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The *C. elegans* G-protein-coupled receptor SRA-13 inhibits RAS/MAPK signalling during olfaction and vulval development

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SUMMARY

In *C. elegans*, the RAS/MAPK pathway is used in different tissues to regulate various cell fate decisions. Several positive and negative regulators tightly control the activity of the RAS/MAPK pathway at different steps. We demonstrate a link between a G-protein-coupled receptor signalling pathway and the RAS/MAPK cascade. SRA-13, a member of the SRA family of chemosensory receptors, negatively regulates RAS/MAPK signalling during vulval induction and the olfaction of volatile attractants. Epistasis analysis indicates that SRA-13 inhibits the RAS/MAPK pathway at the level or upstream of MAPK. In both tissues, the vulval precursor cells and the chemosensory neurones, SRA-13 acts through the GPA-5 Gα protein subunit, suggesting a common mechanism of crosstalk. Moreover, we find that vulval induction is repressed by food withdrawal during larval development and that SRA-13 activity is required for the suppression of vulval induction in response to food starvation. Thus, SRA-13 may serve to adapt the activity of the RAS/MAPK pathway to environmental conditions.

Key words: RAS, MAPK, G protein, *Caenorhabditis elegans*

INTRODUCTION

G-protein-coupled receptors (GPCRs) constitute a large family of seven-pass transmembrane proteins that mediate the cellular responses to a wide array of stimuli, such as hormones, neurotransmitters, chemoattractants, taste ligands, light, phospholipids, pheromones and growth factors (Hamm, 1998; Neer, 1995). The binding of an extracellular ligand to the corresponding GPCR induces a conformational change in the receptor that unmasks an intracellular binding site for a heterotrimeric G protein. The interaction of a G protein with an activated GPCR promotes the exchange of GDP against GTP in the Gα subunit, which activates Gα and at the same time exposes the effector binding sites in the dissociating βγ heterodimer. About 20 different Gα protein subunits that fall into four classes (G_s, G_i, G_q and G_{12}) have been identified in mammals (Wilkie et al., 1992). Each class of Gα proteins transduces GPCR signals by controlling the activity of a different intracellular second messenger system, such as the adenylate cyclase, which is stimulated by G_s and inhibited by G_i (Simonds, 1999), the phosphatidylinositol-specific phospholipase, which is activated by G_q (Rhee and Bae, 1997), or the Rho and Rap small GTPases, which are regulated by G_{12} and G_i (Kozasa et al., 1998).

Several GPCRs and heterotrimeric G proteins were found to control cellular proliferation and differentiation (Dhanasekaran et al., 1995; Lorenz et al., 2000; Moolenaar, 1991; Post and Brown, 1996; Pouyssegur et al., 1988; van Corven et al., 1989). Moreover, activated Gα proteins can cause malignant transformation of cultured cells and have been implicated in the formation of human cancer (Dhanasekaran et al., 1995). These observations have suggested a possible link between GPCR signalling pathways and the RAS/mitogen-activated protein kinase (MAPK) pathway that is a key regulator of cellular proliferation and differentiation (Peyssonnaux and Eychene, 2001; Schramek, 2002). Different pathways linking GPCR signalling to the RAS/MAPK cascade have been reported (Belcheva and Coscia, 2002; Gutkind, 2000; Hur and Kim, 2002; Lowes et al., 2002; Luttrell et al., 1997; Marinissen and Gutkind, 2001; Sugden and Clerk, 1997). For example, in *Saccharomyces cerevisiae* MAPK is regulated by a heterotrimeric G protein when the mating pheromone binds to a GPCR (Herskowitz, 1995; Metodiev et al., 2002). In mammalian cells, the autophosphorylation of the PDGFR or the EGFR can be induced by stimulation of the angiotensin II receptor or by GPCR agonists like endothelin 1, lysophosphatic acid and thrombin (Daub et al., 1996; Linseman et al., 1995). Furthermore, heterotrimeric G proteins can control RAS signalling by activating a RAS guanine-nucleotide releasing factor through a protein kinase C pathway (Marais et al., 1998; Mattingly and Macara, 1996), via PI3Kγ (Lopez-Ilasaca et al., 1997) or by regulating the RAP-1 small GTPase that acts as a RAS antagonist (Kitayama et al., 1989). However, the exact biochemical routes linking the various GPCR pathways to the RAS/MAPK cascade and the in vivo significance of many of the postulated interactions remain to be investigated.

In *C. elegans*, the RAS/MAPK pathway is used multiple

times during development to control diverse processes (Kayne and Sternberg, 1995; Riddle, 1997; Sternberg and Han, 1998). In particular, RAS-mediated signalling controls the cell fate specification during the development of the hermaphrodite vulva, and it is required for the olfaction of volatile attractants (Hirotsu et al., 2000). During vulval development, a signal from the gonadal anchor cell induces three out of six equipotent vulval precursor cells (VPCs) to adopt the vulval cell fates (Kornfeld, 1997; Sternberg and Han, 1998; Wang and Sternberg, 2001). The anchor cell signal activates in the three proximal VPCs (P5.p, P6.p and P7.p) the conserved RTK/RAS/MAPK signalling pathway to specify the primary (1°) and secondary (2°) vulval fates (Hill and Sternberg, 1992). The remaining VPCs that do not receive the inductive signal (P3.p, P4.p and P8.p) adopt the non-vulval tertiary (3°) cell fate and fuse with the hypodermis. After the vulval fates have been specified, P6.p, P5.p and P7.p divide in an invariant pattern and differentiate yielding 22 descendants that form the vulva. Mutations that reduce the activity of the RAS pathway cause a vulvaless (Vul) phenotype because P5.p, P6.p or P7.p adopt the 3° uninduced instead of the 1° or 2° vulval cell fates. Conversely, mutations that hyperactivate the RAS pathway result in a multivulva (Muv) phenotype where more than three VPCs can adopt 1° or 2° vulval cell fates.

C. elegans can detect various water-soluble and volatile substances in its environment (Bargmann et al., 1993; Dusenbery, 1974; Ward, 1973). Two pairs of bilaterally symmetric neurones in the head, the AWA and AWC neurones, mediate the response to volatile attractants (Bargmann et al., 1993). When an animal is exposed to volatile attractants such as isoamylalcohol and diacetyl, which are sensed by the AWC and AWA chemosensory neurones, respectively, MPK-1 MAPK is rapidly activated in these neurones (Hirotsu et al., 2000). Mutations that reduce the activity of the RAS/MAPK pathway at the level or downstream of RAS decrease the response of the animals to volatile attractants (Hirotsu et al., 2000). Thus, RAS-mediated signalling in AWA and AWC is required to elicit a prolonged response that results in the chemotaxis towards the source of the attractant. A large number of GPCRs that are expressed in sensory neurones and are candidate chemosensory receptors have been identified in *C. elegans* (Bargmann and Kaplan, 1998; Troemel et al., 1995). They have been divided into six gene families (*sra*, *srb*, *srd*, *sre*, *srg* and *sro*). It was estimated that a single chemosensory neurone expresses up to 20 GPCRs (Mombaerts, 1999). In addition, the *C. elegans* genome encodes 20 Gα, two Gβ and two Gγ subunit proteins (Jansen et al., 1999). One homologue exists for each of the four classes of Gα proteins found in mammals, plus 16 Gα genes that do not belong to a specific class. Although little is known about the function of the putative GPCRs in chemosensation, except for the diacetyl receptor ODR-10 (Sengupta et al., 1996), several of the Gα subunits have been shown to regulate olfaction (Jansen et al., 1999). However, if and how G proteins regulate the RAS/MAPK signalling pathway in the olfactory neurones is not known.

Here, we report the identification of the GPCR SRA-13 as a negative regulator of the RAS/MAPK pathway during vulval induction and olfaction. SRA-13 acts predominantly through the GPA-5 Gα protein to inhibit MAPK activation in the AWA and AWC chemosensory neurones. Surprisingly, SRA-13 and GPA-5 play a similar role in negatively regulating the RAS/MAPK pathway during vulval induction, suggesting that a common mechanism of crosstalk functions in both tissues. During vulval development, the SRA-13/GPA-5 signal may serve to adapt the activity of the RAS/MAPK pathway to environmental conditions. Our data demonstrate the in vivo significance of the connection between GPCR signalling pathways and the RAS/MAPK cascade.

MATERIALS AND METHODS

General methods and strains used

Standard methods were used for maintaining and manipulating *Caenorhabditis elegans* (Brenner, 1974). The *C. elegans Bristol* strain, variety N2, was used as the wild-type reference strain in all experiments. Unless noted otherwise, the mutations used have been described previously (Riddle et al., 1997) and are listed below by their linkage group.

LGI: *mek-2(n2678)*, *che-3(e1124)*

LGII: *ga145* (G.B. and A.H., unpublished), *sra-13(zh13)* (this study), *let-23(sa62)*, *let-23(sy1)*

LGIII: *unc-119(e2498)*, *daf-2(e1370)*

LGIV: *let-60(n2021)*, *let-60(n1046)*, *let-60(n2031dn)* (Beitel et al., 1990), *let-60(n1876)* (Beitel et al., 1990), *let-60(ga89)* (Eisenmann and Kim, 1997), *lin-45(sy96)*, *gpa-7(pk610)* (Jansen et al., 1999)

LGV: *gpa-2(pk16)*, *gpa-3(pk35)* (Jansen et al., 1999), *gaIs36[hs::mpk-1]* (Lackner and Kim, 1998)

LGX: *sem-5(n2019)*, *gap-1(ga133)* (Hajnal et al., 1997), *gpa-5(pk376)* (Jansen et al., 1999), *osm-5(p813)*, *syIs1[lin-3(xs)]*

Unless noted in the table legends, all experiments were conducted at 20°C. Transgenic lines were generated by injecting the DNA at a concentration of 100 ng/μl into both arms of the syncytial gonad as described (Mello et al., 1991). pUNC119 (20 ng/μl) and pTG96 (100 ng/μl) were used as a transformation markers (Maduro and Pilgrim, 1995; Yochem et al., 1998).

Identification of sra-13

During the positional cloning of a gene defined by the *ga145* and *zh17* mutations that cause a Muv phenotype in a *gap-1(0)* background and map on chromosome II between *dpy-10* and *unc-4*, we observed that multicopy extrachromosomal arrays consisting of the YAC Y24H1 and the cosmid F49E12 partially rescued the *ga145; gap-1(0)* Muv phenotype, reducing the penetrance from 77% to 25%. However, the subsequent genetic mapping and the molecular cloning indicated that the *ga145* and *zh17* mutations are alleles of the *puf-8* gene located on the YAC Y7B4 (Fig. 1A; G. Battu and A. Hajnal, unpublished).

Isolation of the *sra-13(zh13)* deletion

The *sra-13(zh13)* deletion mutant was isolated from an EMS-mutagenized library consisting of ~10^6 haploid genomes as previously described (Berset et al., 2001; Jansen et al., 1997). DNA pools were screened by nested PCR with primers OGB29 (5′GTC CAC CGA ATT GGC AAA GAA ATG G3′) and OGB30 (5′GGA TGA ACA GTT GAA GCT CCT GCT3′) in a first PCR reaction and OGB30 (5′GAG AAA ATG TTG GGG AAC AGG TGG3′) and OGB31 (5′GGG ACA TTT GAT TAG TGG CCA GAA G3′) in a second PCR reaction. The *zh13* deletion removes 1756 base pairs (bp), including 396 bp of 5′ sequences and 1360 bp of the F49E12.5 open reading frame (positions 29811 to 31567 in the cosmid F49E12). The *sra-13(zh13)* strain was backcrossed eight times to N2 before the phenotypic analysis was performed.

Plasmid constructs

The *sra-13::gfp* translational reporter was generated by PCR amplification of a 2.4 kb genomic *sra-13* fragment containing 900 bp

of 5′ promoter sequences and the entire open reading frame, using the primers OGB61(AAT CTA GAA TTC GTG AAA AAG TCC ACC) and OGB62 (CGT CTA GAT ATT TCC ACG CTG AAT TG) followed by *Xba*I restriction and ligation into the *Xba*I site of the vector pPD95.75 (a gift from Andrew Fire). The transcriptional *sra-13::nls::gfp* reporter was generated by ligating a *Xba*I-*Bam*HI restricted 900 bp genomic fragment containing the 5′ promoter elements of F49E12.5 into the *Xba*I-*Bam*HI site of pPD96.04 (a gift from Andrew Fire). The 900 bp fragment was PCR amplified using the primers OGB61 and OAH150 (TTT GGA TCC GTT GAG GAA GGA GTT GCC AT). The *sra-13(xs)* fragment was generated by PCR amplification using the primers OGB 7 (GTC CAC CGA ATT GGC AAA GAA ATG G) and OGB 29 (ATG CGT GTC AGT CAT CCT TTT GG) of a 3.7 kb genomic fragment encompassing the entire *sra-13* ORF, 0.9 kb of upstream and 1.3 kb of downstream sequences. To generate the *sra-13(mut)* construct, two complementary primers, OGB 138 (GTT AAG TCA ACC GGA TCC GTG CTT) and OGB139 (AGC ACG GAT CCG GTT GAC TTA AC), bearing a stop mutation after residue 21 followed by a *Bam*HI site were used in two separate PCR reactions with primers OGB7 and OGB29 (see above), respectively, to generate two DNA fragments, one containing the upstream sequences, the first 21 amino acids and a stop codon, and the other starting from the stop codon and containing the remaining *sra-13* open reading frame and downstream sequences. These two fragments were ligated at the *Bam*HI sites and cloned into the pGEMT vector (Promega), yielding an insert identical to the *sra-13(xs)* fragment, except for the stop mutation and a *Bam*HI site. The *hs::sra-13* construct was generated by ligating a 1.9 kb *Bam*HI-*Kpn*I fragment amplified with the primers OGB113 (AAT ATC GGA TCC ATG GCA ACT CCT TCC TCA ACT) and OGB140 (AAA AAA GGT ACC CGG TGT GAT TGA TGA GTT GGA) covering the *sra-13*-coding sequence and 350 bp of the 3′ UTR into the *Bam*HI-*Kpn*I site of vector pJK465, which contains a *hsp16* heat shock promoter upstream of the *Bam*HI site.

Chemotaxis assays

Chemotaxis assays were performed as described (Bargmann et al., 1993). Around 100 animals that had been washed three times with M9 buffer were placed along a line in the centre of a 90 mm phosphate-buffered agar plate onto which the attractant and a neutral chemical, each diluted in ethanol, had been spotted together with 1 M sodium azide on either side of the plate and equidistant from the worms. After allowing the animals to crawl for 1 hour, the numbers of animals at the attractant and at the neutral chemical were counted. The chemotaxis index was calculated as I=(the number of animals at the attractant)–(the number of animals at the neutral chemical)/(total number of animals on the plate). Chemotaxis towards NaCl was assayed as described (Bargmann and Horvitz, 1991). All experiments were performed at least in triplicate and the mean index and standard deviation were calculated. Except for the assays shown in Fig. 3A,B, isoamylalcohol and diacetyl were used at dilutions of 10^{-2} and 10^{-3}, respectively.

Immunostaining and microscopy

The animals were washed three times with water and left at room temperature for 4 hours before a 10 second stimulation with a 1:50 dilution of isoamlyalcohol in water. They were immediately fixed using Bowins fixative (a mixture of 75 ml saturated picric acid, 25 ml of formalin and 5 ml of glacial acetic acid) as previously described (Hirotsu et al., 2000; Nonet et al., 1993), and stained with DP-ERK antibodies (Sigma, diluted 1:100) and anti-GFP antibodies (Clontech, diluted 1:200) to detect SRA-13::GFP-expressing cells. Fluorescent images were recorded with a Leica TCS4 confocal microscope (Fig. 2A) or a Leica DMRA microscope equipped with a coded CCD camera (Hamamatsu ORCA-ER) controlled by the Openlab 3.0 software package (Improvision). The images shown in Fig. 4 were processed by volume deconvolution (Improvision) to remove out-of-focus light.

Vulval induction and starvation assays

Vulval induction was scored by examining worms at the L4 stage under Nomarski optics as described (Sternberg and Horvitz, 1986). The number of VPCs that had adopted a 1° or 2° vulval fate was counted for each animal and the induction index was calculated by dividing the number of 1° or 2° induced cells by the number of animals scored. Statistical analysis was performed using a *t*-test for independent samples.

For the starvation assays, the worms were hypochlorite treated to release the eggs, which were allowed to hatch overnight in M9 buffer as described (Riddle et al., 1997). The synchronized L1 larvae were fed with *E. coli* OP50 for 3.5 hours until they reached the end of the L1 stage. The late L1 larvae were washed 3 times with M9 buffer and put on NGM plates containing 50 μg/ml ampicillin (to prevent the growth of attached *E. coli*) without food for 36 hours. The larvae were then re-fed until they reached the L4 stage and their vulval induction index could be scored as described above. At the late L1 to early L2 stage, vulval induction is sensitive to food starvation, although in larvae that had been grown in the presence of food until they reached the mid- to late-L2 stage (9 hours or longer), vulval induction was insensitive to starvation. For example, in *let-60(n1046)* L1 larvae that were fed for 3 to 7 hours before starvation, the average number of induced VPCs per animal ranged from 3.5 to 3.6, but in larvae fed for 9 hours or longer before starvation, the average number of induced VPCs was between 4.5 and 4.4. (Induction in unstarved *let-60(n1046)* larvae was 4.4 induced VPCs per animal.)

RESULTS

Identification of SRA-13 as an inhibitor of the RAS signalling pathway

To identify inhibitors of the RTK/RAS/MAPK signalling pathway, we used vulval induction in *C. elegans* as an in vivo assay for RAS-mediated signal transduction. While generating multicopy extrachromosomal arrays consisting of YAC and cosmid clones spanning the *dpy-10* to *unc-4* interval on chromosome II (see Materials and Methods) we observed that the cosmid F49E12 reduced the penetrance of the Muv phenotype caused by mutations that hyperactivate the RAS signalling pathway (Fig. 1A; Table 1). Cosmid sub-fragments were injected and the inhibitory activity was narrowed down to a single open reading frame (F49E12.5, Fig. 1A). These observations suggested that overexpression of the protein encoded by the F49E12.5 open reading frame decreases the activity of the RAS signalling pathway during vulval induction.

We named the corresponding gene *sra-13* because it encodes a seven-pass transmembrane protein belonging to the SRA family of G-protein-coupled chemosensory receptors (Fig. 1B,C). SRA-13 exhibits no obvious homology to proteins from other species. Most of the over 40 *sra* genes predicted by the genome project are found in clusters of two to nine genes (Troemel et al., 1995). *sra-13* is most similar to *sra-11* (33% identity, 58% similarity), but it does not belong to the *sra-10* to *sra-12* or the *sra-1* to *sra-9* cluster on LGII (Troemel et al., 1995). As extrachromosomal arrays encoding other GPCRs located in the *dpy-10* to *unc-4* interval (e.g. *srd-54*, *srd-55*, *sre-1*, *sre-2* and *sro-1*; Fig. 1A) did not suppress vulval induction and the cosmid F49E12 does not encode another GPCR, the effect caused by overexpression of SRA-13 is likely to be specific.

To test if endogenous SRA-13 inhibits RAS signalling, we isolated the *sra-13* loss-of-function mutation *zh13* (see

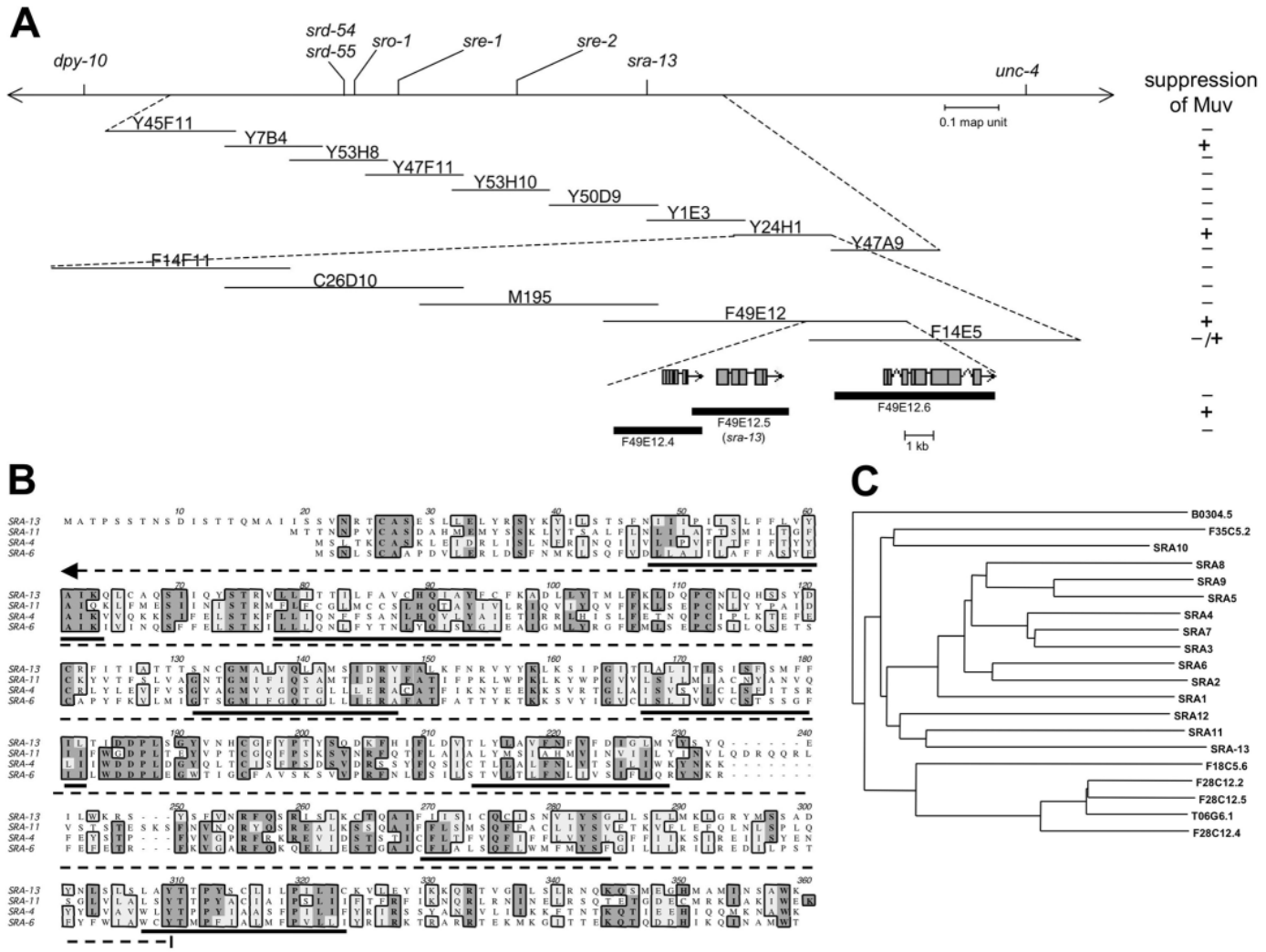

Fig. 1. Identification of *sra-13*. (A) Physical map of the *dpy-10 unc-4* interval on chromosome II. The YAC and cosmid clones tested are shown. The three long-PCR fragments derived from the cosmid F49E12 that were tested are shown at the bottom. The F49E12.4, F49E12.5 (*sra-13*) and F49E12.6 PCR products spanned positions 27,000 to 30,140; 29,500 to 33,085; and 34,600 to 38,300 in the F49E12 cosmid sequence, respectively. '–' indicates no suppression and '+' indicates suppression of the Muv phenotype. (B) Sequence alignment of SRA-13 with SRA-11 (gi2500866), SRA-4 (gi1176649) and SRA-6 (gi1176651). Conserved residues are indicated by the grey shading. The regions underlined in bold are the predicted transmembrane domains. The broken line indicates the extent of deletion in the *sra-13* open reading frame in the *zh13* allele. (C) Dendogram of the SRA family of GPCRs calculated with the neighbour-joining method using the CLUSTALX software (Thompson et al., 1997).

Materials and Methods). *sra-13(zh13)* animals carry a deletion of 1756 bp that removes 396 bp of 5′ promoter sequences and all but the last exon of SRA-13 (Fig. 1B). Thus, the *zh13* mutation is most probably a null allele. Henceforth, we refer to this allele as *sra-13(0)*. *sra-13(0)* single mutant animals are healthy and exhibit no obvious morphological defects. In particular, vulval development in *sra-13(0)* animals appeared to occur normally (Table 1, row 2). However, this does not exclude the possibility that *sra-13(0)* may negatively regulate RAS-mediated signalling (see below).

SRA-13 is expressed in the AWA and AWC chemosensory neurones

The SRA proteins are expressed in chemosensory neurones, interneurones and various other tissues, including muscle and hypodermal cells (Troemel et al., 1995). To examine the expression pattern of SRA-13, we built translational and transcriptional GFP reporter constructs. The translational *sra-13::gfp* reporter was generated by fusing a 2.4 kb genomic fragment containing 900 bp of 5′ promoter sequences reaching up to the 3′ end of the neighbouring gene (F49E12.4) and the entire open reading frame of *sra-13* to a *gfp* cassette. The C-terminal fusion to GFP is likely to inactivate SRA-13, as animals carrying the *sra-13::gfp* reporter transgene did not exhibit any of the phenotypes caused by SRA-13 overexpression (see below). For the transcriptional reporter, the 900 bp of 5′ promoter sequences were fused to a *gfp::lacZ* fusion gene (a gift from A. Fire).

Both *gfp* reporter transgenes showed strong expression in two bilaterally symmetric pairs of neurones in the head of the animal (Fig. 2A). We identified these neurones as the AWAL/R and AWCL/R chemosensory neurones by comparing the *sra-*

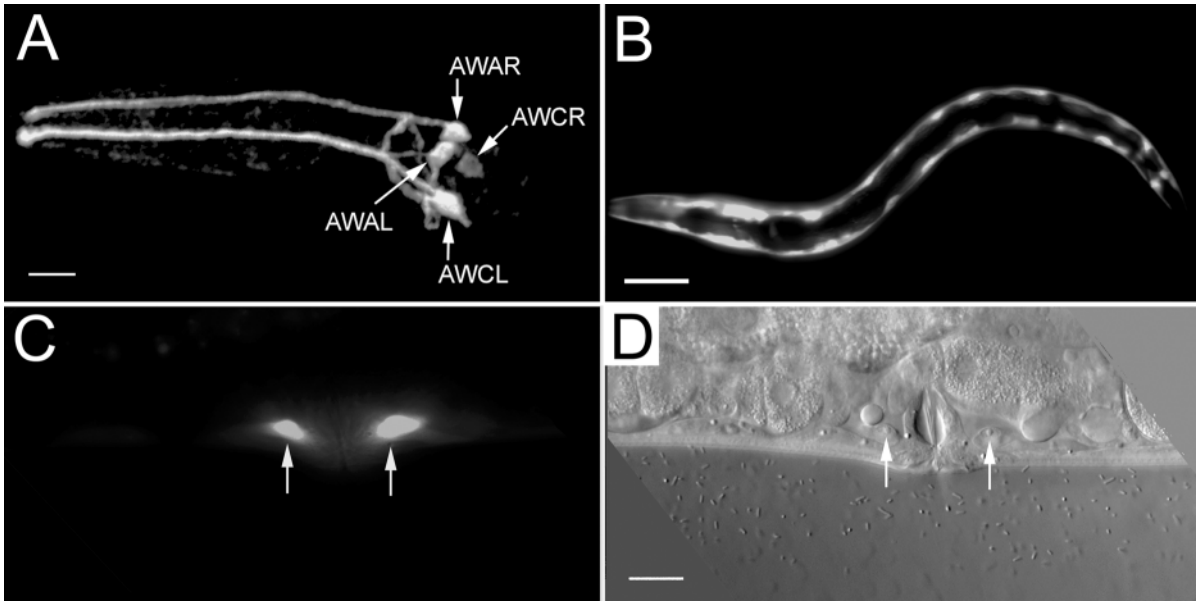

Fig. 2. SRA-13 expression pattern. (A) Expression of the *sra-13::gfp* translational reporter in the two pairs of AWA and AWC chemosensory neurones in the head of an adult animal (arrows). A 3D reconstruction of confocal sections through the head region is shown. (B) Expression of the *sra-13::gfp* transcriptional reporter in the body wall muscles and hypodermis of an L4 animal and (C) in the vulval muscles that control egg-laying in the adult hermaphrodite. (D) Nomarski image of the animal shown in C. Scale bars: 10 μm in A,D; 50 μm in B.

13::gfp expression pattern with the expression pattern of the *gcy-10::gfp* reporter, which is expressed in the AWB and AWC chemosensory neurones and interneurone I1 (Yu et al., 1997), and by staining *sra-13::gfp* worms with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), which stains the ASK, ADL, ASI, AWB, ASH and ASJ, but not the AWA, neurones. In the AWA and AWC neurones, the translational SRA-13::GFP fusion protein was present in the cell body, the axons and the dendrites (Fig. 2A). In addition to the four chemosensory neurones, the transcriptional fusion was expressed in muscle and hypodermal cells throughout the animal (Fig. 2B-D). The expression of SRA-13 in the chemosensory neurones suggested that, like the other SRA proteins, SRA-13 might be involved in sensing the environment.

SRA-13 negatively regulates olfaction

The AWA and the AWC neurones are required for sensing a variety of volatile attractants present in the environment (Bargmann et al., 1993). Upon exposure to a volatile attractant, the RAS/MAPK pathway is rapidly activated in the chemosensory neurone that is used to detect the attractant (Hirotsu et al., 2000). Mutations that reduce the activity of the RAS/MAPK pathway severely compromise the ability of the animals to respond to a volatile attractant, resulting in a reduced chemotaxis towards the source of the attractant. For example, animals carrying reduction-of-function mutations in *let-60 ras*, *lin-45 raf* or *mek-2 mapkk* exhibit a reduced chemotaxis index to the volatile attractants isoamylalcohol and diacetyl, which are sensed by AWC and AWA, respectively (Hirotsu et al., 2000). We thus speculated that SRA-13 in the AWA and AWC neurones might act antagonistically to the RAS/MAPK pathway during olfaction. Moreover, *sra-13(0)* animals were not defective for chemotaxis to isoamylalcohol or diacetyl. On the contrary, they displayed stronger chemotaxis to limiting concentrations of isoamylalcohol and diacetyl than did wild-type animals (Fig. 3A,B), suggesting that SRA-13 negatively regulates olfaction. Furthermore, loss of *sra-13* function partially suppressed the chemotaxis defects caused by reduction-of-function mutations in components of the RAS/MAPK pathway. For example, in *let-60 ras(n2021rf)* animals olfaction is severely compromised, causing an approximately threefold reduction in the chemotaxis index towards isoamylalcohol or diacetyl (Fig. 3C,D) (Hirotsu et al., 2000). By contrast, *sra-13(0); let-60(rf)* double mutants exhibited a twofold higher chemotaxis index to isoamylalcohol than *let-60(rf)* single mutants and nearly wild-type chemotaxis to diacetyl. Similar results were obtained using reduction-of-function mutations in *lin-45 raf* and *mek-2 mapkk* (Fig. 3C,D).

Overexpression of SRA-13 caused a phenotype opposite to that observed in *sra-13(0)* animals. To increase SRA-13 activity, we used the *sra-13(xs)* strain that carries multiple copies of the *sra-13* genomic region on an extrachromosomal array. *sra-13(xs)* animals were defective in chemotaxis towards isoamylalcohol and diacetyl (Fig. 3E,F), while *sra-13(xs)* animals could detect NaCl as well as wild-type animals, indicating that their chemotaxis defect was specific for volatile chemicals and that the general locomotion was not compromised (data not shown). Animals carrying a *sra-13* transgene with a mutation truncating the protein after the first 21 amino acids (*sra-13(mut)*) did not exhibit a significant decrease in chemotaxis to isaomylalcohol and diacetyl (Fig. 3E,F).

Olfaction seems to require just the right amount of RAS/MAPK signalling, as mutations that decrease or increase the activity of the LET-60 RAS protein both affect the ability of the animals to sense volatile attractants (Hirotsu et al., 2000). For example, in *let-60(n1046gf)* animals, chemotaxis to

isoamylalcohol is severely compromised (Fig. 3E), possibly because perpetual RAS/MAPK signalling might trigger a desensitizing mechanism. We next tested if overexpression of SRA-13 overcomes the chemotaxis defect of *let-60(gf)* mutants. The *sra-13(xs)* transgene partially rescued the strong chemotaxis defect of *let-60(gf)* animals to isoamylalcohol (Fig. 3E). By contrast, chemotaxis to diacetyl was only moderately affected by the *let-60(gf)* mutation, and *let-60(gf); sra-13(xs)* animals showed a further reduction in the chemotaxis index to diacetyl (Fig. 3F) (Hirotsu et al., 2000). It thus appears that the olfaction of diacetyl tolerates higher levels of RAS/MAPK activation, while the *sra-13(xs)* transgene may reduce RAS pathway activity below wild-type levels.

To exclude the possibility that SRA-13 is required for the development of the chemosensory circuit we expressed SRA-13 under control a heat-shock inducible promoter (*hs::sra-13*) (Fig. 3I,K). *hs::sra-13* animals that were grown at the permissive temperature (20°C) until they reached adulthood and then subjected to heat-shocks to induce SRA-13 expression were severely defective in chemotaxis to both isoamylalcohol and diacetyl. By contrast, wild-type animals that were subjected to the same heat-shock regime and *hs::sra-13* control animals that were kept at 20°C showed no significant reduction in chemotaxis.

The experiments presented so far suggested that SRA-13 antagonizes RAS-mediated signalling during olfaction. However, these results did not allow us to distinguish whether SRA-13 signalling directly inhibits the activity of the RAS/MAPK pathway or if SRA-13 acts in a parallel pathway that antagonizes the response to the RAS/MAPK signal.

SRA-13 inhibits MAPK activation during olfaction

To address this point, we tested if the *sra-13(0)* mutation causes an increased activity of the RAS/MAPK pathway in the chemosensory neurones. For this purpose, we determined the levels of activated MPK-1 MAPK in the AWC neurones after stimulation with a volatile attractant (Hirotsu et al., 2000). Animals carrying the *sra-13::gfp* reporter to label the AWA and AWC neurones were briefly stimulated with isoamylalcohol and then stained with an antibody specific for the diphosphorylated, activated form of MPK-1 (DP-MPK-1) and an anti-GFP antibody to visualize the AWA and AWC neurones (see Materials and Methods). In most wild-type

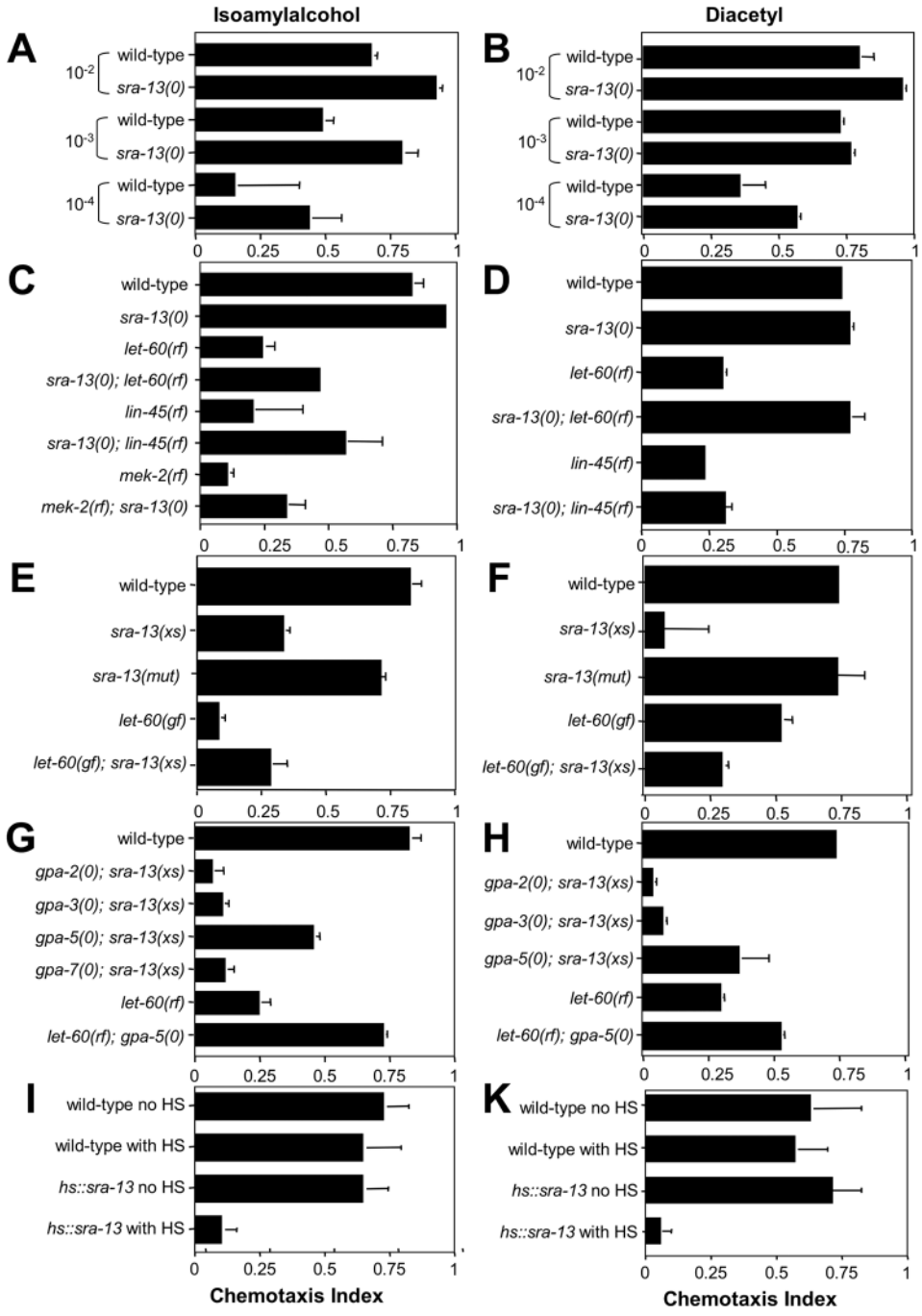

Fig. 3. *sra-13* inhibits the RAS/MAPK pathway during olfaction. The chemotaxis indices were calculated as described in the Materials and Methods. An index of 0 indicates no attraction and 1 indicates maximal attraction. The error bars indicate the standard deviation of the mean observed in at least three independent trials. 'with HS' in I,K indicates that young adult animals were heat shocked twice at 33°C for 30 minutes with a 3 hour interval and chemotaxis was assayed after a 4 hour recovery period. Alleles used: *sra-13(zh13), let-60(n2021), lin-45(sy96), mek-2(n2678), let-60(n1046gf), gpa-2(pk16), gpa-3(pk35), gpa-5(pk376), gpa-7(pk610).*

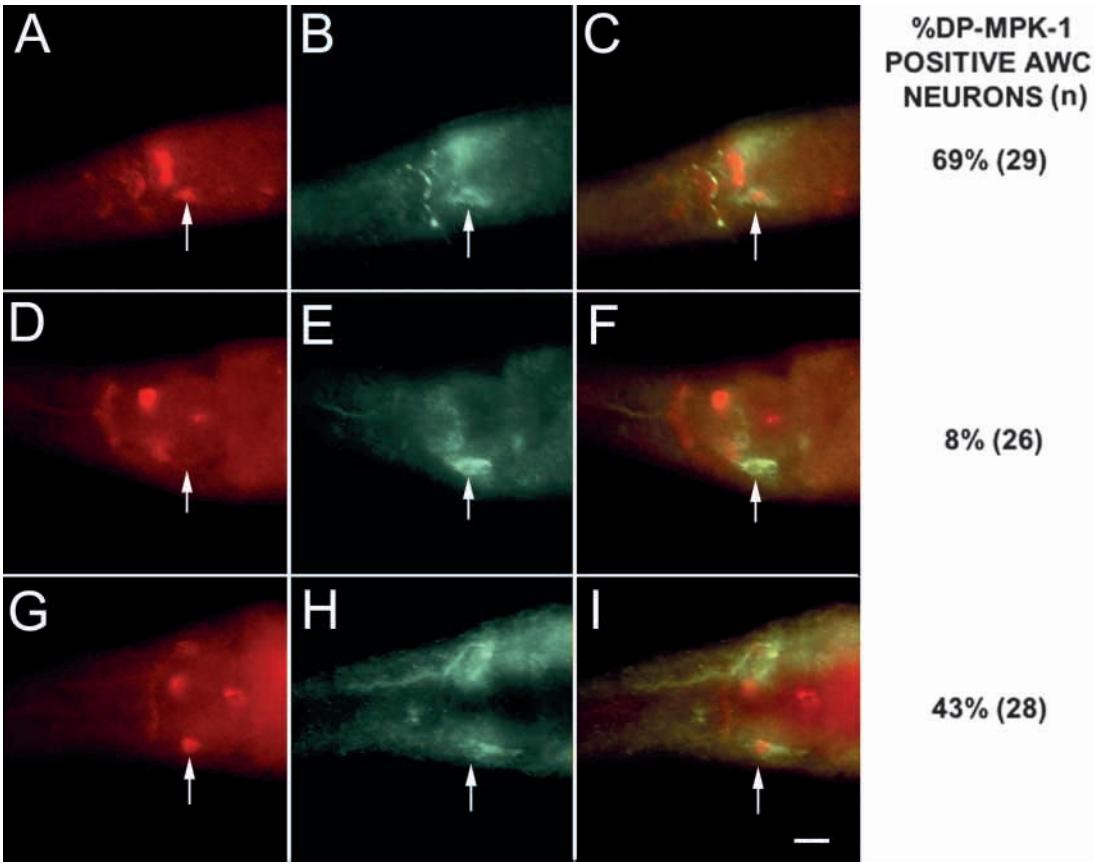

Fig. 4. DP-MPK-1 staining in AWC after stimulation with isoamlyalcohol. (A-C) Wild type; (D-F) *let-60(rf)*; (G-I) *sra-13(0); let-60(rf)* animal. DP-MPK-1 staining in A,D,G is shown in red, GFP staining in B,E,H is in green. (C,F,I) Merged images. Anterior is towards the left and dorsal is upwards in all panels. The arrows indicate the AWC neurones. The frequency at which DP-MPK-1 staining was observed in each strain is given on the right. (n) refers to the number of animals scored. Scale bar: 10 μm.

animals, a sharp increase in DP-MPK-1 staining in the AWC neurones could be observed after stimulation (Fig. 4A), while in most *let-60(rf)* animals no or very little of DP-MPK-1 staining was detected in AWC (Fig. 4D). However, in isoamylalcohol stimulated *sra-13(0); let-60(rf)* double mutants we found an increased frequency of strong DP-MPK-1 staining in the AWC neurones, indicating that a loss of *sra-13* function partially restores MPK-1 activation in *let-60(rf)* mutants (Fig. 4G). Thus, SRA-13 negatively regulates the activity of the RAS/MAPK pathway at the level or upstream of MPK-1.

SRA-13 signals via the GPA-5 Gα subunit

To examine whether SRA-13 signals through a specific Gα protein subunit, we tested if loss-of-function mutations in different Gα genes suppressed the *sra-13(xs)* phenotype. We tested *gpa-2*, *gpa-3*, *gpa-5* and *gpa-7* because these four Gα genes had previously been shown to inhibit chemotaxis to volatile attractants (Jansen et al., 1999). Of the four *gpa* mutants tested, only the *gpa-5(0)* mutation suppressed the chemotaxis defect of *sra-13(xs)* animals (Fig. 3G,H). Similar to *sra-13(0)*, the *gpa-5(0)* mutation enhanced chemotaxis towards isoamylalcohol and diacetyl (data not shown), and suppressed the chemotaxis defect of *let-60(rf)* mutants (Fig. 3G,H). From these experiments, we conclude that GPA-5 probably acts downstream of SRA-13 to transduce an inhibitory signal. However, GPA-5 may not be the only Gα protein acting downstream of SRA-13, as chemotaxis was not restored to wild-type levels in *gpa-5(0); sra-13(xs)* animals. We also noticed that the *gpa-5(0)* mutation suppressed the chemotaxis defect of *let-60(rf)* mutants to isoamylalcohol more efficiently than the *sra-13(0)* mutation, suggesting that GPA-5 may transduce signals from additional GPCRs (Fig. 3G).

SRA-13 and GPA-5 inhibit vulval induction

We had originally identified SRA-13 because its overexpression partially suppressed the Muv phenotype of mutants that exhibit a hyperactivation of the RAS/MAPK pathway. To investigate if endogenous SRA-13 and GPA-5 inhibit vulval induction, we performed epistasis analysis by combining the *sra-13(0)* and *gpa-5(0)* mutations with reduction-of-function mutations in the RTK/RAS/MAPK pathway that cause a partial Vul phenotype. Vulval induction in *sra-13(0)* single mutants was normal (Table 1, row 2), but loss of *sra-13* function significantly suppressed the Vul phenotype caused by the *let-60 ras(rf)* (Table 1, rows 6,7) or the dominant negative *let-60 ras(n2031dn)* mutation (Table 1, rows 9,10). However, the *sra-13(0)* mutation did not suppress the stronger Vul phenotype caused by the *sem-5 grb2(n2019)*, *let-23 egfr(sy1)* and *lin-45 raf(sy96)* mutations or the *let-60(n1876)* mutation that completely blocks the RAS signalling pathway in the VPCs and causes a 100% penetrant Vul phenotype (Table 1, rows 3,4 and data not shown). Loss of *gpa-5* function efficiently suppressed the Vul phenotype of *let-60(rf)* mutants as well as the stronger Vul phenotype caused by the *sem-5(rf)* mutation, suggesting that also during vulval induction GPA-5 may transduce inhibitory signals from additional GPCRs (Table 1, rows 5,8).

Next, we tested if excess SRA-13 inhibits vulval induction. Overexpression of SRA-13 under control of its own promoter (*sra-13(xs)*, Table 1, rows 11,12) as well as from a heat-shock

Table 1. Genetic interaction between *sra-13* and RAS/MAPK pathway mutants during vulval induction

Row	Genotype	% Vul†	% Muv‡	Induced VPCs/animal§	*n*¶
1	Wild type	0	0	3.0	Many
2	*sra-13(0)*	0	0	3.0	50
3	*sem-5(rf)*	72	0	1.1	71
4	*sra-13(0); sem-5(rf)*	70	0	1.3	53
5	*sem-5(rf) gpa-5(0)*	41	0	2.4*** (3)	51
6	*let-60(rf)*	46	0	2.2	41
7	*sra-13(0); let-60(rf)*	16	0	2.8** (6)	56
8	*let-60(rf); gpa-5(0)*	3	0	2.9*** (6)	59
9	*let-60(dn)/+*	49	0	2.2	70
10	*sra-13(0); let-60(dn)/+*	13	0	2.8** (9)	67
11	*let-60(gf)*	0	83	4.4	29
12	*let-60(gf); sra-13(xs)*	0	44	3.6*** (11)	57
13	*let-60(gf); sra-13(mut)*	0	73	4.3	30
14	*hs::sra-13*	0	0	3.0	36
15	*hs::sra-13* +HS††	3	0	2.9	61
16	*sra-13(0); let-60(rf); hs::sra-13*	17	0	2.8	30
17	*sra-13(0); let-60(rf); hs::sra-13* +HS††	57	0	1.9*** (7)	21
18	*sra-13(0); let-60(gf)*	0	83	4.6	127
19	*sra-13(0); let-60(gf); hs::sra-13*	0	40	3.5	28
20	*sra-13(0); let-60(gf); hs::sra-13*+HS††	0	9	3.1*** (18)	35

†The fraction of animals with fewer than three induced VPCs.
‡The fraction of animals with more that three induced VPCs.
§The average number of induced VPCs per animal was scored and statistical analysis was carried out as described in Materials and Methods. ***$P \leq 0.0001$, **$P \leq 0.001$ and *$P \leq 0.02$. The numbers in brackets indicate the row with which a dataset was compared.
¶Number of animals scored.
††The animals were heat-shocked for 30 minutes at 33°C every 20-24 hours beginning in the early L1 stage until the L4. Alleles used: *sra-13(zh13), let-60(n2021rf), let-60(n1046gf), let-60(n2031^{dn}), sem-5(n2019)* and *gpa-5(pk376)*.

promoter (*hs::sra-13*, Table 1, rows 18-20) reduced the penetrance of the Muv phenotype caused by the *let-60 ras* gain-of-function mutation, whereas the *sra-13(mut)* transgene had no effect (Table 1, row 13). Moreover, *hs::sra-13* animals exhibited a weak Vul phenotype in a wild-type background (Table 1, rows 14,15). In addition, in *sra-13(0); let-60(rf); hs::sra-13* animals, the levels of vulval induction were comparable with those observed in *let-60(rf)* single mutants (Table 1, compare rows 6, 7 and 17). Taken together, these results indicated that SRA-13 and GPA-5 negatively regulate vulval induction.

SRA-13 links vulval induction to growth conditions

sra-13(xs) animals exhibited pleiotropic phenotypes besides the chemotaxis defect described above. Fourteen percent of *sra-13(xs)* animals (n=169) developed into dauer larvae when grown in the presence of abundant food at 25°C, whereas wild-type animals did not form any dauer larvae under the same conditions (n=423). Moreover, adult *sra-13(xs)* animals displayed a reduced rate of egg laying. They contained on the average 13±11 (n=19) fertilized eggs in the uterus while wild-type animals contained 8±5 (n=25) eggs. As enhanced dauer formation and a reduced egg-laying rate are usually observed when wild-type animals are deprived of food (C. Trent, PhD thesis, MIT, 1982), the hyperactivation of the SRA-13 pathway appeared to mimic the effects of food starvation.

We also noticed that vulval induction is sensitive to food levels. The effect of food on vulval induction becomes apparent in mutants that exhibit a hyperactivation of the RAS/MAPK pathway. For example, when *let-60 ras(gf)* animals were grown in the absence of food during the L2 and L3 stages, which is the period when vulval induction occurs, the penetrance of the Muv phenotype was decreased compared with non-starved control animals (Table 2, rows 3,4, for the starvation protocol see Materials and Methods). A similar reduction of vulval induction by food starvation was observed in *ga89*, a temperature-sensitive *let-60 ras* gain-of-function allele, and in *hs::mpk-1* animals (Table 2, rows 11,12,13,14). However, *lin-3 egf(xs)* and *let-23 egfr(gf)* animals were insensitive to food starvation, suggesting that the overproduction of the inductive anchor cell signal or the constitutive activation of LET-23 EGFR can overcome the inhibitory starvation signal (Table 2, rows 7-10). Furthermore, the *e1370ts* mutation that reduces the activity of the DAF-2 insulin receptor without causing entry into the dauer stage at 20°C (Dorman et al., 1995) caused a reduction in the penetrance of the *let-60(gf)* Muv phenotype comparable to food starvation (Table 2, row 15). By contrast, in *sra-13(0); let-60(gf)* double mutants, vulval induction remained unchanged after starvation during the L2 and L3 stages (Table 2, rows 16,17), but dropped to the levels observed in food-starved *let-60(gf)* single mutants when the *sra-13(xs)* transgene was introduced (Table 2, rows 18,19). Thus, loss of *sra-13* function appears to uncouple vulval induction from environmental conditions. To test if the reduction in vulval induction by starvation requires the input from the sensory system, we used the *che-3(e1124)* and *osm-5(p813)* mutations that disrupt the structure and activity of the sensory neurones, respectively, and hence make the animals insensitive to the environment (Perkins et al., 1986). As in *sra-13(0)* animals, no drop in vulval induction could be observed in *che-3(e1124); let-60(n1046gf)* and *let-60(n1046gf); osm-5(p813)* double mutants when the animals were deprived of food during vulval induction (Table 2, rows 20-23). Thus, under starvation conditions, SRA-13 is required to transmit a starvation signal from the sensory system to the VPCs or to produce an inhibitory signal in the VPCs.

Table 2. Loss of *sra-13* function renders vulval induction insensitive to starvation

Row	Genotype	Food	% Muv	Induced VPCs/animal	*n*
1	Wild type	+	0	3.0	Many
2	Wild type	–	0	3.0	74
3	*let-60(gf)*	+	83	4.4	29
4	*let-60(gf)*	–	45	3.5*** (3)	43
7	*lin-3(xs)*	+	80	5.1	60
8	*lin-3(xs)*	–	82	5.1	50
9	*let-23(gf)*	+	66	4.0	64
10	*let-23(gf)*	–	73	4.2	45
11	*let-60(ga89)*†	+	26	3.4	63
12	*let-60(ga89)*†	–	11	3.1* (11)	61
13	*hs::mpk-1*‡	+	90	5.2	52
14	*hs::mpk-1*‡	–	68	4.6* (13)	25
15	*daf-2(rf); let-60(gf)*	+	7	3.1*** (3)	56
16	*sra-13(0); let-60(gf)*	+	83	4.6	127
17	*sra-13(0); let-60(gf)*	–	76	4.4	107
18	*sra-13(0); let-60(gf); sra-13(xs)*	+	22	3.2§	9
19	*sra-13(0); let-60(gf); sra-13(xs)*	–	15	3.2	47
20	*che-3(0); let-60(gf)*	+	72	4.5	29
21	*che-3(0); let-60(gf)*	–	78	4.4	60
22	*let-60(gf); osm-5(0)*	+	76	4.2	42
23	*let-60(gf); osm-5(0)*	–	88	4.6	69

Vulval induction was scored as described in the legend to Table 1, and in the Materials and Methods. ***$P \leq 0.0001$ and *$P \leq 0.02$. The numbers in brackets indicate the row with which a dataset was compared.

†The strain was grown at 25°C.

‡L1 larvae were heat-shocked for 30 minutes at 33°C and grown at 25°C until L4.

§Induction in this strain was not significantly different from the *let-60(gf); sra-13(xs)* strain shown in Table 1, row 12 ($P=0.14$). Alleles used: *che-3(e1124), sra-13(zh13), let-23(sa62), daf-2(e1370), let-60(n1046gf), gaIs36[hs::mpk-1], syIs1[lin-3(xs)]* and *osm-5(p813).*

DISCUSSION

Several studies using vertebrate cells have shown different pathways by which GPCRs can regulate the activity of the RAS/MAPK pathway (Belcheva and Coscia, 2002; Gutkind, 2000; Hur and Kim, 2002; Lowes et al., 2002; Luttrell et al., 1997; Marinissen and Gutkind, 2001; Sugden and Clerk, 1997). However, the in vivo significance of these reported interactions has remained unclear in many cases. Using *C. elegans* as a model, we provide genetic and biochemical evidence linking the GPCR SRA-13 to the RAS/MAPK signalling pathway. The SRA-13 protein negatively regulates RAS/MAPK signalling through the GPA-5 G protein α subunit during olfaction and vulval development. The signal transduced by SRA-13 and GPA-5 directly inhibits RAS-mediated signalling at the level or upstream of MAPK. As the GPA-5 protein belongs to a novel class of Gα subunits whose downstream effectors are unknown (Jansen et al., 1999), it is difficult to postulate a specific mechanism of crosstalk.

SRA-13 and GPA-5 negatively regulate olfaction in the AWA and AWC chemosensory neurones

RAS-mediated signalling in the chemosensory AWC and AWA neurones is necessary for the response to volatile attractants (Hirotsu et al., 2000). RAS/MAPK signalling may be directly involved in controlling synaptic transmission, for example by regulating synaptic vesicle supply (Hirotsu et al., 2000), or the MAPK signal could be required to elicit a prolonged response by inducing the expression of specific genes that mediate the long-term effects necessary for chemotaxis. It is not currently known how RAS is activated in chemosensory neurones when an odorant binds to a GPCR. In particular, RAS activation in the chemosensory neurones does not appear to involve an RTK or an adaptor protein like SEM-5 GRB2 (Hirotsu et al., 2000). Instead, GPCRs may trigger through the action of heterotrimeric G proteins the influx of Ca^{2+} ions that stimulate a Ca^{2+}-activated guanine nucleotide release factors similar to mammalian RAS-GRF (Farnsworth et al., 1995).

To our knowledge, SRA-13 is the first member of the SRA family of chemosensory receptors for which an inhibitory function during olfaction has been demonstrated. The SRA-13 signal attenuates the response to volatile attractants, probably by activating a heterotrimeric G protein consisting of the GPA-5 Gα subunit. Accordingly, GPA-5 has previously been found to negatively regulate olfaction of volatile attractants (Jansen et al., 1999), while other Gα subunits, such as ODR-3, positively regulate the response to volatile attractants (Roayaie et al., 1998). Achieving the right balance between stimulatory and inhibitory G proteins may be important to set a threshold level for the detection of odorants and allow the animals to detect odorant concentration gradients. Moreover, negative regulation of RAS/MAPK signalling during olfaction may be necessary to discriminate between different odorants (Wes and Bargmann, 2001) or to adapt olfaction to changes in the environment. We do not know if SRA-13 functions as a chemosensory receptor because we have not identified its ligand(s). SRA-13 could function as a constitutive, ligand-independent inhibitor of olfaction. However, overexpression of SRA-13 induces phenotypes reminiscent of food starvation, suggesting that SRA-13 is activated when food becomes limiting. Thus, SRA-13 may suppress the olfaction of certain odorants under adverse growth conditions.

SRA-13 and GPA-5 negatively regulate vulval induction

During vulval induction, the activity of the RTK/RAS/MAPK pathway is tightly controlled to ensure that only one VPC (P6.p) adopts the 1° cell fate (Kornfeld, 1997; Sternberg and Han, 1998; Wang and Sternberg, 2001). For this purpose, several negative and positive regulators act at different steps of the pathway. In particular, the activity of the RAS protein LET-60 appears to be controlled by a number of factors in addition to the guanine nucleotide exchange factor SOS-1 that transduces the signal from the LET-23 RTK (Belcheva and Coscia, 2002; Chang et al., 2000; Gutkind, 2000; Lowes et al., 2002). Our observation that the GPCR SRA-13 negatively regulates RAS/MAPK signalling via a heterotrimeric G protein containing GPA-5 fits well into this picture. Moreover, a gain-of-function mutation in the $G_q\alpha$ protein EGL-30 causes excess vulval induction (N. Moghal, L. R. Garcia, L. Khan, K. Iwasaki and P. W. Sternberg, unpublished). Thus, the balance between the inhibitory SRA-13/GPA-5 signal and a stimulatory signal that is received by an as yet unidentified GPCR and transduced by EGL-30 may control RAS-mediated signalling in the VPCs.

In contrast to RAS/MAPK signalling during olfaction, the RAS/MAPK pathway in the VPCs evokes a binary all-or-none cell fate choice with a relatively high threshold level. Under normal growth conditions when food is abundant, the *sra-13(0)* mutation slightly increases the activity of the RAS/MAPK pathway, but not above the threshold required to change the normal pattern of vulval induction. For this reason, the inhibitory activity of SRA-13 and GPA-5 manifests only in a sensitized genetic background. The same observation has been made for other inhibitors of the RTK/RAS/MAPK pathway, such as *sli-1* (Jongeward et al., 1995), *gap-1* (Hajnal et al., 1997) or *lip-1* (Berset et al., 2001).

Cell-autonomous or non-autonomous function of SRA-13 during food starvation

The overexpression phenotype has suggested that SRA-13 may send an inhibitory signal during food starvation or under other unfavourable conditions. Interestingly, vulval induction is sensitive to food starvation in *sra-13(+)* animals but becomes insensitive to starvation in *sra-13(0)* animals. One model predicts that during food starvation, SRA-13 acts cell-autonomously in the VPCs to send an inhibitory signal. It is possible that SRA-13 is expressed in the VPCs at relatively low levels that were not detected with the translational *sra-13::gfp* reporter because the SRA-13 protein may be unstable in non-neuronal cells and the transcriptional reporter is expressed in muscle and hypodermal cells. Experiments using ectopic expression of SRA-13 in the VPCs (data not shown) do not prove a cell-autonomous function of *sra-13* as RAS signalling may be repressed when artificially high levels of SRA-13 are produced in the VPCs, even though endogenous SRA-13 may not act in these cells. Another equally likely possibility is that the activation of SRA-13 in the sensory neurones leads to the production of a secondary signal that globally downregulates RAS/MAPK signalling in the animal. The observation that mutations in *osm-5* and *che-3*, which perturb the function of the sensory neurones, uncouple vulval induction from food starvation similar to loss of *sra-13* function is consistent with such a cell non-autonomous model. However, the close lineage relationship between the VPCs and the chemosensory neurones make it difficult to perform a genetic mosaic analysis that could determine the cellular site of SRA-13 action during vulval development.

Finally, it should be noted that GPCRs are the targets for many (up to 60%) of the drugs currently used in medicine. Therefore, by controlling GPCR signalling it may be possible modulate the activity of the RAS/MAPK signalling pathway in order to control cellular proliferation and differentiation.

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