Molecules and Cells

Neuropeptide Signaling Regulates Pheromone-Mediated Gene Expression of a Chemoreceptor Gene in *C. elegans*

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Animals need to be able to alter their developmental and behavioral programs in response to changing environmental conditions. This developmental and behavioral plasticity is mainly mediated by changes in gene expression. The knowledge of the mechanisms by which environmental signals are transduced and integrated to modulate changes in sensory gene expression is limited. Exposure to ascaroside pheromone has been reported to alter the expression of a subset of putative G protein-coupled chemosensory receptor genes in the ASI chemosensory neurons of *C. elegans* (Kim et al., 2009; Nolan et al., 2002; Peckol et al., 1999). Here we show that ascaroside pheromone reversibly represses expression of the *str-3* chemoreceptor gene in the ASI neurons. Repression of *str-3* expression can be initiated only at the L1 stage, but expression is restored upon removal of ascarosides at any developmental stage. Pheromone receptors including SRBC-64/66 and SRG-36/37 are required for *str-3* repression. Moreover, pheromone-mediated *str-3* repression is mediated by FLP-18 neuropeptide signaling via the NPR-1 neuropeptide receptor. These results suggest that environmental signals regulate chemosensory gene expression together with internal neuropeptide signals which, in turn, modulate behavior.

Keywords: chemoreceptor, gene expression, neuropeptide signaling, pheromone, plasticity

INTRODUCTION

Proper chemosensory gene expression and its flexible modulation are essential to generate and shape behaviors of animals. A key feature of chemosensory gene expression is that it is highly dynamic and is extensively modulated by changes in external and internal conditions (Barth et al., 1996; Fox et al., 2001; Sengupta, 2013; Ryu et al., 2017). It is now well-established that plasticity of chemosensory gene expression mediates behavioral changes and thus plays a pivotal role in the ability to find food sources or avoid predators. However, the mechanisms underlying this type of gene expression plasticity are still not well-known and require further detailed genetic, physiological, and behavioral analyses.

The nematode *Caenorhabditis elegans* is an excellent model system in which to study plasticity of chemosensory gene expression. The *C. elegans* genome encodes over 1,500 predicted GTP-binding protein (G protein)-coupled receptor (GPCR) genes, most of which appear to be putative chemoreceptor genes (Robertson and Thomas, 2006; Sengupta et al., 1996; Troemel et al., 1995). Gene expression of these chemoreceptor genes appears to be plastic and can be altered by external environmental conditions and/or the internal metabolic state including the presence of pheromones, upon starvation, or by alteration of the ambient

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temperature (Gruner et al., 2014; 2016; Kim et al., 2009; Nolan et al., 2002; Peckol et al., 2001; Ryan et al., 2014; Satterlee et al., 2004; Suo et al., 2006). For example, the expression of a *srh-234* chemoreceptor gene in the ADL chemosensory neurons is down-regulated in starved animals (Gruner et al., 2014; 2016) and *odr-10* diacetyl receptor expression in the AWA chemosensory neurons is modulated by the feeding state and somatic sex (Ryan et al., 2014). However, the molecular and neuronal mechanisms underlying plasticity of chemosensory gene expression have yet to be fully determined.

C. elegans secretes a complex cocktail of small chemicals collectively called dauer pheromone (Butcher et al., 2007; Edison, 2009; Golden and Riddle, 1982; Jeong et al., 2005; Zhou et al., 2018). Distinct components of ascaroside pheromone appear to affect many aspects of *C. elegans* development and behavior (Ludewig and Schroeder, 2013). For example, at the early larval developmental stage, a set of ascaroside pheromones act as a population density indicator to determine dauer formation (Butcher et al., 2007; 2008; Cassada and Russell, 1975; Hirsh and Vanderslice, 1976; Jeong et al., 2005). In addition, ascarosides elicit acute behavioral changes (Greene et al., 2016; Jang et al., 2012; Park et al., 2017; Srinivasan et al., 2008; 2012). For example, acute exposure to ascr#3 (asc-ΔC9, C9 ascaroside, daumone-3) causes an avoidance behavior in adult animals, which is further modulated by previous experience and feeding state (Hong et al., 2017; Jang et al., 2012; Ryu et al., 2018). It was previously shown that chronic exposure to ascaroside pheromone down-regulates expression of putative G protein-coupled chemosensory receptor genes including *str-3* in the chemosensory ASI neurons (Kim et al., 2009; Neal et al., 2016; Nolan et al., 2002; Peckol et al., 2001). Here, we attempted to further investigate conditions in which *str-3* expression is affected. We found that *str-3* expression is repressed by the presence of ascaroside but not by the feeding state or ambient temperature. Pheromone exposure at the L1 larval stage was required for repression of *str-3* GPCR expression, which could be de-repressed at any developmental stage when the pheromone was removed. Moreover, the down regulation of *str-3* expression upon pheromone exposure was dependent on FLP-18 neuropeptide and its receptor, NPR-1. This study hence indicates that chemoreceptor expression in these chemosensory neurons is modulated by secreted pheromone cues that may reflect the internal metabolic and physiological conditions of the worms and is further influenced by endogenous neuropeptide signaling pathways.

MATERIALS AND METHODS

Strains and Genetics

The *C. elegans* N2 strain was used as wild-type. All strains were maintained at 20℃ on *Escherichia coli* OP50-seeded NGM plates. The mutants and transgenic strains used in this study included: CX3596 *kyIs128[str-3*p*::gfp]* X, KHK742 *srbc-64(tm1946); srbc-66(tm2943); kyIs128[str-3*p*::gfp]* X, KHK787 *srg-36 srg-37(kyIR88); kyIs128[str-3*p*::gfp]* X, KHK487 *flp-18(tm2179); kyIs128[str-3*p*::gfp]* X, KHK1355 *npr-1(ad609); kyIs128[str-3*p*::gfp]* X, KHK488 *unc-31(e169); kyIs128[str-3*p*::gfp]* X, KHK 485 *npr-4(tm1782); kyIs128[str-3*p*::gfp]* X, KHK486 *npr-5(rb1393); kyIs128[str-3*p*::gfp]* X, and KHK1763 *flp-18(tm2179);npr-1(ad609); kyIs128[str-3*p*::gfp]* X.

Crude pheromone and synthetic ascarosides

Crude pheromone was prepared following the protocol described in by Golden and Riddle (1984). The ascaroside pheromone components including ascr#2 (asc-C6-MK, C6 ascaroside, daumone-2), ascr#3 (asc-ΔC9, C9 ascaroside, daumone-3) and ascr#5 (asc-ωC3, C3 ascaroside), were chemically synthesized according to Butcher et al. (2007; 2008). Before use, pheromone was diluted with dH_2O from 3 mM stock solution of pheromone in 100% ethanol.

Preparation of the *str-3* repression assay plates

Crude pheromone plates were prepared by spreading 20 μl of diluted crude pheromone onto the assay plates, which were then incubated at 25℃ for 3-4 h. The synthetic pheromone plates contained 1 μM ascr#2, ascr#3, and ascr#5 ascarosides. Aliquots (50 μg) of live *E. coli* OP50 or heat-killed *E. coli* OP50 were then seeded on the plates and dried in a hood overnight or for 3-4 h, respectively, prior to the assay. Well-fed 5-10 adults were placed on the plates and discarded when 60-80 eggs were obtained. The eggs were grown at 25℃ until worms developed at each developmental stage.

Quantification of *str-3* expression levels

For the GFP quantification of *str-3* expression, the worms were anesthetized in 0.5 M or 1 M sodium azide (NaN_3) on an agar pad, and the GFP fluorescence was observed with a Zeiss Axio Imager using 40x (for adult stage) and 63x (for L1) objectives and a CCD camera (Hamamatsu). The relative expression level of *str-3*p*::gfp* was measured at each developmental stage. The relative GFP levels of *str-3* in the ASI sensory neurons were rated from 1 (dim) to 5 (bright) by the naked eye, and these values were confirmed using image J software.

Molecular biology

Genomic regions of the *flp-18* gene were amplified by nested PCR and sequenced. The PCR products were then directly injected at a concentration of 10 ng/μl or 50 ng/μl with 50 ng/μl *unc-122*p*::dsRed* as a co-injection marker. The outer forward primer was tggatgcgtcaaatgttgtg, the outer reverse primer was gtcagtttgttccagtatccttc, the inner forward primer was ccactccgaaacatacggtac, and the inner reverse primer was cctgacagtcatcacatcacc.

RESULTS AND DISCUSSION

Pheromone-mediated *str-3* repression in the ASI chemosensory neurons was imposed from egg to the L1 larval stage

To investigate the effects of dauer pheromones on *str-3* repression at the different developmental stages, we first exposed worms expressing the *str-3*p*::gfp* transgene to crude

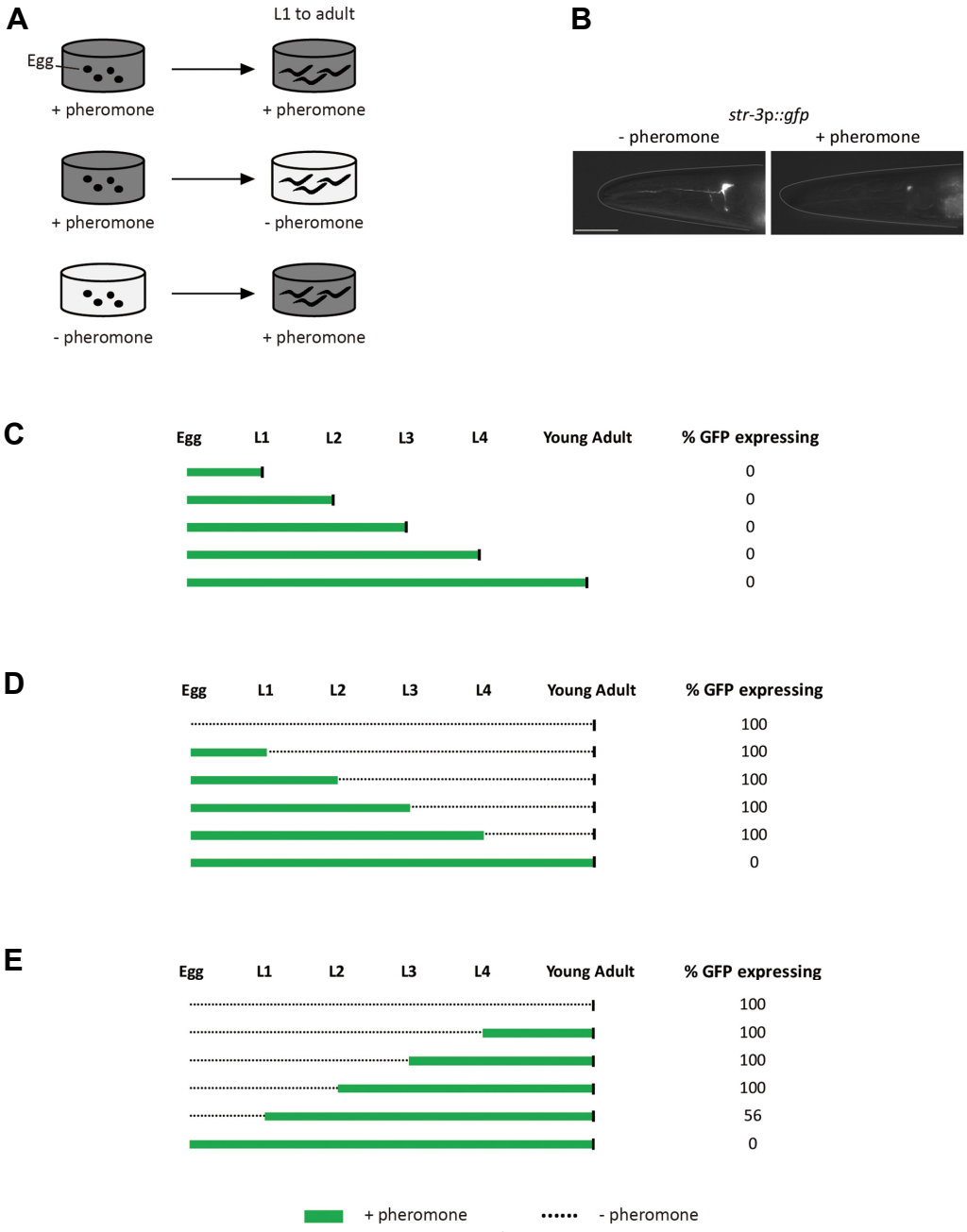

Fig. 1. *str-3* repression upon crude pheromone exposure is imposed from egg to the L1 stage. (A) Experimental diagram of *str-3* repression by crude pheromone exposure at each developmental stage. (B) Fluorescence images of GFP in the ASI neurons of *str-3*p::*gfp* transgenic animals taken at the adult stage in the absence or presence of crude pheromone. (C) Relative percentage of *str-3*p::*gfp* expression in the ASI neurons of animals at each developmental stage after exposure to crude pheromone during the indicated stages of development. (D-E) Relative percentage of *str-3*p::*gfp* expression in the ASI neurons of young adult animals that were exposed to crude pheromone during the indicated stages of development. (C-E) Black vertical bars indicate time of observation. n ≥ 30 for each. The scale bar represents 10 μm.

pheromone from egg to each developmental stage. We then observed expression of the *green fluorescent protein* (*gfp*) in the ASI neurons by imaging of the GFP with a dissection microscope equipped for epifluorescence detection (Fig. 1A). Previously, down-regulated expression of *gfp* reporter gene under the control of *str-3* promoter was validated via quantitative RT-PCR data in which endogenous *str-3* message levels were also decreased upon addition of crude pheromone in a dose-dependent manner (Kim et al., 2009). We found that crude pheromone strongly down-regulated

*str-3*p::*gfp* expression in the ASI neurons when animals were exposed to pheromone from egg to each developmental stage (Figs. 1B and 1C). We next removed the pheromone by transferring *str-3*p::*gfp*-repressed worms at each developmental stage onto plates seeded with 50 μg of live *E. coli* OP50 that did not contain pheromone, and then examined *gfp* expression as adults (Fig. 1A). *str-3*p::*gfp* expression was de-repressed, resulting in *str-3*p::*gfp* expression levels that were comparable to those of worms that had not been pre-exposed to pheromone (Fig. 1D). We then tested the critical period for pheromone-mediated *str-3* repression by exposing worms to crude pheromone starting from each develop-

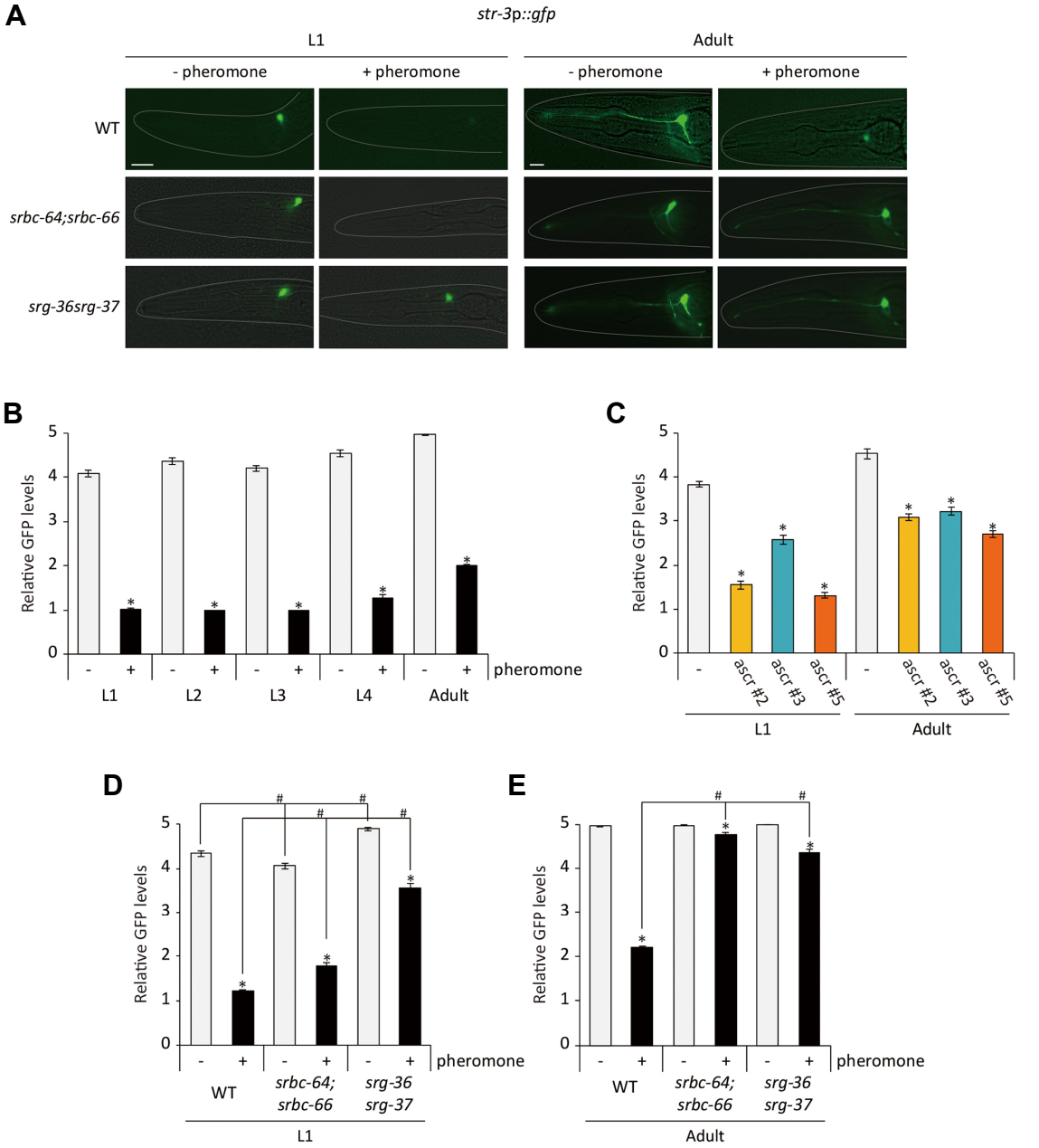

Fig. 2. Exposure to ascaroside pheromone components causes *str-3* repression via pheromone receptors in L1 larvae and adults. (A) Representative images of *srbc-64;srbc-66* and *srg-36 srg-37* double mutants expressing the *str-3*p::*gfp* transgene in the ASI neurons at the L1 (left panels) and adult stage (right panels) upon exposure mixtures of 1 μM ascr#2, ascr#3, and ascr#5. (B-C) Relative GFP levels are shown upon exposure to mixtures of 1 μM ascr#2, ascr#3, and ascr#5 (B) or individual 1 μM ascarosides (C). (D-E) Relative GFP levels of *str-3*p::*gfp* expression in *srbc-64;srbc-66* and *srg-36 srg-37* double mutants at the L1 (D) and adult stage (E) upon 1 μM ascaroside pheromone mixtures. n ≥ 30 for each. The scale bar represents 10 μm. Error bars represent the SEM. * and # indicate different from the controls (absence of pheromone and wild-type animals, respectively) at $P < 0.001$ (*Bonferroni* t-test).

mental stage through the young adult stage (Fig. 1A). We found that *gfp* expression in the ASI neurons was repressed only when the worms were exposed starting from the first L1 stage through the adult stage (Fig. 1E). *gfp* expression was not fully repressed in animals that were exposed to pheromone after the second L2 stage (Fig. 1E). Taken together, these results suggest that *str-3* expression can be repressed by pheromone in the developmental window of egg to L1 and de-repressed at any developmental stages.

Crude pheromone contains ascaroside pheromone components including ascr#2, ascr#3, and ascr#5 (Supplementary Fig. S1)(Butcher et al., 2007; 2008). We previously showed that these chemically synthesized ascaroside pheromone components down-regulated *str-3* expression in the ASI neurons when worms were exposed to each of these pheromone components from egg to the adult stage (Kim et al., 2009). We next determined whether these pheromone components could repress *str-3*p::*gfp* expression at the different developmental stages. Similar to crude pheromone, mixtures of ascr#2, ascr#3, and ascr#5 down-regulated *str-3*p::*gfp* expression in the ASI neurons when animals were exposed to pheromone mixtures from egg to the each developmental stage in a dose-dependent manner (Figs. 2A and 2B, Supplementary Fig. S2)(Kim et al., 2009). Moreover, similar to crude pheromone, each pheromone component significantly repressed *str-3* expression when animals were exposed from egg to the L1 stage or the adult stage (Fig. 2C)(Kim et al., 2009). We noted that the GFP levels in the adults were higher than those in the L1 worms in the presence or absence of pheromone (Fig. 2C). Taken together, these results indicate that repression of *str-3* in the ASI neurons requires early exposure to pheromone components.

Mutations in the pheromone receptor genes abolished pheromone-mediated regulation of *str-3* gene expression

It was previously shown that the SRBC-64 and SRBC-66 GPCRs and the SRG-36 and SRG-37 GPCRs mediate developmental roles of ascr#2/#3 and ascr#5, respectively (Kim et al., 2009; McGrath et al., 2011). Moreover, mutations in the *srbc-64* and *srbc-66* genes significantly suppressed pheromone-mediate down-regulation of *str-3* expression in the ASI neurons when worms were exposed to 3 μM of each synthetic pheromone component from egg to adults (Kim et al., 2009). We found that mixtures of 1 μM ascr#2, ascr#3, and ascr#5 were unable to repress *str-3* expression in the ASI neurons of *srbc-64 (tm1946);srbc-66 (tm2943)* double mutants, and the defects in *str-3* repression were more robust in adults than in L1 larvae of *srbc-64;srbc-66* double mutants (Figs. 2A, 2D and 2E).

We next examined pheromone-mediated *str-3* regulation in *srg-36 srg-37* (*kyIR88*) double mutants. Similar to *srbc-64;srbc-66* double mutants, mixtures of 1 μM ascr#2, ascr#3, and ascr#5 did not repress *str-3* expression in the ASI neurons of *srg-36 srg-37* mutants, and these defects were also more robust in adults (Figs. 2A, 2D and 2E). These results support the notion that pheromone signals are transmitted to repress *str-3* expression in the ASI neurons via *srbc-64*, *srbc-66*, *srg-36*, and *srg-37* pheromone receptors.

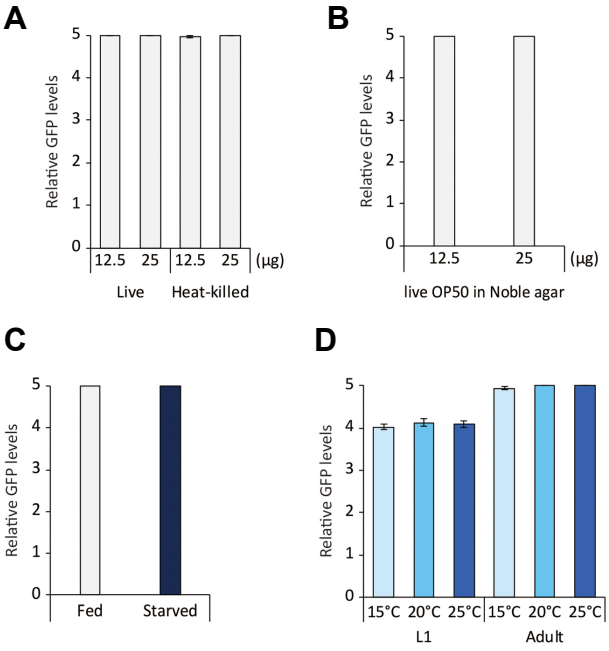

Fig. 3. *str-3* expression is not modulated by the quality or the quantity of food or ambient temperature. (A) Relative GFP levels of *str-3*p::*gfp* expression upon exposure to 12.5 μg or 25 μg live or heat-killed OP50. (B) Relative GFP levels of *str-3*p::*gfp* expression upon exposure to 12.5 μg or 25 μg live OP50 cultivated on noble agar plates. (C) Relative GFP levels of *str-3*p::*gfp* expression after starvation for 24 h. (D) Relative GFP levels of *str-3*p::*gfp* expression following cultivation at 15℃, 20℃, or 25℃ at the L1 or adult stage. n≥ 30 for each. Error bars represent the SEM.

Food availability, internal feeding state, and ambient temperature did not affect *str-3* expression

We next sought to further define conditions that affect *str-3* expression. We first tested whether food quantity and/or quality could influence *str-3* expression. Decreasing the amount of live OP50 from 50 μg to either 25 μg or 12.5 μg did not affect the level of *str-3* expression (Fig. 3A). We next incubated *str-3*p::*gfp* transgenic worms on plates seeded with heat-killed OP50, which represents low-quality food used in dauer inducing conditions (Jeong et al., 2005). We found that *str-3* expression was not affected by exposure to low-quality food (Fig. 3A). Incubation of *str-3*p::*gfp* transgenic worms on peptone-free noble agar plates, in which growth of OP50 is limited (Hosono et al., 1989), did not change the level of *str-3*p::*gfp* expression (Fig. 3B). These results imply that *str-3* expression is not regulated by food availability.

To investigate whether *str-3* expression is affected by the feeding state, we either fed or starved young adult *str-3*p::*gfp* transgenic animals for 24 h. We found that chronic starvation for 24 h did not alter the level of *str-3* expression (Fig. 3C), indicating that the internal feeding state does not play a role in *str-3* expression.

We next examined whether the cultivation temperature

affected *str-3* expression by incubating the *str-3*p::*gfp* transgenic worms at 15℃, 20℃, and 25℃. These different cultivation temperatures did not affect the level of *str-3* expression (Fig. 3D). Taken together, these results suggest that *str-3* expression is regulated by pheromone exposure but not by changes in the feeding status or the ambient temperature.

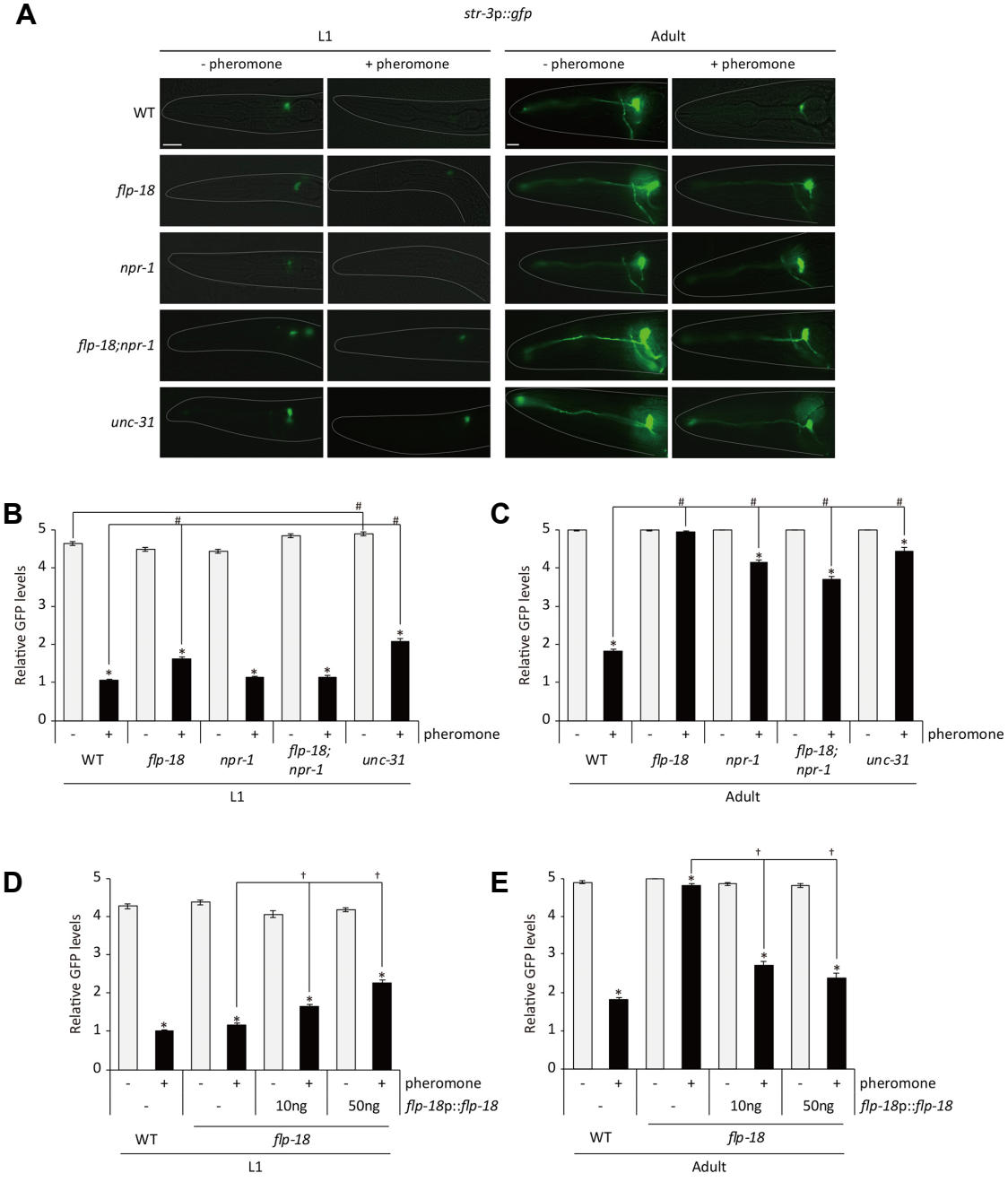

Fig. 4. FLP-18 neuropeptide and NPR-1 neuropeptide receptor are required for pheromone-mediated *str-3* repression. (A) Representative images of *flp-18, npr-1,* and *flp-18;npr-1* mutants expressing the *str-3*p::*gfp* transgene in the ASI neurons at the L1 (left two panels) and adult stage (right two panels) upon exposure to mixtures of 1 μM ascaroside pheromone. (B-C) Relative GFP levels of *str-3*p::*gfp* expression in *flp-18, npr-1,* and *flp-18;npr-1* mutants at the L1 (B) and adult stage (C) upon exposure to mixtures of 1 μM ascaroside pheromone. (D-E) Relative GFP levels of *str-3*p::*gfp* expression in *flp-18* mutants expressing *flp-18* genomic DNA driven by its own promoter upon exposure to mixtures of 1 μM ascaroside pheromone. n≥ 30 for each. Error bars represent the SEM. *, #, and † indicate different from the controls (absence of pheromone, wild-type animals, and no transgene, respectively) at $P < 0.001$ (*Bonferroni t*-test). The scale bar represents 10 μm.

FLP-18 neuropeptide signaling was required for pheromone-mediated regulation of *str-3* gene expression via a NPR-1 neuropeptide receptor

We next performed a candidate mutant screen to identify genes required for pheromone-mediated *str-3* repression. First, we found that *unc-31* mutants exhibited defects in *str-3* repression, resulting in *str-3*p::*gfp* still being strongly expressed in *unc-31* (*e169*) mutants grown on plates containing mixtures of ascr#2, ascr#3, and ascr#5 (Figs. 4A-4C). The defect of *unc-31* mutants was severe in adults than in L1 larvae (Figs. 4A-4C). The *unc-31* gene encodes a calcium-dependent activator protein (CAPS) that is required for dense-core vesicle exocytosis (Sieburth et al., 2007; Speese et al., 2007). These results suggest that neuropeptide signaling plays a role in pheromone-mediated *str-3* repression.

As it has been reported that a *flp-18* FMRFamide neuropeptide gene regulates dauer formation (Cohen et al., 2009), we examined pheromone-mediated *str-3* repression in *flp-18* (*tm2179*) null mutants. Similar to what we found with *unc-31* mutants, pheromone did not repress *str-3* expression in adult *flp-18* mutants (Figs. 4A-4C). To confirm that defects in *flp-18* mutants were caused by loss-of-function mutation of the *flp-18* gene, we expressed *flp-18* genomic DNA under the control of the *flp-18* promoter in a *flp-18* mutant background. The expression of *flp-18* genomic DNA rescued the defects of adult *flp-18* mutants (Figs. 4D and 4E). These results indicate that *flp-18* neuropeptide signaling mediates pheromone-mediated *str-3* repression.

FLP-18 neuropeptides play various physiological and developmental roles via neuropeptide receptors including NPR-1, NPR-4, and NPR-5 (Cohen et al., 2009; Rogers et al., 2003). We next asked which neuropeptide receptors couple to FLP-18 neuropeptide to mediate pheromone-mediated *str-3* repression. We found that *str-3* expression was not down-regulated upon pheromone exposure in *npr-1* (*ad609*) mutants (Figs. 4A-4C). Although *npr-5* mutants exhibited defects in dauer formation (Cohen et al., 2009), *str-3* repression was either weakly or not affected in *npr-4* (*tm1782*) or *npr-5* (*rb1393*) mutants (Supplementary Fig. S3). Taken together, FLP-18 mediates *str-3* repression at least partially via the NPR-1 neuropeptide receptor but not the NPR-4 and NPR-5 receptors.

Concluding remarks

In this study, we further analyzed conditions in which expression of a chemoreceptor *str-3* gene is modulated via pheromone together with FLP-18 neuropeptide signaling. Since *srbc-64/66* pheromone receptors act in the ASK chemosensory neurons to detect ascr#2 and ascr#3 (Kim et al., 2009) and *str-3* is expressed in the ASI chemosensory neurons, FLP-18 could transmit signals from the ASK to the ASI neurons to regulate *str-3* expression. However, *flp-18* is not expressed in the ASK and other chemosensory neurons (Rogers et al., 2003), suggesting that FLP-18 play a different role in pheromone-mediated *str-3* expression. Since expression of several chemoreceptor genes including *str-3* is altered in the presence of pheromone, it is plausible that pheromone could eventually change chemosensory behavior(s) which the chemoreceptors play roles in. We examined attraction behavior to odorants including benzaldehyde and diacetyl (Bargmann et al., 1993) and found that compared to control animals, pheromone-treated animals did not exhibit altered chemotactic behavior to these odorants (Supplementary Fig. S4). These results indicate that chemosensory behaviors are not broadly affected by addition of pheromone but *str-3*-mediated chemosensory behavior(s) may be modulated in the presence of pheromone. Investigating site of FLP-18 action and identifying *str-3* gene function would be the next step to understand this pheromone-mediated gene expression plasticity.

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Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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