: 24±2.9 s; $P<0.001$; Oct:

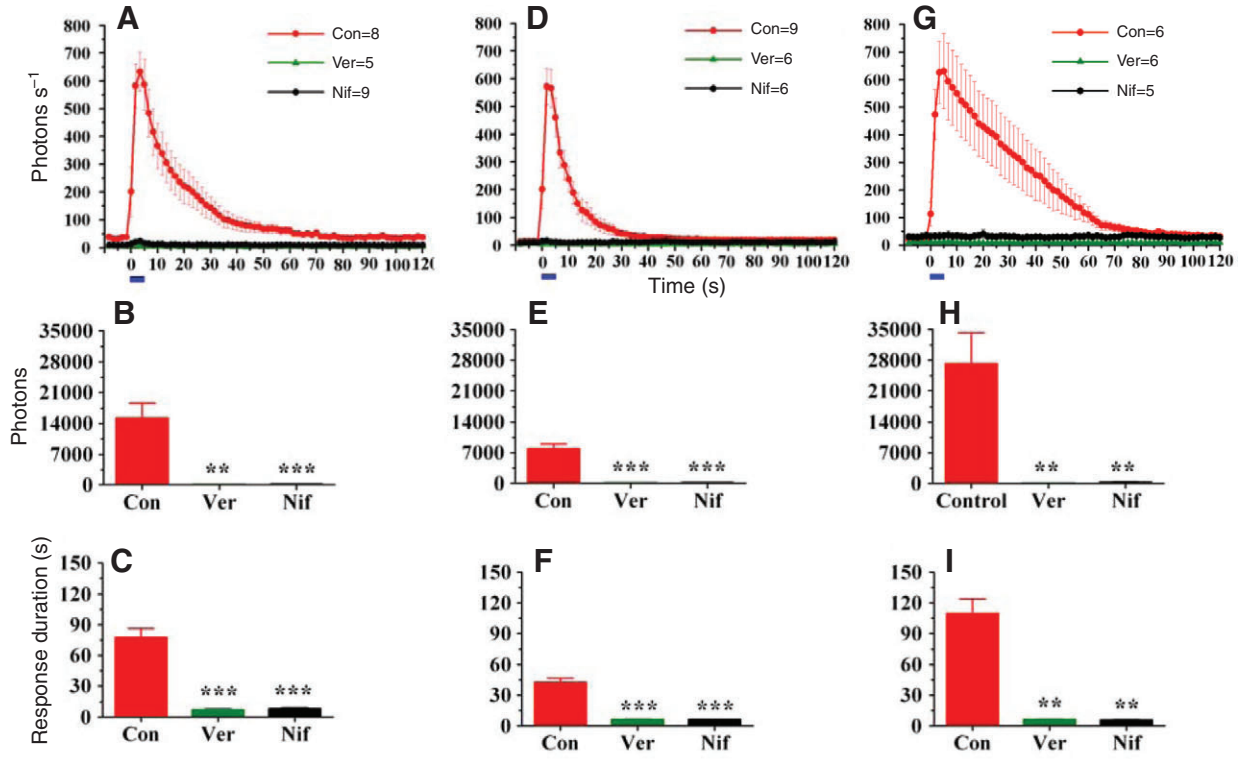

Fig. 3. Effects of blocking the VGCC on the odor-induced Ca^{2+} response within axon terminals of the ORNs. (A,D,G) Effect of verapamil (Ver, green) and nifedipine (Nif, black) on the Ca^{2+}-induced responses collected from the ROI, evoked by 5 s applications (blue bar) of spearmint (A–C), citronella (D–F) or octanol (G–I); control experiments (Con) are shown in red. (A,D,G) Mean amplitude of the overall responses (photons s^{-1} *versus* time), for the two tested conditions: Ver or Nif, *versus* controls (5 s). (B,E,H) Means of the total photons for each condition. (C,F,I) Mean response duration (s). Notice that the VGCC blockers, Ver and Nif, completely block the Ca^{2+} response. *N*=5–9 flies for each condition. Values are means ± s.e.m. Statistics: same tests as for Fig. 2. For spearmint (A) Ver *versus* Con: $P<0.05$; Nif *versus* Con: $P<0.05$. For citronella (D) Ver *versus* Con: $P<0.05$; Nif *versus* Con: $P<0.05$. For octanol (G) Ver *versus* Con: $P<0.05$; Nif *versus* Con: $P<0.05$. For B,C,E,F,H,I, two-tailed *t*-test: $^{**}P<0.001$; $^{***}P<0.0001$.

30±1.3 s; $P<0.0001$). Moreover, as observed with ryanodine, the amplitude of the first component was not significantly affected in the case of citronella, whereas it was affected for spearmint ($P<0.05$) and octanol ($P<0.05$; Fig. 5A,D,G). These suggest that the RyR might also contribute to the first component, or else, that knocking down the RyR has already caused changes in Ca^{2+} homeostasis that disturbs the 'basic resting state' of the ORNs. In conclusion, the similar results obtained with two independent approaches, genetic and pharmacological, confirm that InsP_3R as well as RyR, and consequently the Ca^{2+} stores released from the ER, contribute to the olfactory response.

Disrupting presynaptic Ca^{2+} stores hampers olfactory behavior

According to the above observations, disrupting presynaptic Ca^{2+} stores is likely to have behavioral correlates, which can be evaluated using olfactory T-maze tests (Störtkuhl et al., 1999). Flies were exposed to an odor and then tested with the same odor (at the same concentration) in the T-maze and their responses were compared with that of control flies (Fig. 6). Olfactory responses were recorded from sibling flies of the three genotypes used for brain imaging: Or83b,GA/CS (control), Or83b,GA/InsP_3R-RNAi, Or83b,GA/RyR-RNAi (pharmacologically manipulated flies are not usable in this test), as well as for the two appropriate controls (each of the two RNAi in heterozygote: InsP_3R-RNAi/CS, RyR-RNAi/CS). Naïve flies (all controls as well as the flies genetically expressing a RNAi) preferred the control arm (without odor), meaning that they were repelled by the odorants (RI value: –0.3 to –0.7 depending on the condition tested; Fig. 6). Since the results of this test (which measures the odor acuity) did not reveal a striking difference between the controls and the flies with genetically disturbed intracellular Ca^{2+} stores, we wondered if in a different behavioral context, the outcome would be similar. Notably, mutations in the InsP_3R affect olfactory adaptive response to the stimuli (Deshpande et al., 2000). Thus, to determine if a pre-exposure to an odor affects the response to this odor, the flies were pre-exposed to the odorants for 5 min. We found that after pre-exposure, there was a significant change in the response of the control flies (Or83b,GA/CS, Cont.InsP_3R-RNAi/CS and Cont.RyR-RNAi/CS): the preference toward the control-arm disappeared and the control flies now preferred the odor-arm for the three odors (mean RI value: 0.4 to 0.5; Fig. 6). We ascribed this 'pre-exposure' effect to an adaptation process (change of the sensitivity to an odor, or in other words, a change of the threshold). Conversely, we found that the inversion in the olfactory response, as a result of the 'pre-exposure' was significantly different in the Or83b,GA/InsP_3R-RNAi and Or83b,GA/RyR-RNAi flies, compared with control flies, for the three tested odors (except for the Or83b,GA/RyR-RNAi flies tested with octanol: although the mean is importantly reduced, it is not statistically different from the two appropriated control groups). As shown in Fig. 6, these RNAi-expressing flies had mean RI values around 0, indicating that they had become almost indifferent to the

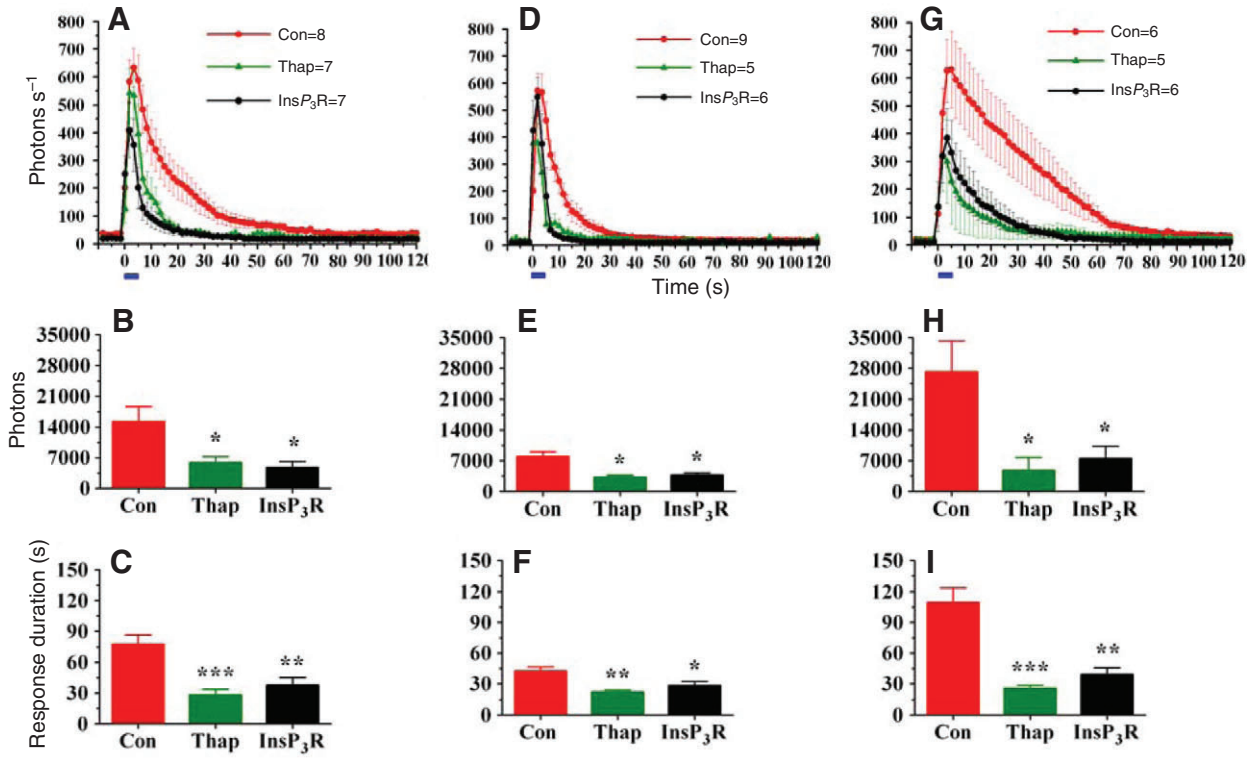

Fig. 4. Odor-induced Ca^{2+} response is reduced in flies lacking InsP_3R or when blocked pharmacologically. (A–C) Spearmint, (D–F) citronella and (G–I) octanol. (A,D,G) Mean amplitude of the responses of thapsigargin (Thap, green)-treated flies and the Or83b,GA/InsP_3R-RNAi (InsP_3R, black) flies, to spearmint (A), citronella (D) or octanol (G) for 5 s (blue bar). (B,E,H) Total amount of photons emitted for the different fly groups (as in A). (C,F,I) Response duration for the different flies groups (as in A). For all conditions, the total photons is decreased by about half as well as the response duration. *N*=5–9 flies for each condition. Values are means ± s.e.m. Statistics: same tests as for Fig. 2. For spearmint (A) Thap *versus* Con: $P<0.05$; InsP_3R *versus* Con: $P<0.05$. For citronella (D) Thap *versus* Con: NS; InsP_3R *versus* Con: NS. For octanol (G): Thap *versus* Con: $P<0.05$; InsP_3R *versus* Con: $P<0.05$. For B,C,E,F,H,I: Two-tailed *t*-test: *$P<0.05$; **$P<0.001$; ***$P<0.0001$.

odor after the pre-exposure. Thus, in contrast to the controls that were attracted to the three odorants after pre-exposure, these flies were unable to choose (or had difficulty in discerning) between air and odor and hence were randomly distributed. The fact that without pre-exposure all groups of flies preferred the control arm and were repelled by the odorants seems to indicate that the odor acuity of these flies is intact, but unlike control flies they become unresponsive to a long (5-min) exposure. Altogether, these results illustrate that the olfactory behavior is severely impaired in flies lacking InsP_3R or RyR, specifically in the ORNs.

DISCUSSION

We have used an *in vivo* bioluminescence Ca^{2+}-imaging technique to record the response of the axon terminals of the ORNs, within the antennal lobes, to application of three different odors, spearmint, citronella and octanol. This technique has been used to characterize, at the cellular and molecular level, the contribution of the presynaptic intracellular Ca^{2+} stores of the ORNs to the response to these selected odors. Indeed, the bioluminescent GFP–aequorin Ca^{2+} sensor expressed in the ORNs, and thus in their synaptic terminals, is sensitive enough to monitor the graded [Ca^{2+}] changes in response to various protocols of odor application. For all three tested odors, short 1 s applications induce a brief and slight response in the ORN terminals, which was easily detected over the background noise. These responses, which last for about 5 s for spearmint and citronella are in the same time range and are comparable to the responses already described using various other fluorescent reporter probes, such as pH-sensitive green fluorescent protein [synapto-pHluorin; see fig. 2 in Shang et al. (Shang et al., 2007)] or G-CaMP [see figs 4 and 8 in J. W. Wang et al. (J. W. Wang et al., 2003)], confirming that the GA bioluminescent probe is sensitive and fast enough to follow synaptic activity. The duration of the octanol response is longer than the response to the other odors, as already observed with other Ca^{2+}-imaging approaches, such as Ca^{2+} dyes (Sachse and Galizia, 2002). Finally, applications lasting 3 s induce a larger response, which further increases during a 5 s odor stimulation. These results are consistent with former studies performed on the olfactory receptor cells of tiger salamanders, in which the intensity of the response was correlated with the strength and/or duration of the stimulus (Getchell and Shepherd, 1978a; Getchell and Shepherd, 1978b; Firestein et al., 1993).

We have chosen these three odors because, (1) two of them are natural odors (spearmint and citronella) and have already been used in different studies, both in *Drosophila* (e.g. Ng et al., 2002) and in other insects such as locusts (e.g. Stopfer and Laurent, 1999; Perez-Orive et al., 2002). The 3-octanol has been selected because it is a commonly used odor in the learning and memory studies of the mushroom bodies, and it has also been used in several other studies (e.g. Wilson et al., 2004; Turner et al., 2008). (2) In a pre-trial test, these three odors gave a strong and reliable response in a behavioral olfactory adaptation test, which make them suitable for our study. Unfortunately, according to the electrophysiological

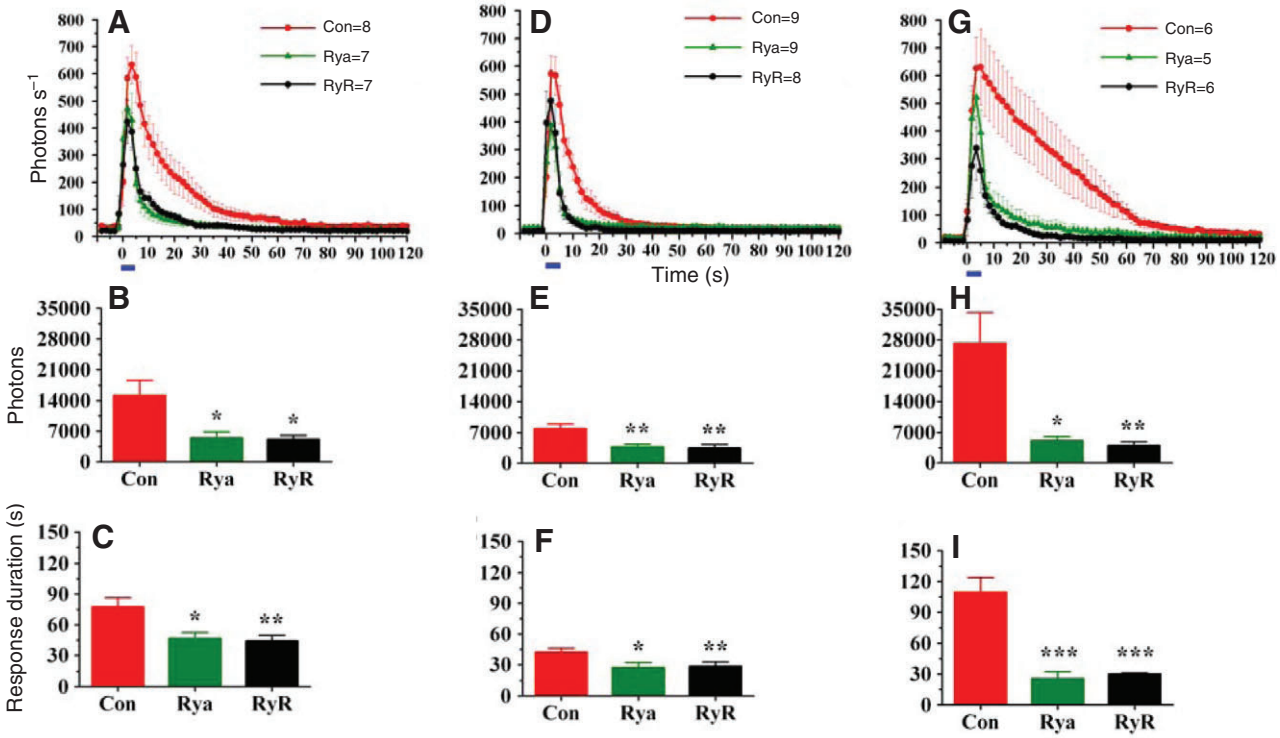

Fig. 5. Odor-induced Ca^{2+} response is reduced in flies lacking RyR, or when blocked pharmacologically. (A,B,C) Spearmint, (D,E,F) citronella and (G,H,I) octanol. (A,D,G) Mean amplitude of the responses of ryanodine (Rya, green)-treated flies and the Or83b,GA/RyR-RNAi (RyR, black) flies to exposure to the odors for 5 s (blue bar). (B,E,H) Total photons emitted by the different fly groups (as in A). (C,F,I) Response duration of the different fly groups (as in A). For all conditions, the total photons is decreased by about half as well as the duration of the response. *N*=5–9 flies for each condition. Values are means ± s.e.m. Statistics: same tests as for Fig. 2. For spearmint (A): Rya *versus* Con: $P<0.05$; RyR *versus* Con: $P<0.05$. For citronella (D): Rya *versus* Con: NS; RyR *versus* Con: NS. For octanol (G): Rya *versus* Con: $P<0.05$; RyR *versus* Con: $P<0.05$. For B,C,E,F,H,I: two-tailed *t*-test: *$P<0.05$; **$P<0.001$; ***$P<0.0001$.

studies performed by J. Carlson and his colleagues (Hallem et al., 2004; Hallem and Carlson, 2006), it is not possible to determine which precise olfactory receptors neurons (ORNs) and subsequently, which precise glomeruli are activated by these three respective odors (even for octanol which is a pure odor). Indeed, as reported (Hallem et al., 2004; Hallem and Carlson, 2006), the precise identity and number of different receptors that can be activated strongly depend on the odor concentration for the majority of odors. Here, in this first study using the GFP–aequorin bioluminescent approach, at the given (relatively high) concentration, each odor obviously activates more than a single and specific ORN and consequently more than a single glomerulus. Nevertheless, as can be seen in Fig. 1A–C, the spearmint clearly does not activate the same glomeruli as the citronella (although some overlaps between glomeruli could not be excluded), and similarly with octanol. Moreover, the response is much bigger with octanol (as revealed by the total number of photons; Fig. 2) than with the two other odors, whereas the smallest response is seen with citronella (although the amplitude is similar to the response to spearmint, the duration of the response is much shorter).

The Ca^{2+} response induced by a 5 s odor application comprises two phases

In contrast to 1 s or 3 s odor applications, a longer odor application, such as 5 s revealed that the response comprised two components, a first, fast rising and fast decay phase, and a delayed second phase of smaller amplitude, but with a relatively slow rising and slow decay. This second component is already visible in some flies with 3 s applications of spearmint and octanol, but not with citronella. This suggests that for odor applications longer than 3 s the second phase starts to be induced. To quantify the odor-induced Ca^{2+} response three parameters have been considered: the total light emission (photons), the amplitude (photons s^{-1}) and the duration of the response. Pharmacological manipulation has revealed that the response is composed of two components (or phases). First, TTX (which inhibits sodium channels and thus spike propagation) abolishes the Ca^{2+} response, showing that it results from activation of spiking activity induced by odors in the distal part of the ORNs. Second, the action of nifedipine and verapamil demonstrates that the Ca^{2+} response in the axon terminal is initiated by Ca^{2+} entry through the VGCC, activated by the spikes. The shoulder visible in the 5 s application records (Fig. 1Bii,Cii,Dii) which prevents fitting the decay with a single or a double exponent reveals the presence of a second component slightly delayed compared with the first one. The two components can be separated using a deconvolution analysis based on the Hodgkin–Huxley equations (Hodgkin and Huxley, 1952) describing activation and deactivation of an ionic current. The two components differ in their amplitude, their duration and their kinetics. Also, the duration of the first component is relatively similar for the three odors, thus suggesting that the duration of the overall response depends on the second phase. Interestingly, such biphasic Ca^{2+} responses resemble the fast rising Ca^{2+} phase followed by a slow phase described in the long term depression (LTD) protocol of the CA3 pyramidal neurons of the hippocampus, which is also implicated in presynaptic control of neurotransmitter release and long-term synaptic plasticity (Unni et al., 2004), or else

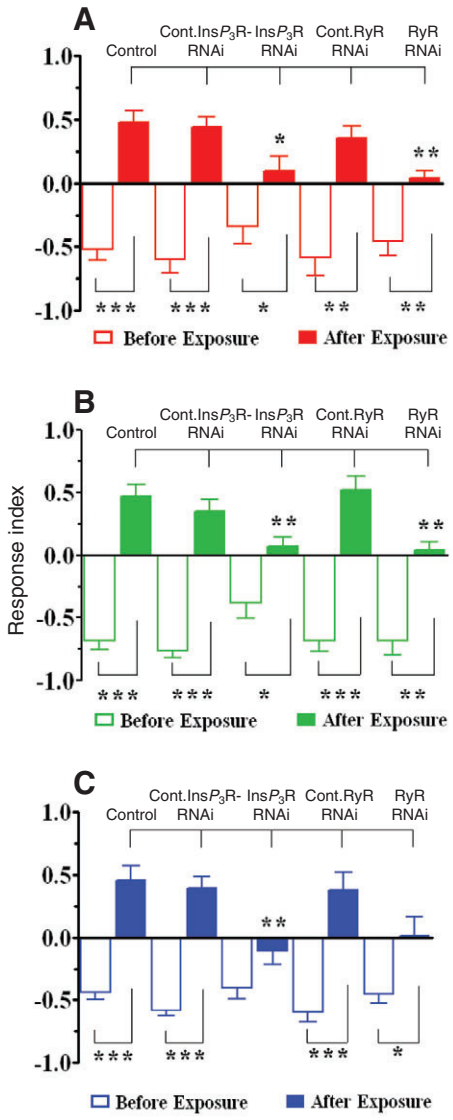

Fig. 6. Behavioral defects caused by the disruption of intracellular Ca^{2+} stores as revealed by T-maze test. (A) Spearmint, (B) citronella and (C) octanol. Without pre-exposure, in an instantaneous T-maze choice, all groups of flies preferred the control airstream (without odor) thereby avoiding the odor-arm for the three tested odors (control: Or83b,GA/CS; Cont.InsP_3R-RNAi/CS; Cont.RyR-RNAi/CS; Or83b,GA/InsP_3R-RNAi; Or83b,GA/RyR-RNAi). This suggests that disturbing the intracellular Ca^{2+} stores does not interfere significantly with odor acuity. However, for the two groups expressing an RNAi (Or83b,GA/InsP_3R-RNAi; Or83b,GA/RyR-RNAi), in which the intracellular Ca^{2+} stores are defective, after a 5 min pre-exposure, the flies are not able to distinguish the odor from the control air (not different from 0). For all groups, N=10 batches of 10 flies/batch, for a total of 100 flies per group. Values are means ± s.e.m. Three types of comparison have been performed: (1) comparison within the same genotype between before and after pre-exposure (using Mann–Whitney test); (2) comparison between control and the different genotypes for the 'without pre-exposure condition' (all groups are non-significant); and (3) comparison between control and the different genotypes for the 'after pre-exposure condition' (*P<0.05; **P<0.001; ***P<0.0001; using one-way ANOVA, followed by a Newman–Keuls comparison test).

in the large-amplitude miniature inhibitory post-synaptic currents (IPSCs) of the Purkinje cells (Galante and Marty, 2003). Therefore, at this stage of our understanding of synaptic function in flies, this effect could not be assigned to a form of LTP and LTD. Nevertheless, it is tempting to speculate that this biphasic Ca^{2+} response, resulting from the mobilization of the pre-synaptic Ca^{2+} stores, could be implicated in synaptic plasticity which has been related, in *Drosophila*, to olfactory adaptation (Devaud et al., 2001) and/or perception (Acebes and Ferrus, 2001).

Olfactory responses involve intracellular Ca^{2+} stores

Two independent approaches, pharmacological and genetic (based on targeting RNAi specifically in ORNs), converge to show that odors induce specific responses that rely on InsP_3R and RyR. These results suggest that the intracellular Ca^{2+} stores importantly contribute to the ORN responses. Indeed, following application of thapsigargin or knocking down InsP_3R by RNAi, the second component of the response is almost absent, suggesting that the second phase comes from the activation of InsP_3-sensitive stores. Similarly, blocking the CICR by ryanodine or specifically knocking down the RyR by RNAi produces similar results, indicating that ryanodine-sensitive stores are also involved. This is the first time that presynaptic intracellular Ca^{2+} stores within the ORN terminals of *Drosophila* have been shown to participate in olfactory responses. InsP_3Rs have already been shown to function in neurons required for flight (Banerjee et al., 2004), as well as to contribute to calcium signaling in other tissues, such as, the renal epithelia (Pollock et al., 2003). Two former reports have shown that the InsP_3 signaling pathway is specifically implicated in olfaction: one using an InsP_3R mutant (Deshpande et al., 2000) and another using a mutation (gain of function) of the inositol (1,4,5)-trisphosphate kinase 1 gene (Gomez-Diaz et al., 2006). However, neither of these two studies determined precisely in which neurons, and where in the neurons such an effect occurs. In mammals, the InsP_3 pathway has been implicated in the signal transduction in the dendrites following the olfactory receptor activation by odors (Breer et al., 1990). Moreover, it has been reported in CA3 pyramidal neurons of the hippocampus and in basket cell–Purkinje cell synapses (Unni et al., 2004; Collin et al., 2005; Galante and Marty, 2003) that presynaptic Ca^{2+} stores contribute to synaptic transmission. However, in *Drosophila* this second-messenger signaling pathway within the ORNs has not been clearly demonstrated, although its existence had been suspected. Here, we provide evidence that presynaptic Ca^{2+} stores within the axon terminal contribute to the odor-induced response. However, the direct involvement of the Ca^{2+} from internal stores in the neurotransmission, as for instance contributing to facilitation, augmentation or depression, remains to be investigated. Although the difference in the responses between odors (amplitude, total photons, duration), remains to be determined, we speculate that these differences could come from the neurophysiological properties of each specific activated ORN, perhaps related to different expression levels of InsP_3R and/or RyR (in other words, from a combinatorial level of expression conferring a different and specific kinetic of adaptation to a given ORN).

Disrupting intracellular Ca^{2+} stores results in defective olfactory behavior

To explore the behavioral and functional consequences of disturbing the intracellular Ca^{2+} homeostasis we have studied flies in which InsP_3R and RyR were genetically knocked down specifically in the ORNs. Both groups of flies presented strong behavioral deficits since flies with disrupted intracellular Ca^{2+} stores are not able to discern between odors *versus* air after a pre-exposure (5 min) to odor. Interestingly, control flies reverse their choice (they prefer odor) after a 5-min pre-exposure, suggesting that in these experimental conditions, the meaning of the odor has changed in the pre-exposed

flies. These results resemble those of some former studies suggesting that pre-exposure (comparable to an adaptation process) serves to extend the operating range of sensory systems over different stimulus intensities (Torre et al., 1995). In other words, pre-exposure modifies the sensitivity (threshold) to the odor, as reported in different organisms, such as nematodes (Colbert and Bargmann, 1995) and vertebrates (Zufall and Leinders-Zufall, 1997), including humans (Ekman et al., 1967). It has been reported that odors could be repulsive (at high concentration) or attractive (at low concentration) (Ayyub et al., 1990; Acebes and Ferrus, 2001; Heimbeck et al., 2001; Wang et al., 2001; Y. Wang et al., 2003). In our experimental conditions (a relatively high odor concentration) the odors are repulsive to the control flies. However, after 5 min of pre-exposure, the flies adapt to this odor concentration and, when tested in the T-maze at the same concentration, odors are then only weakly perceived and therefore might correspond, for the flies, to an attractive 'weak odor concentration'. To confirm that the ability of an odor to act as attractant or repellent is concentration dependent, we recorded the olfactory response of control flies using odors at lower concentration (10^{-1}, by dilution in mineral oil). Our results indicate that it is indeed the case since without pre-exposure, the control flies preferred the odor arm (positive RI value; supplementary material Fig. S2) which is the inverse of the case where an odorant is used at higher concentration. Although the change from repulsion toward attraction remains largely unexplained, Acebes and Ferrus (Acebes and Ferrus, 2001) have suggested that the number of synapses within the antennal lobes modify the olfactory perception. It is tempting to speculate that when we disrupt the pre-synaptic Ca^{2+} stores, we disrupt the strength (efficiency) of the synaptic transmission (possibly less synapses are functional or the same number function for a shorter period). Thus, functionally, this could be similar to a morphological change in the number of synapses [as shown by Acebes and Ferrus (Acebes and Ferrus, 2001)]. Subsequently, the odor sensitivity might be modified and consequently the behavior (repulsion to attraction) of the fly.

In conclusion, this is the first demonstration that odor-induced responses occurring specifically in the axon terminals of the ORNs depend on the intracellular Ca^{2+} stores. Complementary to the functional *in vivo* brain imaging data, we have demonstrated, using a genetic approach targeting specific RNAi to knock-down InsP_3R or RyR specifically in the ORNs, a functional role for the internal Ca^{2+} stores. Thus, disrupting presynaptic Ca^{2+} stores leads to olfactory-led behavioral deficits, and more specifically, leads to adaptive process following a relatively long odor exposure (as for instance longer than about 3 s, as shown here). Therefore, our results suggest that an olfactory integration process occurs immediately after odor application, directly into the ORNs, that can be ascribed to a form of neuronal plasticity and/or sensory memory.

ACKNOWLEDGEMENTS

We are indebted to R. Ueda, NIG, Japan, for the InsP_3R-RNAi and RyR-RNAi lines. We thank G. Ouanounou for constructing the deconvolution equation and helping to program it in Origin software; S. O'Regan for help with the English, L. Rabinow for critical reading of the manuscript, and G. Baux and M. Ruat for their support. This work was supported by ANR-Neurosciences and by the CNRS.

LIST OF ABBREVIATIONS

CICR	calcium-induced calcium release
GA	GFP–aequorin
InsP_3R	inositol (1,4,5)-trisphosphate receptor
ORNs	olfactory receptor neurons
PNs	projections neurons
RNAi	RNA interference
ROI	region of interest
RyR	ryanodine receptor
VGCC	voltage-gated calcium channels

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