The *Inositol 1,4,5-triphosphate kinase1* Gene Affects Olfactory Reception in *Drosophila melanogaster*

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The Inositol 1,4,5-triphosphate (IP3) route is one of the two main transduction cascades that mediate olfactory reception in *Drosophila melanogaster*. The activity of IP3 kinase1 reduces the levels of this substrate by phosphorylation into inositol 1,3,4,5-tetrakiphosphate (IP4). We show here that the gene is expressed in olfactory sensory organs as well as in the rest of the head. To evaluate *in vivo* the olfactory functional effects of up-regulating IP3K1, individuals with directed genetic changes at the reception level only were generated using the UAS/Gal4 method. In this report, we described the consequences in olfactory perception of overexpressing the *IP3Kinase1* gene at eight different olfactory receptor-neuron subsets. Six out of the eight studied *Gal-4/UAS-IP3K1* hybrids displayed abnormal behavioral responses to ethyl acetate, acetone, ethanol or propionaldehyde. Specific behavioral defects corresponded to the particular neuronal olfactory profile. These data confirm the role of the *IP3kinase1* gene, and consequently the IP3 transduction cascade, in mediating olfactory information at the reception level.

KEY WORDS: *Drosophila melanogaster*; Gal-4/UAS gene-expression system; IP3 cascade; olfaction; olfactory reception; sensory transduction.

INTRODUCTION

The IP3 transduction cascade mediates olfactory transduction in vertebrates as well as invertebrates as deduced from molecular, cellular and electrophysiological data (see the reviews by Hildebrand and Shepherd (1997), Schild and Restrepo (1998), Prasad and Reed (1999), Ronnett and Moon (2002), Breer (2003)). In species where mutant stocks can be generated systematically, like the worm *Caenorhabditis elegans* (see for example Bernhard and van der Kooy, 2000) or the fly *Drosophila melanogaster*, behavioral data revealing sensorial perception can be added to the former information.

In *Drosophila*, the IP3 signaling cascade has been directly linked to olfaction. The inositol 1,4,5-trisphosphate-receptor gene has been cloned and characterized. Strong expression of the mRNA in the adult retina and antenna suggests that it is involved in visual and olfactory transduction (Hasan and Rosbash, 1992; Yoshikawa *et al.*, 1992). Electrophysiological data indicate that the IP3 receptor is required for normal response to odorants (Deshpande *et al.*, 2000). Partial requirement for a phospholipase C, encoded by the *norpA* gene, in odor response has been also reported using genetic and molecular data. Gene expression has been shown at the maxillary palps, the secondary olfactory receptor organs of *Drosophila*. Null mutants of this gene displayed abnormal electrophysiological responses to odorants of the maxillary palps but not of the antennae, the main olfactory organs (Riesgo-Escovar *et al.*, 1995). The *rdgB* (*retinal degeneration B*) gene encodes a membrane-associated phosphatidylinositol transfer protein involved ultimately in IP3 formation. It has been shown to

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affect the IP3 cascade for visual (Harris and Stark, 1977) as well as olfactory reception (Riesgo-Escovar *et al.*, 1994; Woodard *et al.*, 1992).

However, only the studies in the *rdgB* mutant have been able to relate the IP3 transduction cascade with abnormal olfactory perception deduced from behavioral data. Further studies on the effects of IP3-mediated transduction in olfactory perception of *Drosophila* should overcome the lack of mutant stocks for genes directly related to the level of the second messenger IP3.

Phospholipase C releases IP3 from the plasma membrane by hydrolysis of the phosphatidyl inositol 4,5-bisphosphate (Nalaskowski and Mayr, 2004). Three genes encode in *Drosophila* for a phospholipase C, *norpA* (Pak *et al.*, 1970), *Plc21C* (Shortbridge *et al.*, 1991) and *sl* (Thackeray *et al.*, 1998). However, only the *norpA* gene has been related partially to olfactory reception in *Drosophila* according to elec-trophysiological data in the maxillary palps (Riesgo-Escovar *et al.*, 1995).

Inositol phosphate 5-phosphatases remove IP3 by dephosphorylation, but although some genes in *Drosophila* have been related with this activity (see for example gene CG31110), mainly by sequence similar-ity, effects in olfactory reception have not been studied.

A third group of enzymes eliminate IP3 by further phosphorylation to Inositol 1,3,4,5 tetrakiphosphate (IP4), the inositol 1,4,5 triphosphate kinases, corre-sponding to two genes in *Drosophila*, *IP3K1* and *IP3K2*. The *IP3K1* gene has been shown to control oxidative stress resistance (Monnier *et al.*, 2002). Its extended expression, confirmed by our results, in the head fraction together with antennal and maxillary palp fractions, is coincident with the generalized expression pattern of IP3kinase isoforms in vertebrates and agrees with the expected extended role of the IP3 transduction cascade. However, it seriously prevents correlation of behavioral differences due to *IP3K1* mutants with changes exclusively at olfactory reception.

In this report we tested the role of IP3K1 in olfactory reception *in vivo* at the behavioral level using directed overexpression of the *IP3K1* gene only in olfactory receptor neurons to generate mutants. The Gal-4/UAS method has been proven efficient to obtain directed dominant mutations (Brand and Perrimon, 1993). Recently, some UAS transgenic lines were generated containing a construct with the *inositol 1,4,5-triphosphate kinase1* gene (*IP3K1*) (Monnier *et al.*, 2002). Generation of specific mu-tants for different olfactory receptor-neuron subsets was approached by using Gal-4 enhancer-trap lines.

Eight Gal-4 lines with restricted expression patterns to the olfactory receptor organs drove overexpression of the normal *IP3K1* allele in eight different subsets of olfactory receptor neurons, in living animals.

Responses to several odorants were studied, revealing an extensive role of the IP3 transduction cascade in mediating olfactory reception.

MATERIAL AND METHODS

Fly Stocks

Canton-S flies (provided by the Bloomington stock centre, Indiana, USA) were used to test gene expression.

The Gal-4 line 208a (provided by B. Hovemann, Rurh-Universität-Bochum, Germany) was selected as reference line for quantitative estimation of *IP3K1*-mRNA overexpression, electroantennogram (EAG) and behavioral analysis. A set of eight Gal-4 en-hancer-trap lines: 345, 131a, 148a, 179a, 272, 250, 555, 588 (also provided by B. Hovemann) with spe-cific reporter-gene expression at different subsets of olfactory receptor neurons (Gomez-Diaz *et al.*, 2004) were used to direct *IP3K1* gene overexpression.

One stock containing a $P\{UAS-IP3K1\}$ insert in the second chomosome in a w^{1118} background (Monnier *et al.*, 2002) and the w^{1118} line were pro-vided by H. Tricoire (University of Paris, France).

Eight groups of heterozygous flies, overexpress-ing the *IP3kinase1* gene with the same restricted pattern as the corresponding Gal-4 line, were gener-ated by crossing each Gal-4 line and the *UAS-IP3K1* stock. The control flies in each case were the hetero-zygous flies between the correspondent Gal-4 line and the w^{1118} stock, which shares genetic background with the *UAS-IP3K1* strain.

The homozygous Gal-4 lines and the w^{1118} stock by themselves were discarded as appropriate controls because they displayed recessive abnormal behavioral phenotypes (see the odorants and concentrations section) that disappeared in the experimental as well as in control hybrid flies (Gomez-Diaz *et al.*, 2004).

Expression of the reporter gene *LacZ* was ob-tained by crossing each Gal-4 line with the stock w[*]; $P\{w[+mC] = UAS-lacZ.B\}Bg4-2-4b$ provided by the Bloomington stock center.

Expression of the *IP3 Kinase1* Gene

A reverse transcriptase (RT) experiment was performed to test the presence of native *IP3K1*

mRNA at the head, antenna and maxillary palp fractions of normal Canton-S individuals. About 50 heads deprived of olfactory organs (antennae and maxillary palps), 300 third antennal segments and 50 maxillary palps were collected after sieving complete flies freezed in liquid nitrogen. Total RNA was isolated with Nucleospin RNA II (Macherey-Nagel, Hoerdt, France) according to manufacturer's instructions, followed by an additional acid phenol/cloroform extraction step and RNA precipitation. First strand cDNA was synthesized from the whole amount of the isolated RNA using the Superscript^{TM} first strand synthesis system for RT-PCR (Invitrogen, Barcelona, Spain) with random primers.

PCR was carried out in a final volume of 20 μl in the presence of 1 μl of head cDNA, 1 μl of antenna cDNA, 4 μl of palp cDNA or 1 μl of the genomic DNA (used as control) and the Taq polymerase (Promega, Wisconsin, USA). Samples were subjected to 40 cycles PCR. Each cycle included 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C and 1.5 minutes elongation at 72°C. After amplification, 10 μl aliquots were analyzed by agarose gel electrophoresis for each sample except 20 μl for the palp sample. The sequences of the primers used were: forward 5′ GCGCCGAAGAATCACATC 3′ and reverse 5′ GTGGCTTCGCCTGCTTGT 3′ for the *IPK1* gene (FlyBase accession number FBgn0032147) and forward 5′ AGTCGCCTACAAT GGTCTGC 3′ and reverse 5′ GTTCGAATCG TTG CTAACGG 3′ for the *G6PD* gene (FlyBase accession number FBgn0004057) used as a control housekeeping gene (Fouts *et al.*, 1988).

Quantitative RT-PCR

Line 208a, with generalized Gal-4 expression at the third antennal segment, was used to measure *IP3 Kinase* overexpression in the olfactory tissue comparing *IP3K1* m-RNA amounts in Gal-4/UAS-*IP3K* (experimental, E) and Gal-4/w^{1118} (control, C) hybrids by quantitative RT-PCR, following the previously described protocol (Gomez-Diaz *et al.*, 2004). After reverse transcription (RT), Real Time PCR was performed. Each sample was analyzed for *glucose-6-phosphate dehydrogenase (G6PD)* RNA, as control to normalize for RNA input amounts, and the *IP3K1* RNA.

The sequences of the primers used for *G6PD* (FlyBase accession number FBgn0004057) were: forward 5′ CGAGGAGGTGACTG-TCAACATC 3′ and reverse 5′ CAACCGCAGACCGACATG 3′. Primers generated for th7e *IP3K1* gene (FlyBase accession number FBgn0032147) were as follows: forward 5′ GCAATCGAACAACAATAACGAGC 3′ and reverse 5′ CAAATAGTCGCAGTTCTC GTT GG 3′. Melting curve analysis showed a single sharp peak with the expected T_m (melting time constant) for all samples. The complete experiment was repeated proving the accuracy of the measurements.

Data were analyzed using the relative standard curve method to quantify gene expression (Del Toro *et al.*, 2003; Dorak, 2003; Giuletti *et al.*, 2001). The expression level of the *IP3K1* gene at control condition (hybrids Gal-4/W^{1118}) was used as reference for calibration purposes.

EAG Recording

EAGs are extracellular measurements of voltage changes produced in the antennal surface in response to odorant stimulation. The recording method as well as odorant delivery system and data analysis has been already described (Alcorta, 1991). Odorant pulses were generated by changing airflow direction from a control bottle containing paraffin oil to a stimulus bottle with a certain dilution of ethyl acetate in paraffin oil using an electric activated valve. Voltage recordings in response to odorant stimulation were amplified and stored by computer at 50 Hz sampling rate. Five EAGs were recorded for each fly during 150 seconds (30 seconds/repetition).

A total number of 20 flies were recorded for each phenotype in response to ethyl acetate 10^{-2} and 10 in response to ethyl acetate 10^{-1}.

Behavioral Tests

A double-choice, horizontally placed Y maze was used to measure olfactory preference (Alcorta and Rubio, 1989; Martin *et al.*, 2002). In short, 40 three- to four-day-old females starved for 24 hours chose during 30 minutes between a stimulus tube containing filter paper soaked with 0.5 ml of a certain concentration of odorant and a control tube with 0.5 ml of solvent. An olfactory index (IO) was calculated as the number of flies in the stimulus tube compared to the total number of flies reaching the end of the maze either at the stimulus or the control tube. According to this algorithm, IO values ranged from 0 (maximal repulsion) to 1 (maximal attraction), marking the threshold of indifference at 0.5.

As a rule, 15 replicate tests were performed for each line and stimuli. The number of replicate tests was increased in those cases where differences were at the limit of statistical significance. The number of

257 replicate tests in these cases will be indicated in the
258 text.

Odorants and Concentrations

260 Three odorants out of five were tested at a
261 single concentration for each group of experimen-
262 tal/control hybrid flies: ethyl acetate, acetone, eth-
263 anol, acetic acid and propionaldehyde, according to
264 previous data. In short, Gal-4 homozygous lines
265 131a, 148a, 179a, 250 and 555 displayed abnormal
266 response to ethanol. The other three lines showed
267 abnormal behavior in response to two odorants,
268 ethyl acetate and acetic acid for line 272, acetone
269 and ethanol for line 345 and acetone and propi-
270 onaldehyde for line 588. Although abnormal phe-
271 notypes were only observed in homozygous lines,
272 due to the recessive character of the mutation, this
273 information was used to further determine the
274 olfactory specificity spectra. At the cellular level,
275 the olfactory profile of the affected receptor-neuron
276 subsets for each line was deduced by using the
277 same 8 Gal-4 lines as expression drivers of the
278 tetanus toxin light-chain (TNT) gene that blocks
279 synapses, using the Gal-4/UAS method (Gomez-
280 Diaz et al., 2004).

281 The chosen concentration evoked intermediate
282 repellent responses in control flies (around $IO = 0.2$–
283 0.3), so as to identify changes in both directions,
284 decreasing or increasing odorant sensitivity (Martin
285 et al., 2002). This is not possible for concentrations
286 eliciting attractive responses.

287 The following concentrations were tested: ethyl
288 acetate 10^{-2}, acetone $10^{-1.25}$ or $10^{-1.5}$, ethanol $10^{-0.5}$
289 or $10^{-1.5}$ and propionaldehyde $10^{-1.75}$ expressed as
290 volume/volume dilutions. For those odorants where
291 two concentrations are indicated, the particular
292 concentration tested in each case will depend on the
293 Gal-4 line and indicated in the text.

294 Ethyl acetate 10^{-2} and 10^{-1} were used as odorant
295 stimuli for EAG recording in hybrids with the 208a
296 line.

Statistical Analysis

298 EAG responses, measured at two different
299 odorant concentrations, were analyzed by a two-way
300 ANOVA.

301 Behavioral IOs were corrected using the arcsine
302 transformation ($y = \arcsine\sqrt{IO}$) to normalize data
303 (Martin et al., 2002).

304 Statistical significance was established using the
305 Student's t-test for comparison between each Gal-4/
306 UAS-IP3K1 heterozygous flies and the corresponding
307 Gal-4/w^{1118} group.

RESULTS

Expression of the *IP3K1* Gene at the Olfactory Receptor Organs

311 The *IP3K1* gene encodes for an Inositol 1,4,5
312 triphosphate kinase that mediates IP3 degradation. If
313 the IP3 transduction cascade functions at olfactory
314 reception, expression of this gene at the main and
315 secondary olfactory receptor organs, antennae and
316 maxillary palps respectively, would be expected. We
317 carried out a RT-PCR experiment to check for native
318 *IP3K1* mRNA expression in head, antennae and
319 maxillary palps of standard Canton-S flies. A
320 housekeeping gene, *G6PD*, was used as control. For
321 both genes, primer pairs that span introns were used
322 in order to distinguish PCR bands amplified from
323 cDNA from those amplified from any remaining
324 genomic DNA during m-RNA extraction.

325 The amplified fragment of the *IP3K1* gene is
326 located in the zone between exons 2 and 3 that ap-
327 pears in the single transcript IP3K1-RA (release 3.2
328 of the *Drosophila melanogaster* genome). The ex-
329 pected sizes of *IP3K1* and *G6PD* cDNA fragments
330 are 144 and 959 b.p., respectively. The sizes of the
331 amplified genomic fragments are 491 b.p. for *IP3K1*
332 and 1081 b.p. for *G6PD*.

333 Both *IP3K1* and the control *G6PD* amplified
334 products appeared (Fig. 1) in the head cDNA (lanes*I
335 P3K1* H, *G6PD* H), antennae cDNA (lanes*IP3K1* A,
336 *G6PD* A) and maxillary palps (lanes *IP3K1* P, *G6PD*
337 P). Products amplified from cDNA have different
338 length that the PCR products amplified from geno-
339 mic DNA (lanes *IP3K1* G, *G6PD* G), as expected.
340 The sizes of the amplification products obtained with
341 our *IP3K1* specific primers coincided with the
342 predicted sizes for this gene. In summary, these data
343 demonstrated that the *IP3K1* gene is expressed in his
344 native form at the *Drosophila* olfactory organs, the
345 third antennal segment and the maxillary palp, in
346 wild type flies. Expression at the head fraction sug-
347 gested that this gene participates not only in olfactory
348 reception but also at other intermediate steps of
349 olfactory information integration. This fact advises
350 against using traditional mutants of this gene to test
351 the effect of the IP3 cascade in olfactory reception at
352 the perception level.
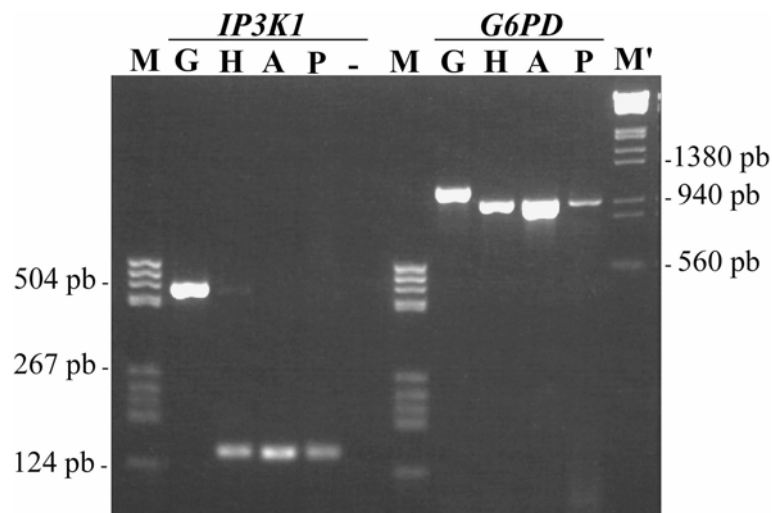
Fig. 1. RT-PCR analysis from Canton-S flies. (M) and (M′) size markers. Amplification products of *IP3K1* and *G6PD* (control) genes, respectively: (G) Genomic DNA. (H) Head (deprived of antennae and maxillary palps) cDNA. (A) Antennal cDNA (P) maxillary palp cDNA. The – mark indicates the RT-PCR negative control.

Overexpression of the *IP3K1* Gene in Hybrids Gal-4/ UAS-*IP3K1*

Line *208a* drives extensive Gal-4 expression in the third antennal segment, the main olfactory organ of *Drosophila* (Fig. 2a, left). Therefore, it has been chosen as the reference line to test *IP3K1* gene overexpression that was measured in control (C) and experimental (E) hybrids of the *208a* Gal-4 line and the w^{1118} or *UAS-IP3K1* stocks, respectively.

A quantitative real time RT-PCR experiment was carried out to answer whether or not the amount of *IP3K1* m-RNA increased due to the Gal-4 driven expression. The expression level of the housekeeping gene *G6PD* in each sample was used to normalize for cDNA input.

Since the UAS-IP3K1 insert contains the IP3K1 cDNA, a control experiment was performed to evaluate the amount of genomic DNA contamination in the samples, which could induce overestimation of IP3K1 m-RNA in the experimental hybrid group. Quantitative analysis of experimental samples with (RT+) and without (RT−) RT yields a cycle threshold difference of 6.6 units, corresponding to 97 times more cDNA amplified from the RT+ sample (that includes m-RNA expression) that from the RT-one (referring to genomic DNA contamination). Taking this into account, overestimation was considered negligible and was ignored in the following measurements.

Using the relative standard curve method for analyzing data (Giuletti et al., 2001), a 2.9-fold increase of the *IP3 Kinase 1 gene* m-RNA was found in the antennae of the experimental hybrids compared to the controls (Fig. 2a, right). Therefore, hybrids 208a/UAS-*IP3K1* showed a 290% of *IP3K1* mRNA compared to the 100% of the control 208a/w^{1118} flies. For this line, with generalized Gal-4 expression in the third antennal segment, Gal-4 driven UAS-*IP3K1* expression accounts for an extra 190% of *IP3K1* mRNA.

Since RNA samples were extracted from complete third antennal segments, this measurement can be taken as representative of the overexpression level in olfactory receptor neurons only for those lines with extensive Gal-4 expression at this organ. Hence, determination of *IP3K1* mRNA level was restricted to the 208a E and C hybrids and was not performed for hybrids with each one of the 8 Gal-4 lines that affected different neuronal subsets and were used for behavioral analysis. In these last lines increase of *IP3K* mRNA due to Gal-4 driven overexpression would appear more o less diluted depending on the ratio of olfactory neurons expressing Gal-4 in each case.

Electrophysiological Changes Associated to *IP3K1* Overexpression

If the IP3 transduction cascade is involved in olfactory transduction at the receptor level we would expect changes in the electrical signal produced at the third antennal segment in response to odorants, as a consequence of increasing the IP3K1 levels.

(a)

208a/UAS-*LacZ*

(b) Electroantennograms

(c) Behavioral response to ethyl acetate $10^{-1.5}$
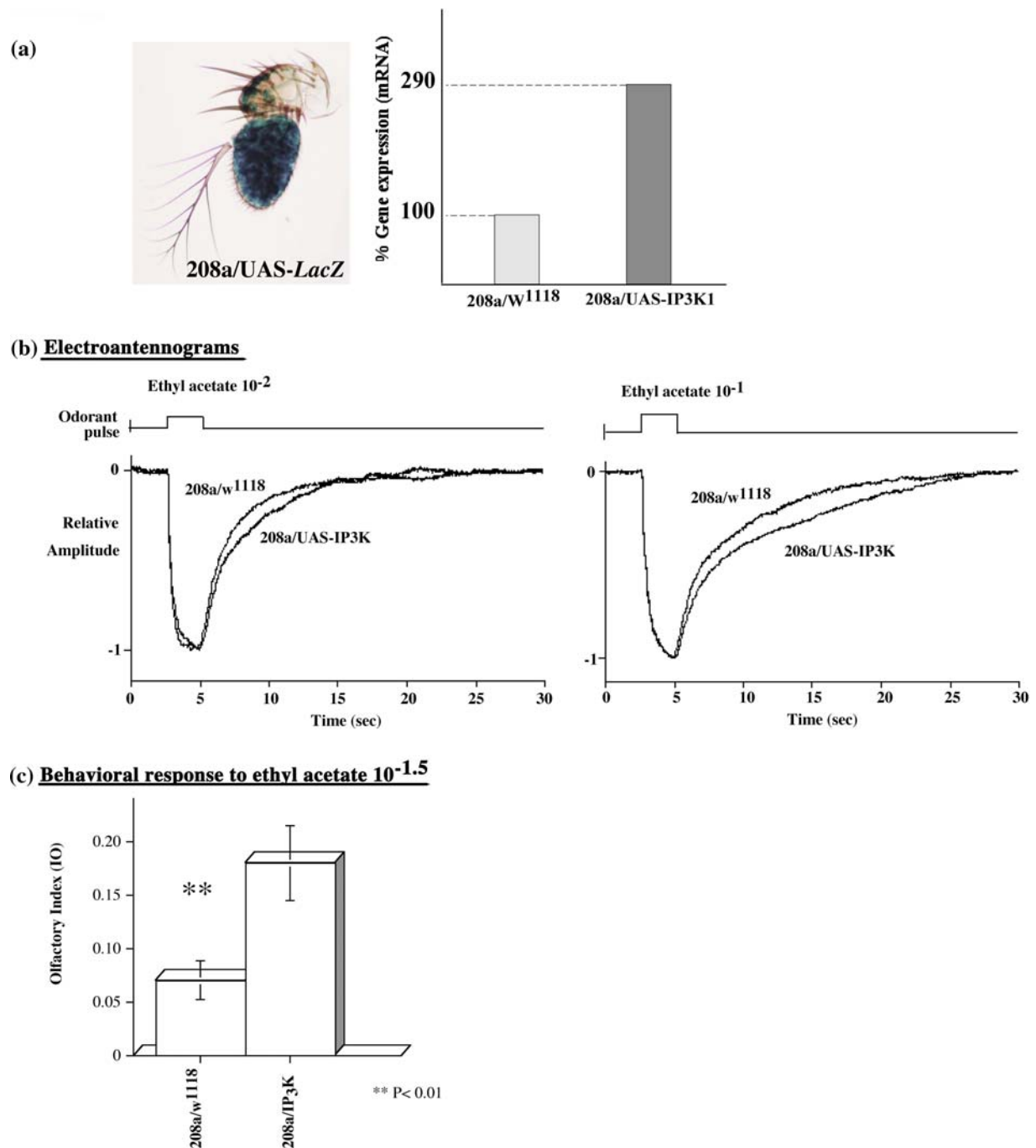
Fig. 2. Summary of data comparing experimental *208a/UAS-IP3K1*, E, and control *208a/w*[1118], C, hybrid flies at different levels. (a) left, *LacZ* reporter-gene expression at the antennae of hybrids 208a/UAS-lacZ , right, quantitative RT-PCR. (b) Average traces of the normalized EAGs obtained from E and C hybrid flies in response to ethyl acetate 10^{-2} and 10^{-1} (vol/vol). Fall time values were measured in each fly's EAG and used for establishing statistically significant differences. (c) Behavioral responses of the same flies to ethyl acetate $10^{-1.5}$.

To directly address this subject EAG measurements were performed using the same 208a reference stock. EAG recordings of hybrids of the 208a and the *UAS-IP3K1* lines were compared with these of the control hybrids *208a/w*[1118] in response to ethyl acetate at two concentrations, 10^{-2} and 10^{-1} (Fig. 2b).

Table I. Two-way ANOVA of the EAG Fall Time Values Obtained for Hybrids *208a/UAS-IP3K1* and *208a/w*[1118] at Two Different Concentrations of Ethyl Acetate

Source	df	Sum of squares	*F*-test	*p* value
Stock	1	27.78	5.24	0.026*
Concentration	1	143.83	27.14	0.0001***
S × C	1	1.89	0.36	0.553 n.s.
Error	55	291.45		

*$p < 0.05$; ***$p < 0.001$.

The same pattern appeared in both cases. Statistically significant differences between lines were observed in recovery kinetics after odorant stimulation (Table I) that added to the significant changes produced in response to increasing odorant concentration, as previously reported (Alcorta, 1991). No significant differences were detected between lines in amplitude or onset kinetics.

The observed differences in EAG paralleled olfactory behavior changes of the experimental hybrids *208a/UAS-IP3K1* compared to the control flies *208a/w*[1118] in response to ethyl acetate (Fig. 2c).

Observed differences in EAG's are not likely to result from putative developmental effects of IP3K up-regulation. Instead, they reinforce the idea of an electrical signal change due to the IP3 route adjustment.

As for the quantitative RT-PCR experiment, the observed EAG differences in hybrids of the 208a line were considered representative of the effects of IP3K1 overexpression in olfactory receptor neurons and additional EAG experiments for the other Gal-4 line hybrids presented in this report have not been systematically performed. Since the EAG is a general measurement in the third antennal segment, differences will appear diluted when very few olfactory receptor neurons were affected. However, some EAG measurements were performed for hybrids of line 250 (with extensive Gal-4 expression at the third antennal segment, Fig. 3) in response to ethyl acetate and the results agree with those of 208a hybrids (data not shown).

Olfactory Behavior Changes Associated to *IP3K1* Overexpression

Behavioral responses of the eight groups of hybrid flies, experimental (E) Gal-4 line/*UAS-IP3K1* and control (C) Gal-4 line/*w*[1118], to the three tested odorants are presented in Figure 4.

The Gal-4 expression pattern for each Gal-4 line at olfactory receptor neurons (Fig. 3) and the olfactory specificity profile of the corresponding receptor-neuron subsets has been previously reported (Gomez-Diaz et al., 2004). In short, two different data sets were collected to test if olfactory receptor neurons expressed the Gal-4 gene, axonal staining and olfactory sensitivity changes in response to synaptic blockade produced by the TNT. The effects of TNT in behavior were previously described (Sweeney et al., 1995; Keller et al., 2002; Devaud et al., 2003). The olfactory profile of the correspondent receptor-neuron subsets was deduced from the behavioral changes induced by directed expression of the *TNT* gene in the 8 Gal-4/UAS-TNT hybrids.

Statistically significant differences in behavioral response to odorants due to *IP3K1* gene overexpression appeared in six out of the eight tested groups, corresponding to the following Gal-4 lines, 148a, 179a, 250, 345, 555 and 588. No differences have been detected for lines 131a and 272.

Hybrid flies of lines 148a, 179a, 250, 345 and the *UAS-IP3K1* line showed decreased repellent response to ethanol $10^{-1.5}$ (148a, 250, 345) or ethanol $10^{-0.5}$ (179a) compared to the control heterozygous flies ($t = 2.79$, $df = 27$, $p = 0.009$; $t = 2.49$, $df = 34$, $p = 0.018$; $t = 2.97$, $df = 28$, $p = 0.006$ and $t = 2.34$, $df = 25$, $p = 0.027$, respectively). Though no differences were found in response to ethyl acetate or acetone for hybrids 148a and 179a, experimental hybrids of the 250 line displayed also reduced repellent response to ethyl acetate $10^{-2.25}$ ($t = 2.81$, $df = 45$, $p = 0.007$) and these of the 345 line showed decreased repellent sensitivity for acetone $10^{-1.5}$ ($t = 3.50$, $df = 27$, $p = 0.002$).

Overexpression of the *IP3K1* gene also induced significant changes in response to acetone $10^{-1.5}$ for line 555 ($t = 2.12$, $df = 28$, $p = 0.043$) and acetone 10^{-2} for line 588 ($t = 2.11$, $df = 28$, $p = 0.043$). Finally, changes in response to propionaldehyde 10^{-2} have been observed in flies 588/*UAS-IP3K1* compared to the control 588/*w*[1118] ($t = 2.21$, $df = 28$, $p = 0.035$).

Note that although for some odorants different concentrations have been tested depending on the Gal-4 line, they have been chosen because evoked approximately the same level of response, an intermediate repellent response, where differences between the E and C group should be more easily identified.

Additional information can be obtained comparing the stimuli that were perceived differently by overexpressing the *IP3K1* gene and the olfactory specificity profile of the olfactory-neuron subset affected in each Gal-4 line (Table II). From the 8 Gal-4 lines tested, 6 showed olfactory perception differences by affecting the *IP3K1* gene. Moreover, for 4 lines,
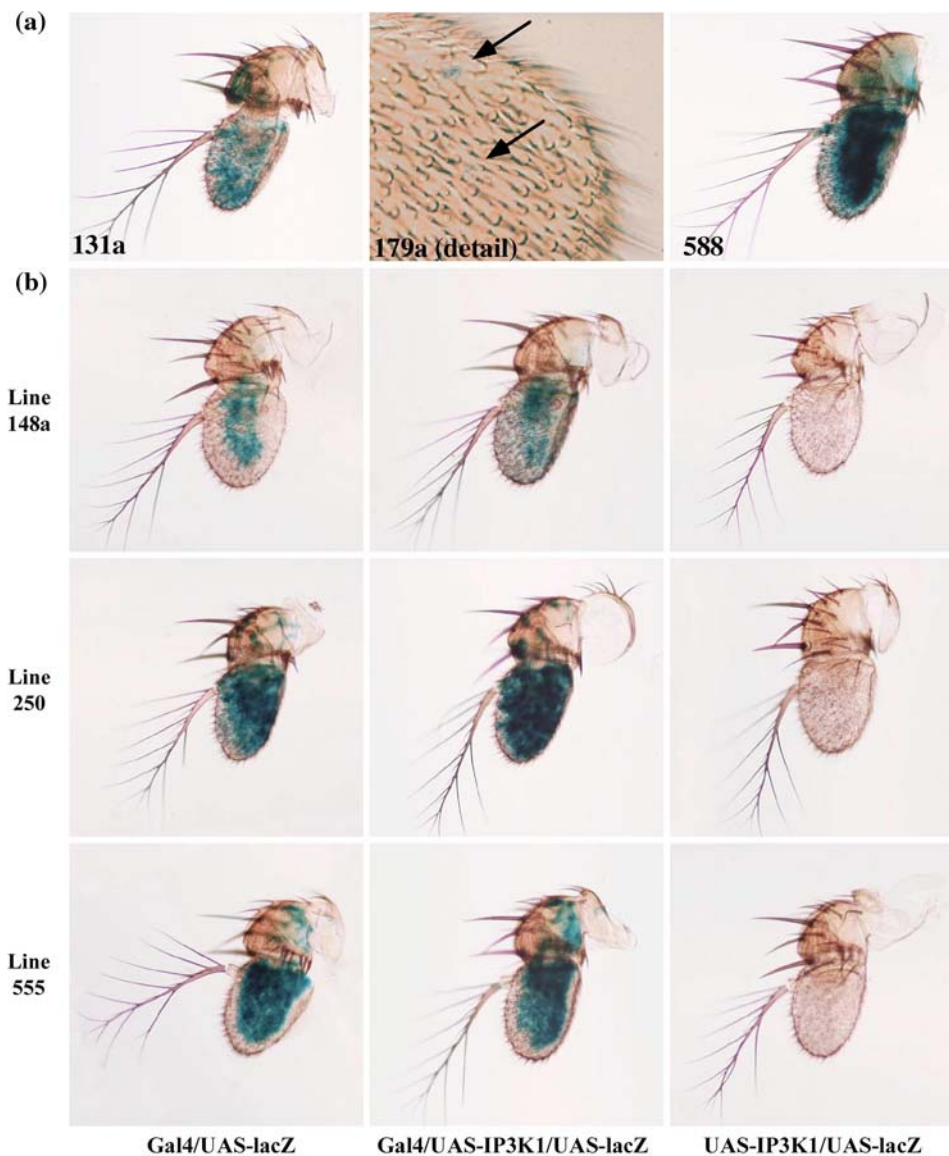
Fig. 3. *LacZ* reporter-gene expression at the antennae of (a) hybrids Gal4/UAS-lacZ of lines 131a,179a (detail) and 588 (lines 272 and 345 displayed a subtle staining similar to 179a) and (b) Gal-4/*UAS-lacZ*, Gal-4/*UAS-IP3K1*/*UAS-lacZ* and the sibling +/*UAS-IP3K1*/*UAS-lacZ* flies for the other three Gal-4 lines: 148a, 250 and 555.

509 148a, 345, 555 and 588, changes associated to modi-
510 fications of the IP3K1 levels exactly matched those
511 due to synaptic blockade. In the other 2 cases, lines
512 179a and 250, correspondence was only partial. All
513 together these results suggest an extensive role of the
514 IP3 cascade in olfactory reception.
515   The effects of the IP3K1 up-regulation, however,
516 are not odorant-specific since for each odorant there
517 were examples of perceptional changes associated to
518 the *IP3K1* overexpression and others where no
519 change was perceived.

Overexpression of the *IP3K1* Gene does not Induce   520
Cell Mortality   521

     The Gal-4 lines used to generate the eight dif-   522
ferent mutant stocks showed preferential Gal-4   523
expression at certain olfactory receptor organ subsets   524
and practically no other brain locations were affected   525
(Gomez-Diaz *et al.*, 2004). Because of this, the   526
olfactory receptor neurons should account for the   527
origin of the perceptional changes observed in   528
behavioral tests, specially when EAG changes have   529
been also reported.   530
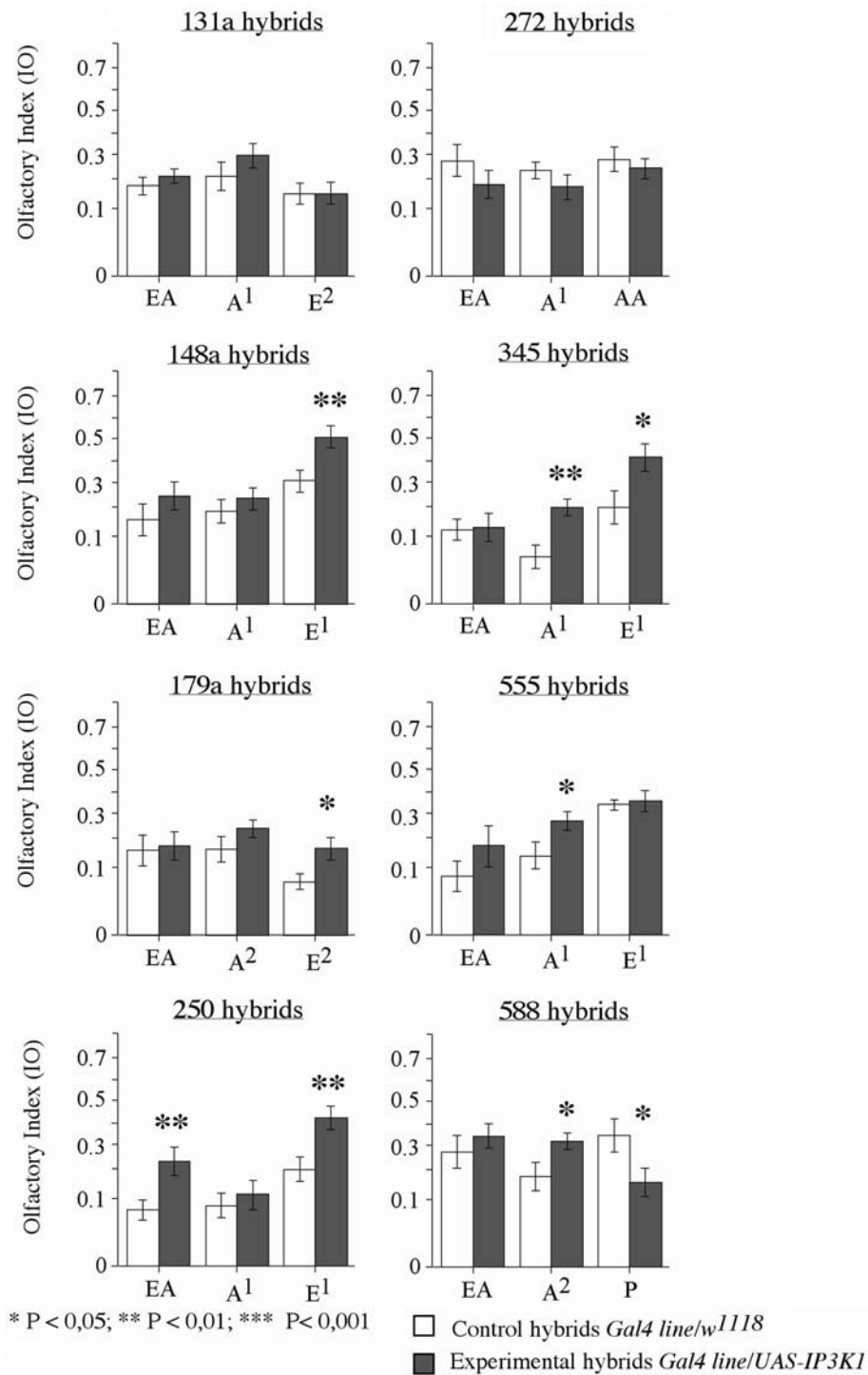
Fig. 4. Behavioral responses to different odorants of eight groups of experimental *Gal-4/UAS-IP3K1* and control *Gal-4/UAS-w*[1118] hybrid flies. EA = ethyl acetate 10^{-2}, A^1 = Acetone $10^{-1.5}$, A^2 = Acetone $10^{-1.25}$, E^1 = ethanol $10^{-0.5}$, E^2 = ethanol $10^{-1.5}$, P = propionaldehyde $10^{-1.75}$. Note that the *Y*-axis scale is not linear but in the arcsine scale, the same scale used to establish statistical significances.

Table II Significant Behavioral Changes, Classified by Odorant and Line, due to the effect of the Tetanus Toxin Light Chain Expression (in Gray) or to *IP3K1* Overexpression (in Black)

Gal-4 LINE	ETHYL ACETATE	ACETONE	ETHANOL	
131a				
148a			■	
179a			■	
250	■		■	ACETIC ACID
272			NO	
345		■	■	
555		■		PROPION-ALDEHYDE
588		■	NO	■

AFFECTED PROCESS:

▨	SYNAPTIC BLOCKAGE		NO : NO TESTED
■	IP3 TRANSDUCTION CASCADE		

532   However, other causes could induce the same
533 outcome. Additional experiments were performed to
534 rule out the possibility of neuronal death due to
535 IP3K1 overexpression as the source of the decreased
536 sensitivity to odorants. By crossing each *Gal-4* line, a
537 reporter line *UAS-lacZ* line (to observe directed Gal-4
538 expression as blue staining) and the *UAS-IP3K1* line
539 during two generations we obtained individuals con-
540 taining the three inserts simultaneously. In this case,
541 cells overexpressing the *IP3K1* gene would also ex-
542 press the reporter *lacZ* gene and, therefore, the pres-
543 ence of blue staining in the corresponding
544 preparations would indicate cell viability after *IP3K1*
545 overexpression. No differences in blue staining were
546 observed between *Gal-4/UAS-lacZ /UAS-IP3K1* and
547 *Gal-4/UAS-lacZ* hybrids either in the antenna or the
548 brain, indicating no cell mortality associated to *IP3K1*
549 overexpression (Fig. 3b). As expected, no blue stain-
550 ing was observed in the sibling control flies +/*UAS-
551 lacZ/UAS-IP3K1* generated from the same crosses
552 that the experimental group for each Gal-4 line.

553 **DISCUSSION**

554   In this report, the *IP3kinase1* gene has been
555 shown to express at the olfactory receptor organs of
556 *Drosophila melanogaster*. According to the proposed
557 role of the IP3Kinase enzyme in switching off signals

558 transmitted by the second messenger IP3 (Brehm
559 *et al.*, 2004) it could be used as indicator of olfactory
560 transduction mediated by the IP3 route. In this case,
561 qualitative or quantitative changes of the IP3K en-
562 zyme by producing mutants in the *IP3K1* gene should
563 affect olfactory reception and, eventually, olfactory
564 behavior responses to odorants. The possibility of
565 affecting olfactory reception by quantitative changes
566 in intermediary products of the IP3 route was already
567 pointed out in heterozygous for an *Itpr* (*IP3 receptor*
568 gene) null mutation (Deshpande, 2000).

569   The availability of mutant stocks in *Drosophila*
570 *melanogaster* allows approaching, in complete living
571 animals, the effects in sensory perception of modi-
572 fying a single step at the reception level. However,
573 this approach applied to the IP3 transduction cas-
574 cade in olfactory reception has been limited by the
575 few described mutations in genes encoding for en-
576 zymes that directly control the level of the second
577 messenger IP3, phospholipase C, inositol phosphate
578 phosphatases and inositol phosphate kinases
579 (IP3Ks) and, only occasionally, mutants for other
580 intermediary genes of the route have been studied
581 for olfaction (Deshpande *et al.*, 2000; Störtkuhl
582 *et al.*, 1999; Woodard *et al.*, 1992). The second
583 problem is the extended expression of these genes
584 in different body structures.

585   In this report, in order to overcome gene
586 expression at locations other than the olfactory
587 receptor organs, directed dominant mutants were
588 generated by the Gal-4/UAS method (Brand and
589 Perrimon, 1993). Overexpression of the *IP3K1* gene
590 was intended by using a transgenic stock bearing the
591 *UAS-IP3K1* construct with an extra dose of the target
592 gene expressed in certain olfactory neuron subsets
593 according to the expression pattern of a Gal-4 line.
594 The neuronal nature of the affected cells has been
595 previously tested (see "olfactory behavior changes
596 associated to IP3K1 overexpression" in the results
597 section and Gomez-Diaz *et al.*, 2004). Eight inde-
598 pendent sets of data were obtained by using eight
599 different Gal-4 lines.

600   The quantitative RT-PCR experiment showed
601 that overexpression was indeed achieved at the third
602 antennal segment, the main olfactory organ of *Dro-
603 sophila*. Although only one extra dose of the gene was
604 present in the *Gal-4/UAS-IP3K1* hybrids, the level of
605 m-RNA corresponded to almost three times the
606 control level of hybrids *Gal-4/w*[1118]. This is possible
607 because the extra expression depends on the Gal-4
608 driver and its enhancer.

609 The applied method was very efficient in induc-
610 ing changes in olfactory perception deduced from
611 behavioral data. Six out of the eight groups of
612 hybrids showed differences in olfactory behavior due
613 to the *IP3K1* overexpression implying an extensive
614 role of the IP3 cascade in olfactory reception.
615 Always, differences corresponded to odorants whose
616 reception was mediated by the affected receptor-
617 neuron subset. No differences were observed in
618 response to other odorants. These results reinforce
619 the idea that the observed changes originate in
620 olfactory receptor neurons and not in other antennal
621 support cells.

622 Since we only augmented the expression of an
623 enzyme that controls the level of the second messen-
624 ger IP3, we will expect that for neurons that do not
625 utilize this transduction cascade for mediating olfac-
626 tory reception no perception changes would appear
627 and, vice versa, for neuronal subsets that use the IP3
628 route, only the odorant information mediated by
629 these neurons and this route should be affected. This
630 could explain those cases where correspondence
631 between the effects of IP3K1 overexpression and
632 synaptic blockade was only partial. On the other
633 hand, if the change induced by altering the *IP3K1*
634 gene was not big enough it could be not observed at
635 the perception level for certain odorants.

636 Reception of some odorants may be mediated in
637 certain olfactory receptor neurons by a transduction
638 cascade different that the IP3 route. The presence of
639 other transduction cascades in olfactory reception of
640 *Drosophila* affecting sensory perception has been
641 previously reported (Gomez-Diaz *et al.*, 2004; Martin
642 *et al.*, 2001). Also the possibility of the same olfactory
643 neuron responding differently depending on the
644 odorant, giving excitatory or inhibitory responses,
645 has been pointed out (de Bruyne *et al.*, 2001). The
646 specificity of the observed differences in behavior,
647 depending on the olfactory profile of the Gal-4 line,
648 speaks in favor of a precise effect of the mutation in
649 the expected neurons and for a particular group of
650 olfactory stimuli.

651 Some experiments have been performed to test the
652 hypothesis of developmental changes in the corre-
653 sponding olfactory neuron subsets accounting for the
654 observed perception defects. Our data did not support
655 this hypothesis. First, no apparent changes in mortal-
656 ity due to *IP3K1* gene overexpression have been
657 observed (Fig. 3b) and antennal morphology seems
658 normal at this level of magnification. Second, anten-
659 nal-electrophysiology changes in signal recovery have

661 been associated to gene overexpression and the level of
662 change depended on odorant concentration. This kind
663 of phenotype affecting the EAG has been always
664 related to mutations in olfactory reception and trans-
665 duction genes (Ayer and Carlson, 1991; Deshpande
666 *et al.*, 2000; Martin *et al.*, 2001; Riesgo-Escovar *et al.*,
667 1994; Woodard *et al.*, 1992) but usually no differences
668 in amplitude or kinetics were found for developmental
669 changes, such as increased synapse number (Acebes
670 and Ferrus, 2001). Only the EAG changes found for
671 the *trp* mutants were related with some developmental
672 alteration since trp channels were not present in the
673 mature antenna of *Drosophila* although they appeared
674 in the developing antenna (Störtkuhl *et al.*, 1999). This
675 is not our case; expression of the *IP3K1* gene has been
676 shown in both adult antennae and maxillary palps.

677 Although nowadays only few transduction
678 mutants related to IP3 have been studied for EAG
679 responses, a common pattern can be established that
680 is coincident with the changes observed in our IP3K1
681 experiments. The *rdgB*, *Itpr* and *trp* mutants showed
682 abnormal recovery kinetics (Deshpande *et al.*, 2000;
683 Störtkuhl *et al.*, 1999; Woodard *et al.*, 1992, respec-
684 tively), that for prolonged exposure to odorants has
685 been interpreted as changes in adaptation.

686 Correspondence analysis between behavioral
687 and electrophysiological data for the IP3K1 mutants
688 showed that increased recovery times correlated to
689 diminished olfactory sensitivity. This effect appears
690 to be opposite to the expected result according to the
691 EAG changes produced with increasing odorant
692 concentrations. However, it could be understood if
693 IP3 plays an active role in maintaining adaptation
694 and the extended EAG recovery process corre-
695 sponded to increase neuronal inactivation time. This
696 hypothesis was proposed for the *Itpr* mutants where
697 decreased number of IP3 receptors correlated to
698 faster recovery kinetics (Deshpande et al., 2000). In
699 fact, olfactory reception does not seem to be a linear
700 process and a single element may act at different
701 timescales as two-faced messenger in transduction
702 and adaptation (Matthews and Reisert, 2003).

703 Although some small developmental changes
704 induced by *IP3K1* overexpression cannot be dis-
705 carded, changes in IP3 mediated signaling can
706 account for the observed changes in olfactory
707 perception in the Y-maze, where behavior is tested
708 during 30 minutes. Therefore, our data strongly
709 suggest that the *IP3K1* gene, expressed at the olfac-
710 tory receptor organs of *Drosophila*, mediate olfactory
711 information transfer at the reception level.

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