Research

Gustatory plasticity in *C. elegans* involves integration of negative cues and NaCl taste mediated by serotonin, dopamine, and glutamate

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While naïve *Caenorhabditis elegans* individuals are attracted to 0.1–200 mM NaCl, they become strongly repelled by these NaCl concentrations after prolonged exposure to 100 mM NaCl. We call this behavior gustatory plasticity. Here, we show that *C. elegans* displays avoidance of low NaCl concentrations only when pre-exposure to NaCl is combined with a negative stimulus, e.g., a repellent, or in the absence of food. By testing serotonin and/or dopamine signaling mutants and rescue by exogenously supplying these neurotransmitters, we found that serotonin and dopamine play a role during the plasticity response, while serotonin is also required during development. In addition, we also show that glutamate plays an important role in the response to NaCl, both in chemoattraction to NaCl and in gustatory plasticity. Thus, *C. elegans* can associate NaCl with negative stimuli using dopaminergic, serotonergic, and glutamatergic neurotransmission. Finally, we show that prolonged starvation enhances gustatory plasticity and can induce avoidance of NaCl in most gustatory plasticity mutants tested. Only mutation of the glutamate-gated Cl[−] channel gene *avr-15* affected starvation-enhanced gustatory plasticity. These results suggest that starvation induces avoidance of NaCl largely independent of the normal gustatory plasticity mechanism.

Salts can signal important cues such as food or osmotic stress. Therefore, sensation of salts is essential for the survival of organisms. However, since environmental conditions can change rapidly, organisms must be able to modulate their response to salt. We study the response to NaCl in the nematode *Caenorhabditis elegans*. *C. elegans* is attracted to 0.1–200 mM NaCl (Ward 1973; Dusenbery 1974) but avoids higher NaCl concentrations (Culotti and Russell 1978). Chemoattraction to NaCl is mediated mainly by one pair of sensory neurons, the ASE neurons (Bargmann and Horvitz 1991), and uses cGMP and Ca²⁺ signaling (Coburn and Bargmann 1996; Komatsu et al. 1996; Kuhara et al. 2002; Hukema et al. 2006). Avoidance of high NaCl concentrations is mediated by the ASH neurons (Bargmann and Horvitz 1991) and requires G protein signaling, TRPV channels, and glutamatergic neurotransmission (Colbert et al. 1997; Berger et al. 1998; Roayaie et al. 1998; Hart et al. 1999; Mellem et al. 2002; Tobin et al. 2002; Fukuto et al. 2004).

Depending on previous experiences, *C. elegans* can modulate its response to salt. We call this gustatory plasticity: The reduced attraction or even avoidance of otherwise attractive NaCl concentrations after pre-exposure to 100 mM NaCl in buffer without food (Jansen et al. 2002; Hukema et al. 2006). This behavior involves signals from at least four pairs of sensory neurons, the ASE, ADF, ASH, and ASI neurons (Hukema et al. 2006). We have identified many genes that function in gustatory plasticity, some of which have been arranged in a genetic pathway, including the TRPV channel subunit *osm-9*, the G protein subunits *gpa-1* and *gpc-1*, the guanylate cyclase *gcy-35*, the arrestin *arr-1*, and the fatty acid desaturase *fat-4* (Hukema et al. 2006). We hypothesize that upon prolonged exposure to NaCl, signals from the ASE neurons sensitize avoidance mediated by one or more of the other sensory neurons, resulting in reduced attraction or even avoidance of otherwise attractive NaCl concentrations.

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A similar salt-plasticity assay has been reported, where animals avoid NaCl after 1–4 h starvation in the presence of 20–50 mM NaCl. This behavior, called “salt chemotaxis learning,” involves signals from the ASE neurons and the AIY and AIA interneurons (Saeki et al. 2001; Ishihara et al. 2002; Tomioka et al. 2006). It is unclear to what extent gustatory plasticity and salt chemotaxis learning overlap.

Our previous findings suggested that gustatory plasticity is precisely regulated. However, it is not yet clear, which environmental cues cause the change from attraction to avoidance, and whether starvation is important. In addition, it remains unknown which neurotransmitters are used. Resolving these issues would provide an important step toward unraveling the cellular circuitry and molecular mechanisms of gustatory plasticity. Here, we show that *C. elegans* avoids low NaCl concentrations only when pre-exposure to NaCl is combined with a negative stimulus, e.g., a repellent, or in the absence of food. In addition, we show that the neurotransmitters dopamine, serotonin, and glutamate are required for gustatory plasticity, and glutamate is also important for attraction to NaCl. Furthermore, prolonged starvation enhances gustatory plasticity and can induce avoidance of NaCl in most gustatory plasticity mutants tested. Only the *avr-15* glutamate receptor mutant affected starvation-enhanced gustatory plasticity.

Results

Gustatory plasticity involves integration of negative cues and NaCl taste

Since pre-exposure results in avoidance rather than reduced attraction, gustatory plasticity probably involves more than only desensitization of attraction and might represent a simple form of associative learning. Previously, we have shown that gustatory plasticity depends on the NaCl concentration during pre-exposure and the duration of pre-exposure. Robust responses,

and no obvious toxicity, were obtained after 15 min of pre-exposure to 100 mM NaCl in a buffer containing no food (Jansen et al. 2002). We wondered whether this response involves additional cues other than NaCl. Both the absence and the presence of food have previously been implicated in behavioral plasticity of *C. elegans* (Colbert and Bargmann 1997; Sawin et al. 2000; Saeki et al. 2001; Nuttley et al. 2002; Chao et al. 2004; Mohri et al. 2005; Kindt et al. 2007). To test if food cues are important in gustatory plasticity, we changed our assay so that animals were pre-exposed to NaCl on agar plates instead of in liquid. After 15, 30, 45, or 60 min of pre-exposure on plates, the response to 25 mM NaCl was tested. Thirty minutes of pre-exposure gave the most reproducible response and was used in all on-plate pre-exposure experiments. Similar to animals pre-exposed in liquid, wild-type animals pre-exposed to 100 mM NaCl on plates, in the absence of food, avoided 25 mM NaCl, while naïve animals were attracted to NaCl (Fig. 1A). In addition, *gpc-1(pk298)* and *odr-3(n1605)* mutant animals, previously shown to have defects in gustatory plasticity (Jansen et al. 2002; Hukema et al. 2006), showed similar defects when pre-exposure was performed on plates (Fig. 1B,C). This alternative method of pre-exposing animals allowed us to test if food signals are important in gustatory plasticity. Interestingly, wild-type animals pre-exposed to 100 mM NaCl in the presence of OP50 bacteria were strongly attracted to 25 mM NaCl, comparable to naïve animals (Fig. 1A). These results suggest that *C. elegans* can associate NaCl with the absence of food, resulting in avoidance of low NaCl concentrations.

To determine if *C. elegans* can also associate NaCl with other negative cues, we tested if avoidance of NaCl can be induced in the presence of food by adding repellents during pre-exposure to 100 mM NaCl. Pre-exposure of wild-type animals in the presence of 500 mM glycerol (osmotic avoidance cue) induced strong avoidance of 25 mM NaCl, while pre-exposure to 500 mM glycerol in the absence of NaCl did not affect chemotaxis to 25 mM NaCl (Fig. 1A). In addition, pre-exposure to 500 mM glycerol in the presence of NaCl did not affect chemotaxis to diacetyl, an unrelated cue (results not shown). Finally, pre-exposure to 700 mM glycerol, in the absence of NaCl, did not affect chemotaxis to 25 mM NaCl (results not shown). Also, pre-exposure to 100 mM NaCl in the presence of 3 μ L of undiluted benzaldehyde induced strong avoidance of 25 mM NaCl. However, this treatment was toxic to the animals and also reduced chemotaxis to NaCl or

diacetyl by naïve animals, although it did not result in avoidance of 25 mM NaCl (Fig. 1A; results not shown). The G γ subunit *gpc-1* is also required for this form of behavioral plasticity (Fig. 1B). *odr-3* mutants have not been tested, since these animals also have defects in osmotic and odorant avoidance (Roayaie et al. 1998). Since both avoidance of glycerol (osmotic avoidance) (Culotti and Russell 1978; Bargmann and Horvitz 1991) and benzaldehyde (Troemel et al. 1997) are mediated by the ASH nociceptive neurons, our results suggest that *C. elegans* can integrate environmental cues mediated by different sensory neurons and change its behavior accordingly.

Prolonged starvation enhances gustatory plasticity

Our results indicate that gustatory plasticity involves the association of negative cues, such as the absence of food or nociceptive stimuli, to NaCl, resulting in avoidance of otherwise attractive NaCl concentrations. Similar results have been presented by Saeki et al. (2001) and Tomioka et al. (2006), who observed avoidance of normally attractive NaCl concentrations after 1–4 h of starvation in the presence of 50 mM NaCl, but attraction to NaCl when animals were pre-exposed in the presence of food. A major difference between the two assays is the pre-exposure time: 1–4 h in the Saeki protocol versus 15 min in our protocol. Since the presence of food influences gustatory plasticity, it seems likely also that prolonged starvation of animals could modulate gustatory plasticity. Therefore, we starved animals up to 4 h on agar plates before testing them in the (liquid) plasticity assay. Fifteen minutes of starvation before the assay increased avoidance after pre-exposure, although not significantly. Longer starvation resulted in significantly stronger avoidance (Fig. 2). We call this behavior starvation-enhanced gustatory plasticity. Starvation did not affect naïve chemotaxis to NaCl (Fig. 2).

Subsequently, we tested the known gustatory plasticity mutant animals *gpc-1* and *odr-3* for starvation-enhanced gustatory plasticity behavior (Jansen et al. 2002; Hukema et al. 2006). Both *gpc-1* and *odr-3* mutant animals showed clear defects in gustatory plasticity, but starvation before the assay strongly enhanced avoidance after pre-exposure (Fig. 2). *gpc-1* animals showed almost wild-type levels of avoidance already after 15 min starvation, while *odr-3* animals required 4 h of starvation. These results indicate that neither *gpc-1* nor *odr-3* mediate the starvation signal. In addition, these results show that neither *gpc-1* nor *odr-3* are required for strong avoidance of low salt concentrations per

Figure 1. Starvation is required for gustatory plasticity. Responses of wild-type (A), *gpc-1* (B), and *odr-3* (C) animals to 25 mM NaCl after various pre-treatments: a 15-min wash in CTX buffer with (gray bars) or without (open bars) 100 mM NaCl (liquid), or 30 min on a CTX plate (on plate) with (gray bars) or without (open bars) 100 mM NaCl, and in the presence or absence of bacteria (food), 500 mM glycerol, or 3 μ L of benzaldehyde (benz). (A) Wild-type animals avoid 25 mM NaCl after pre-exposure to 100 mM NaCl on plates in the absence of food, but not in the presence of food, and they avoid 25 mM NaCl when pre-exposure to NaCl in the presence of food is combined with glycerol or benzaldehyde. (B) *gpc-1(pk298)* animals show a similar gustatory plasticity defect when pre-exposed to NaCl on plates, as in liquid. Conditioning with glycerol or benzaldehyde does not induce avoidance of NaCl. (C) *odr-3(n1605)* animals show no gustatory plasticity. Indicated are the averages of at least four assays $\pm$ SEM. Significance (compared with the response after pre-treatment under the same conditions, but in the absence of NaCl) is indicated: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.005$, (****) $P < 0.001$.

Figure 2. Starvation enhances gustatory plasticity. Responses of wild-type (black and gray lines), *gpc-1* (small dashes), and *odr-3* (large dashes) animals to 25 mM NaCl after 15 min of pre-exposure to 100 mM NaCl in CTX buffer. Before the assays, animals were starved for 0–240 min on CTX plates. Avoidance behavior of wild-type animals was significantly enhanced after 30–240 min of starvation. Avoidance behavior of *gpc-1* and *odr-3* animals was significantly enhanced already after 15 min of starvation. Starvation did not affect chemotaxis of naive animals (shown only for wild-type animals, black line). Indicated are the averages of at least four assays $\pm$ SEM. Significance (compared with 0 min of starvation) is indicated: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.005$, (****) $P < 0.001$.

se, indicating that starvation induces a *gpc-1*- and *odr-3*-independent mechanism that induces avoidance after pre-exposure.

Serotonin is required for gustatory plasticity

Serotonin plays an important role in many plastic behaviors of *C. elegans* that are modulated by starvation or food signals (Colbert and Bargmann 1997; Sawin et al. 2000; Nuttley et al. 2002; Chao et al. 2004; Mohri et al. 2005). To test if serotonin also plays a role in gustatory plasticity, we analyzed the behavior of mutant animals that have defects in the synthesis of serotonin or its transport in the nerve terminal: *tph-1(mg280)*, *bas-1(ad446)* and *(tm351)*, *cat-4(e1141)* and *(ok342)*, and *cat-1(e1111)* and *(ok411)* mutant animals. Mutation of *tph-1* disrupts serotonin synthesis, while mutation of *bas-1*, *cat-4*, and *cat-1* affects both serotonin and dopamine levels (Duerr et al. 1999; Sawin et al. 2000; Sze et al. 2000). Gustatory plasticity was strongly affected in all four mutants (Fig. 3A), suggesting that serotonin plays a role in gustatory plasticity. In addition, *tph-1* animals showed significantly stronger chemotaxis to 1 mM NaCl than wild-type animals (Fig. 3B). The *bas-1* and *cat-4* mutants, but not the *cat-1(e1111)* animals, also showed slightly, but not significantly, increased chemotaxis to NaCl (results not shown). These results suggest that serotonin is involved in the response of *C. elegans* to NaCl, and may affect the sensitivity to NaCl.

To determine when serotonin is required to allow gustatory plasticity, we tested if exogenous serotonin could restore gustatory plasticity of *bas-1* mutant animals. We performed a time series in which *bas-1(ad446)* animals were cultured on plates containing 2 mM seroto-

nin. Exposure to exogenous serotonin did not significantly affect the naïve responses of these mutants to 25 mM NaCl or the behavior of wild-type animals (Fig. 3C; results not shown). Incubation up to 144 h, i.e., including the parental generation, was needed to partially restore gustatory plasticity in *bas-1* mutant animals (Fig. 3C). This result suggests that serotonin is required during embryonic and/or larval development to allow gustatory plasticity. Kindt et al. (2002) have shown that serotonin functions in promoting directed neuronal migration in the developing nervous system. We hypothesize that serotonin is required for the proper development of neurons involved in gustatory plasticity. To determine if serotonin is also acutely needed in the process of gustatory plasticity, we exposed *bas-1* mutant animals to serotonin for 144 h, but not for the last 4 h before the behavioral assays. This treatment did not restore gustatory plasticity, suggesting that serotonin is acutely needed during gustatory plasticity. In addition, withdrawal of excessive serotonin in the last 4 h prior to the assay significantly reduced avoidance after pre-exposure in wild-type animals (Fig. 3C), confirming that serotonin levels are important for gustatory plasticity. On the other hand, this effect of serotonin on the wild-type animals leaves the effect on *bas-1* animals less conclusive. Thus, our results suggest that serotonin is required during development to allow gustatory plasticity, and also plays a role acutely during gustatory plasticity.

Serotonin levels are regulated not only by de novo synthesis, but also by reuptake from the synaptic cleft. Therefore, we tested animals with mutations in the reuptake transporter *mod-5(n822)* and *(n3314)* (Ranganathan et al. 2001). Behavioral phenotypes of *mod-5* mutant animals suggest that these animals are

Figure 3. Serotonin is required for gustatory plasticity. (A) Responses of serotonin synthesis or signaling mutants to 25 mM NaCl, after a 15-min wash in CTX buffer with (gray or black bars) or without (open bars) 100 mM NaCl. (Black bars) Responses after 4 h of starvation prior to the assay. All mutants showed defects in gustatory plasticity, but strong avoidance after starvation. (B) Responses of naive wild-type and *tph-1* animals to 1–100 mM NaCl. Chemotaxis is slightly enhanced in *tph-1* mutants. (C) Responses of wild-type and *bas-1* animals to 25 mM NaCl after a 15-min wash in CTX buffer with (gray bars) or without (open bars) 100 mM NaCl, after 4–144 h of culture on plates containing 2 mM serotonin (5-HT). Gustatory plasticity could be partially restored in *bas-1* animals by exogenous serotonin for 144 h, but not when the animals were transferred to non-serotonin-containing plates for the last 4 h before the assays (144 – 4). Indicated are the averages of at least four assays $\pm$ SEM. Significance (A and B compared with the response of wild-type animals under the same conditions; C compared with *bas-1* animals not cultured on serotonin, or as indicated): (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.005$, (****) $P < 0.001$.

hypersensitive to serotonin, because it is not cleared from the synaptic cleft (Ranganathan et al. 2001). *mod-5* mutant animals showed wild-type chemotaxis to NaCl, but defects in gustatory plasticity (Fig. 3A), suggesting that not only reduced levels of serotonin, but also elevated levels of serotonin affect gustatory plasticity.

To allow identification of possible target cells of serotonin signaling, we tested several serotonin receptor mutants. The *C. elegans* genome contains three to eight serotonin G-protein-coupled receptors (GPCRs) (Carre-Pierrat et al. 2006) and at least one serotonin gated Cl^- channel, *mod-1* (Ranganathan et al. 2000). We tested the behavior of animals that carry mutations in the GPCRs *ser-1(ok345)* (Olde and McCombie 1997) and *ser-4(ok512)* (Hamdan et al. 1999), and in *mod-1(ok103)*. We observed defects in *mod-1* and *ser-1* mutant animals in avoidance after pre-exposure (Fig. 3A). *ser-4* animals showed wild-type responses (results not shown).

Dopamine is involved in gustatory plasticity

Dopamine plays an important role in behavioral plasticity in *C. elegans* (Sawin et al. 2000; Bettinger and McIntire 2004; Sanyal et al. 2004; Kindt et al. 2007). In addition, *bas-1*, *cat-1*, and *cat-4* mutant animals not only lack serotonin, but also have reduced dopamine levels. To determine if dopamine is involved in gustatory plasticity, we tested *cat-2(e1112)* mutant animals, which have reduced dopamine levels but normal serotonin levels (Lints and Emmons 1999). *cat-2* mutant animals showed normal chemotaxis to NaCl, but were strongly defective in gustatory plasticity (Fig. 4A), suggesting that dopamine is needed for gustatory plasticity.

To determine when dopamine is needed for gustatory plas-

ticity, we tested if exogenous dopamine could restore plasticity in *cat-2* and *bas-1* mutant animals. Culturing *cat-2* mutants for only 4 h on plates containing 2 mM dopamine almost fully restored gustatory plasticity (Fig. 4B, not significantly different from the response of wild-type animals exposed to dopamine for 4 h, $P > 0.05$). 72 h of exposure to dopamine fully restored the response of *cat-2* animals (Fig. 4B, not significantly different from the response of wild-type animals exposed to dopamine for 72 h, $P > 0.05$). The responses of *bas-1* animals could only be partially restored by 4 or 72 h of exposure to dopamine. Exposure of wild-type animals to exogenous dopamine did not significantly affect gustatory plasticity or the naïve response to NaCl, nor did exogenous dopamine affect chemotaxis to NaCl of *cat-2* or *bas-1* animals (Fig. 4B; results not shown). These experiments indicate an acute need for dopamine in gustatory plasticity. In addition, the partial rescue of the behavior of *bas-1* animals by both exogenous serotonin and dopamine suggests that these two neurotransmitters function at least partially in separate pathways.

Next, we analyzed the behavior of *dat-1(tm903)* and *(ok157)* mutant animals. *dat-1* encodes a dopamine reuptake transporter; therefore, *dat-1* animals are thought to have potentiated dopamine activity (Jayanthi et al. 1998). Mutation of *dat-1* strongly affected gustatory plasticity (Fig. 4A), confirming that dopamine modulates this behavior.

To allow identification of neurons modulated by dopamine in gustatory plasticity, we determined the responses of *dop-1* and *dop-2* D₂-like dopamine receptor and *dop-3* D₁-like dopamine receptor mutant animals (Chase et al. 2004). We tested two alleles for all three receptor genes: *dop-1(vs100)* and *(ok398)*, *dop-2(vs105)* and *(ok1038)*, and *dop-3(vs106)* and *(ok295)*. All mutants showed a defect in gustatory plasticity (Fig. 4C; results not shown). No significant additive defects were found in double- and triple-mutant animals ($P > 0.05$; Fig. 4A). Taken together, this suggests that both D₁-like and D₂-like dopamine receptors are involved in gustatory plasticity and that they function in the same genetic pathway.

Glutamate is involved in chemotaxis to NaCl and gustatory plasticity

Since glutamate is involved in various sensory responses in several species, including *C. elegans*, we next tested whether glutamate is involved in NaCl chemotaxis or gustatory plasticity, using mutant strains with defects in glutamatergic signaling. First, we tested the role of *eat-4*, which encodes a putative transporter associated with synaptic vesicles and the regulation of transmitter release (Lee et al. 1999). Mutations in the *eat-4* gene, which is expressed in at least 38 neurons, are known to affect behaviors mediated by glutamatergic signaling and seem not to affect behaviors mediated by other neurotransmitters (Lee et al. 1999). Analysis of three alleles (*ad819*, *ky5*, and *n2474*) showed that mutation of *eat-4* strongly affects chemoattraction to NaCl (Fig. 5A; results not shown). In addition, *eat-4* mutant animals did not

Figure 4. Dopamine is required for gustatory plasticity. (A) Responses of dopamine mutants to 25 mM NaCl, after a 15-min wash in CTX buffer with (gray or black bars) or without (open bars) 100 mM NaCl. (Black bars) Responses after 4 h of starvation prior to the assay. Both mutants showed defects in gustatory plasticity, but strong avoidance after starvation. (B) Responses of wild-type, *cat-2*, or *bas-1* animals to 25 mM NaCl after a 15-min wash in CTX buffer with (gray bars) or without (open bars) 100 mM NaCl, after 4–72 h of culture on plates containing 2 mM dopamine (DA). Gustatory plasticity could be fully restored in *cat-2* animals and partially in *bas-1* animals by exogenous dopamine. (C) Responses of dopamine receptor mutants to 25 mM NaCl, after a 15-min wash in CTX buffer with (gray or black bars) or without (open bars) 100 mM NaCl. (Black bars) Responses after 4 h of starvation prior to the assay. All mutants showed defects in gustatory plasticity, but strong avoidance after starvation. Indicated are the averages of at least four assays $\pm$ SEM. Significance (A and C compared with the response of wild-type animals under the same conditions; B compared with *cat-2* or *bas-1* animals not cultured on dopamine): (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.005$, (****) $P < 0.001$.

Figure 5. The role of glutamate in NaCl perception. (A) Responses of naïve wild-type and *eat-4* animals to 0.1–100 mM NaCl revealed a strong chemotaxis defect in *eat-4* mutants. (B) Responses of naïve wild-type, *avr-14*, *avr-15*, and double-mutant animals to 0.1–100 mM NaCl. *avr-14* mutants showed decreased attraction to 1–100 mM NaCl, while *avr-15* mutants showed slightly (but not significantly, $P > 0.05$) enhanced attraction to NaCl. *avr-14; avr-15* double mutants showed wild-type responses. (C) Responses of *avr-14*, *avr-15*, and double-mutant animals to 25 mM NaCl, after a 15-min wash in CTX buffer with (gray or black bars) or without (open bars) 100 mM NaCl. (Black bars) Responses after 4 h of starvation prior to the assay. *avr-14* mutants showed a defect in attraction to 25 mM NaCl. Both *avr-14* and *avr-15* showed defects in gustatory plasticity, but *avr-14; avr-15* double-mutant animals showed wild-type gustatory plasticity. *avr-14* and *avr-15* mutants showed no or strongly reduced avoidance after starvation. (D) Responses of naïve *avr-14*, *avr-15*, and double-mutant animals to 500 mM NaCl. Avoidance is stronger in *avr-14* and *avr-14; avr-15* double-mutant animals, but weaker in *avr-15* animals. (E) Responses of glutamate receptor mutants to 25 mM NaCl, after a 15-min wash in CTX buffer with (gray or black bars) or without (open bars) 100 mM NaCl. (Black bars) Responses after 4 h of starvation prior to the assay. *glr-1*, *glr-2*, and *nmr-1* glutamate receptor mutants showed defects in gustatory plasticity. Double-mutant animals showed very similar plasticity defects as the single mutant; the response of triple-mutant animals was not significantly different ($P > 0.05$) from the wild-type response. These mutants showed wild-type starvation-enhanced gustatory plasticity. Indicated are the averages of at least four assays $\pm$ SEM. Significance (compared with the response of wild-type animals under the same conditions): (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.005$, (****) $P < 0.001$.

show avoidance after pre-exposure, although they were capable of avoiding 1 M NaCl (results not shown). However, this does not necessarily mean that *eat-4* is involved in gustatory plasticity, since these animals have a defect in chemotaxis to NaCl.

C. elegans has four classes of glutamate receptors: AMPA-type glutamate-gated cation channel subunit homologs (*glr-1* to *glr-8*), NMDA-type glutamate-gated cation channels (*nmr-1* and *nmr-2*), metabotropic G-protein-coupled glutamate receptors (*mgl-1* to *mgl-3*), and glutamate-gated chloride channels (*avr-14*, *avr-15* and *glc-1* to *glc-4*) (Hart et al. 1995; Maricq et al. 1995; Dent et al. 1997, 2000; Brockie et al. 2001a; Wolstenholme and Rogers 2005; Dillon et al. 2006). First, we tested mutants for the glutamate-gated chloride channel genes *avr-14* and *avr-15* (Dent et al. 1997, 2000). *avr-14(ad1302)* mutant animals showed a defect in chemotaxis to NaCl (Fig. 5B), very comparable to that found earlier for *tax-2*, *tax-4*, *tax-6*, and *ncs-1* mutant animals (Hukema et al. 2006). In contrast, *avr-15(ad1051)* mutant animals showed wild-type or even stronger chemotaxis to NaCl (Fig. 5B,C). Mutations in both genes also affected avoidance of 1 M NaCl; *avr-15* mutant animals showed reduced avoidance, whereas *avr-14* mutant animals showed stronger avoidance than wild-type animals (Fig. 5D). In addition, mutations in both genes affected gustatory plasticity (Fig. 5C). These results are not conclusive for *avr-14* animals, since these animals also showed defects in chemotaxis to NaCl. However, the gustatory plasticity

defect of *avr-15* animals indicates that glutamate is also involved in this behavior.

Since mutations in the *avr-14* and *avr-15* genes seemed to have opposite effects on the response of *C. elegans* to NaCl, we analyzed *avr-14; avr-15* double-mutant animals for their responses to NaCl. These animals showed wild-type attraction to 0.1–100 mM NaCl and wild-type gustatory plasticity (Fig. 5B,C), but stronger avoidance of 1 M NaCl than wild-type animals (Fig. 5D). Thus, the two subunits *avr-14* and *avr-15* seem to have antagonistic effects on salt perception in *C. elegans*.

Next, we tested mutant animals for the AMPA-type glutamate-gated cation channels *glr-1(n2461)* and *glr-2(tm669)* and (*n2461*) and the NMDA-type glutamate-gated cation channel *nmr-1(ak4)* (Hart et al. 1995; Maricq et al. 1995; Brockie et al. 2001b). Mutations of these receptors did not affect chemoattraction to NaCl, but did affect gustatory plasticity (Fig. 5E). The three mutant strains showed very similar responses to NaCl after pre-exposure. Next, we tested *glr-2; glr-1*, *nmr-1; glr-1*, and *nmr-1; glr-2* double-mutant animals and *nmr-1; glr-2; glr-1* triple-mutant animals to see whether possible stronger defects were masked by functional redundancy. We did not find increased defects in the double-mutant animals (Fig. 5E), suggesting that these glutamate receptor subunits are part of the same genetic pathway involved in gustatory plasticity. However, the response of the *nmr-1; glr-2; glr-1* triple-mutant animals was not significantly different from that of wild-type animals (Fig. 5E), sug-

gesting that some of these subunits might also have antagonistic functions.

avr-15 is required for starvation-enhanced gustatory plasticity

Next, we addressed the question of whether serotonin, dopamine, or glutamate play a role in starvation-enhanced gustatory plasticity. First, we starved the serotonin and dopamine synthesis and signaling mutant animals before testing gustatory plasticity behavior. Remarkably, all mutants with reduced serotonin or dopamine were capable of strong avoidance after pre-exposure under these conditions (Figs. 3A, 4A,C). Only *cat-2* mutant animals showed weakly, but significantly, reduced starvation-enhanced gustatory plasticity compared with wild-type animals (Fig. 4A), suggesting that dopamine might modulate this behavior. Taken together, these results suggest that neither serotonin nor dopamine mediates the starvation signal in this assay, and that these neurotransmitters are not required for avoidance of low NaCl concentrations per se.

Subsequently, we wondered if glutamate mediates the signal that is required for enhanced gustatory plasticity after starvation. The plasticity responses after 4 h of starvation of *glr-1*, *glr-2*, and *nmr-1* mutant animals did not differ significantly from the responses of wild-type animals (Fig. 5E). *avr-15* mutant animals showed enhanced avoidance after pre-exposure when compared

with non-starved *avr-15* mutant animals, but this avoidance did not reach wild-type levels (Fig. 5C). These results indicate that glutamate signaling via AVR-15 plays a role in starvation-enhanced gustatory plasticity.

Discussion

C. elegans shows two different responses to low salt concentrations: Naïve animals are attracted to 0.1–200 mM NaCl, and animals pre-exposed to 100 mM NaCl avoid low salt concentrations (gustatory plasticity). Here, we show that the latter process involves the absence of food, suggesting that *C. elegans* can associate low salt concentrations with this cue. Similar avoidance of low NaCl concentrations can be induced by pre-exposing animals to noxious cues and 100 mM NaCl in the presence of food. This suggests that *C. elegans* can integrate various sensory cues and, by associating the presence of NaCl with noxious cues, such as the absence of food, the presence of benzaldehyde, or osmotic stress, learn to avoid NaCl.

In this study, we show that mutations that affect serotonergic and glutamatergic neurotransmission affect chemotaxis to NaCl. Loss of serotonin synthesis in *tph-1* animals results in a small but significant increase in chemotaxis to NaCl, suggesting that this neurotransmitter suppresses chemotaxis. In addition, we find that glutamate levels play a critical role in the response of *C. elegans* to NaCl. In *eat-4* animals, which lack a putative glutamate transporter, chemotaxis to NaCl was strongly reduced. Interestingly, the phenotypes of *avr-14* and *avr-15* animals that carry mutations in glutamate-gated Cl[−] channel subunits suggest that glutamate can both stimulate and inhibit chemotaxis to NaCl.

Moreover, we show that serotonin, dopamine, and glutamate play important roles in gustatory plasticity. We provide evidence that serotonin is required during development to allow gustatory plasticity. Based on the function of serotonin in neuronal migration, we speculate that serotonin is required for the proper establishment of the circuitry involved in gustatory plasticity. Importantly, both serotonin and dopamine seem acutely needed in the process of gustatory plasticity. Based on our finding that loss-of-function of *tph-1* affects the sensitivity to NaCl and that serotonin affects octanol sensitivity (Chao et al. 2004), we speculate that serotonin and dopamine might modulate the sensitivity of the ASE or ASH sensory neurons, which mediate attraction and osmotic avoidance, respectively (Bargmann and Horvitz 1991); the ASI or ADF neurons, which have also been implicated in gustatory plasticity (Hukema et al. 2006); or unidentified interneurons where these different sensory signals are integrated. Serotonin can be detected in seven neurons and is produced by two cells, and dopamine is found in eight cells: four CEP, two ADE, and two PDE neurons (Sulston et al. 1975; Sze et al. 2000). Since the serotonergic ADF neurons are involved in gustatory plasticity (Hukema et al. 2006), we hypothesize that serotonin released from the ADF neurons is required for gustatory plasticity. It is not clear which of the dopaminergic neurons play a role in gustatory plasticity.

A next step to unravel the cellular circuitry that mediates gustatory plasticity is the identification of serotonin and dopamine receptors involved. Unfortunately, the expression pattern of the *mod-1* serotonin-gated Cl[−] channel involved in gustatory plasticity has not been described precisely. However, the *ser-1* receptor is expressed in the CEP mechanoreceptor neurons, in the RMG, RMH, RMF, and RMD motoneurons, and in pharynx and vulva muscle cells (Xiao et al. 2006). Further cell-specific rescue experiments are required to find out which of these cells are involved in gustatory plasticity. The expression patterns of the dopamine receptors *dop-1*, *dop-2*, and *dop-3* have been described

in some detail (Suo et al. 2003; Tsalik et al. 2003; Chase et al. 2004; Sanyal et al. 2004). Further characterization of these expression patterns and cell-specific rescue should allow the identification of neurons modulated by dopamine in the gustatory plasticity response.

Glutamate plays an important role in gustatory plasticity. Glutamatergic signaling is transduced via NMDA (NMR-1), non-NMDA-like (GLR-1 and GLR-2) ionotropic glutamate receptors, and the glutamate-gated Cl[−] channel subunits AVR-14 and AVR-15. Our results suggest that the *nmr-1*, *glr-1*, and *glr-2* receptors function in the same genetic pathway, since double- and triple-mutant animals for these receptors show very similar defects as the single-mutant animals. It could be that they even function in the same complex, since they are expressed in the same cells: the AVA, AVD, AVE, AVG, and PVC neurons (Hart et al. 1995; Maricq et al. 1995; Brockie et al. 2001a). Our results suggest that AVR-14 and AVR-15 have opposite functions in gustatory plasticity. Interestingly, gustatory plasticity is comparable to wild-type animals in *avr-14*; *avr-15* double-mutant animals and defective in both single-mutant animals. It seems likely that the opposite effects of both channel subunits depend on their expression patterns. Unfortunately, the expression patterns of these genes have not been described to the exact cells.

At a first glance, gustatory plasticity seems very similar to other learning paradigms involving taste (Saeki et al. 2001; Tomioka et al. 2006). However, the major difference between these assays and ours is the length of the pre-exposure time. In our assay, animals avoid low NaCl concentrations after 15 min of exposure to NaCl and a noxious compound, or no food. In the salt learning assays described by Saeki et al. (2001) and Tomioka et al. (2006), animals are starved for 1–4 h in the presence of NaCl. In this study, we show that prolonged starvation in the absence of NaCl, before the gustatory plasticity assay, results in much stronger avoidance of low NaCl concentrations. This response is very similar to that reported by Saeki et al. (2001), suggesting that starvation sensitizes the ability to associate NaCl with negative cues. However, many of the mutants that affect gustatory plasticity do not or only marginally affect starvation-enhanced gustatory plasticity, suggesting that prolonged starvation bypasses the “normal” gustatory plasticity machinery, resulting in strong avoidance even in the absence of, for example, serotonin and dopamine.

We found three mutants that showed significantly reduced starvation-enhanced gustatory plasticity, *cat-2*, *avr-14*, and *avr-15*, suggesting that dopamine and glutamate modulate this behavior. However, mutation of the glutamate-gated Cl[−] channel subunit *avr-14* also affects chemotaxis to NaCl, and these results are therefore not conclusive. Since mutation of *avr-15* also affected gustatory plasticity, it is not clear if AVR-15 mediates the starvation signal or functions in the process that mediates avoidance of low salt concentrations per se. Further studies will have to identify which signals mediate starvation-enhanced gustatory plasticity.

Materials and Methods

Strains and constructs

Strains used in this work were *avr-14(ad1302)*, *avr-15(ad1051)*, *bas-1(ad446)*, *bas-1(tm351)*, *cat-1(e1111)*, *cat-1(ok411)*, *cat-2(e1112)*, *cat-4(e1141)*, *cat-4(ok342)*, *dat-1(tm903)*, *dat-1(ok157)*, *dop-1(vs100)*, *dop-1(ok398)*, *dop-2(vs105)*, *dop-2(ok1038)*, *dop-3(vs106)*, *dop-3(ok295)*, *eat-4(ad806)*, *eat-4(ky5)*, *eat-4(n2474)*, *glr-1(n2461)*, *glr-1(ky176)*, *glr-2(tm669)*, *glr-2(ak10)*, *gpc-1(pk298)*, *mod-1(ok103)*, *mod-5(n822)*, *mod-5(n3314)*, *nmr-1(ak4)*, *odr-3(n1605)*, *ser-1(ok345)* and *ser-4(ok512)*, *tph-1(mg280)*. Wild-type *C. elegans* used was the strain Bristol N2.

Behavioral assays

The response to 25 mM NaCl, with or without pre-exposure to 100 mM NaCl, was assessed as described before (Wicks et al. 2000; Jansen et al. 2002). A chemotaxis index was calculated: $(A - C)/(A + C)$, where A is the number of animals at the quadrants with NaCl, and C is the number of animals at the quadrants without attractant. The behavior of animals was always compared with controls performed on the same day(s). Statistical significance was determined using an ANOVA, followed by a Bonferroni post hoc test. Error bars represent SEM.

Pre-exposure to NaCl on plates was performed on CTX plates (1.7% bacto agar, 5 mM KPO₄ [pH 6], 1 mM CaCl₂, 1 mM MgSO₄), with or without 100 mM NaCl, unseeded or seeded with 200 μ L of 0.5 g/mL OP50 bacteria in H₂O. Adult animals were washed off culture plates using CTX buffer $\pm$ 100 mM NaCl, allowed to sediment for a few minutes, and placed on the pre-exposure plates. After 15, 30, 45, or 60 min pre-exposure, animals were washed off the plates using CTX buffer $\pm$ NaCl, allowed to sediment for 1 min, and placed on a chemotaxis assay plate (Falcon X-plate). Thirty minutes of pre-exposure gave the most reproducible response.

To test if *C. elegans* can learn to avoid NaCl after combined pre-exposure to NaCl and noxious compounds, animals were placed on CTX plates seeded with 200 μ L of 0.5 g/mL OP50 bacteria in H₂O and containing 100 mM NaCl and 0.3, 0.5, or 1 M glycerol, or 0.3, 0.5, 0.7, or 1 M glycerol in the absence of NaCl. After 30 min of pre-exposure, animals were washed off the plates, placed on chemotaxis assay plates, and tested for their response to 25 mM NaCl. Exposure to 300 mM glycerol reduced attraction to NaCl, but did not induce avoidance. Exposure to 1 M glycerol killed the animals. Exposure to 300, 500, or 700 mM glycerol in the absence of NaCl did not affect chemotaxis to 25 mM NaCl. Alternatively, animals were placed on CTX plates seeded with 200 μ L of 0.5 g/mL OP50 bacteria and containing 100 mM NaCl and exposed to 0.3, 1, or 3 μ L of undiluted benzaldehyde on the lid. Exposure to 0.3 or 1 μ L of benzaldehyde did not affect chemotaxis to 25 mM NaCl. Similar experiments were performed with 0.01, 0.1, or 1% SDS in the plates and with 0.3, 1, or 3 μ L of undiluted nonanone on the lids. Exposure to 1% SDS was very toxic and abolished chemotaxis of naïve animals, but resulted in avoidance of 25 mM NaCl after pre-exposure to NaCl. SDS (0.1%) did not affect the naïve response but only mildly decreased attraction to NaCl after pre-exposure, while 0.01% SDS had no effect. Similar results were obtained with nonanone, where exposure to 3 μ L of nonanone killed the animals, 1 μ L had only a mild effect, and 0.3 μ L had no effect.

To test if starvation affected gustatory plasticity, animals were starved on CTX-plates for 15 min up to 4 h before the assay.

Supplementation studies

Rescues of serotonin and dopamine deficiencies were performed by culturing animals for 4, 72, or 144 h before the assays on seeded NGM-plates containing either 2 mM dopamine hydrochloride or 2 mM serotonin creatinine sulfate complex. In case of 144-h serotonin supplementation, the parents of the animals tested were cultured for 72 h on plates containing serotonin, and subsequently their progeny were cultured on serotonin-containing plates for 72 h. The concentration of 2 mM for both substances did not influence the behavior of wild-type animals. Higher concentrations affected locomotion, resulting in poor performance in the behavioral assays, whereas lower concentrations were less effective.

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