Antagonistic sensory cues generate gustatory plasticity in *Caenorhabditis elegans*

Renate K Hukema, Suzanne Rademakers, Martijn PJ Dekkers, Jan Burghoorn and Gert Jansen*

MGC Department of Cell Biology and Genetics, Centre for Biomedical Genetics, Erasmus MC, Rotterdam, The Netherlands

Caenorhabditis elegans shows chemoattraction to 0.1–200 mM NaCl, avoidance of higher NaCl concentrations, and avoidance of otherwise attractive NaCl concentrations after prolonged exposure to NaCl (gustatory plasticity). Previous studies have shown that the ASE and ASH sensory neurons primarily mediate attraction and avoidance of NaCl, respectively. Here we show that balances between at least four sensory cell types, ASE, ASI, ASH, ADF and perhaps ADL, modulate the response to NaCl. Our results suggest that two NaCl-attraction signalling pathways exist, one of which uses Ca^{2+} /cGMP signalling. In addition, we provide evidence that attraction to NaCl is antagonised by G-protein signalling in the ASH neurons, which is desensitised by the G-protein-coupled receptor kinase GRK-2. Finally, the response to NaCl is modulated by G-protein signalling in the ASI and ADF neurons, a second G-protein pathway in ASH and cGMP signalling in neurons exposed to the body fluid.

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Introduction

Salt taste is essential for ion and water homeostasis; however, our understanding of the molecular mechanisms of salt perception is relatively limited (Lindemann, 2001). In rodents and *Drosophila*, salts are thought to be primarily sensed by cation influx through degenerin/epithelial Na^+ channels (DEG/ENaC), leading to membrane depolarisation (Lindemann, 2001; Liu *et al.*, 2003). In humans, the contribution of ENaC channels is less pronounced and salts are thought to be detected by a vanilloid receptor-1 (TRPV1) variant, a member of the transient receptor potential (TRP) cation channel family (Lyll *et al.*, 2004). We use the nematode *Caenorhabditis elegans* to unravel the molecular mechanisms that mediate the response to NaCl.

*Corresponding author. MGC Department of Cell Biology and Genetics, Centre for Biomedical Genetics, Erasmus University Rotterdam, Erasmus MC, PO Box 1738, Rotterdam 3000 DR, The Netherlands. Tel.: +31 10 408 7473; Fax: +31 10 408 9468; E-mail: g.jansen@erasmusmc.nl

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C. elegans shows chemoattraction to low NaCl and avoidance of high NaCl concentrations (Ward, 1973; Dusenbery *et al.*, 1975; Culotti and Russell, 1978). Chemoattraction to salts is mediated by four pairs of amphid sensory neurons (ADF, ASE, ASG and ASI), of which the ASE cells are most important (Bargmann and Horvitz, 1991). Several genes involved in salt detection have been identified. These include the guanylate cyclase DAF-11, the cGMP-gated cation channel subunits TAX-2 and TAX-4, and the calcineurin A subunit TAX-6, suggesting that *C. elegans* uses cGMP and Ca^{2+} signalling in salt detection (Coburn and Bargmann, 1996; Komatsu *et al.*, 1996; Birnby *et al.*, 2000; Kuhara *et al.*, 2002). *C. elegans* avoids NaCl concentrations above 200 mM (RK Hukema and G Jansen, unpublished results). This response is thought to be due to a general avoidance of high osmotic strength. The ASH sensory neurons play a pivotal role in this process (Bargmann *et al.*, 1990). Osmotic avoidance requires the G α protein ODR-3, the TRP channel subunits OSM-9 and OCR-2, the cytoplasmic protein OSM-10, the G-protein-coupled receptor kinase GRK-2 and glutamatergic neurotransmission, involving the glutamate receptors GLR-1 and NMR-1, the glutamate transporter EAT-4 and the proprotein convertase EGL-3 (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).

The behavioural response to salts is plastic and depends on various cues, including exposure time and salt concentration. We use a new chemotaxis assay to analyse both salt detection and its plasticity (Figure 1A; Wicks *et al.*, 2000; Jansen *et al.*, 2002). In the plasticity assay, animals are washed in a buffer containing 100 mM NaCl and subsequently analysed for chemotaxis. These pre-exposed animals show avoidance of otherwise attractive NaCl concentrations. This behaviour is time and concentration dependent, reversible and partially salt specific. Thus far, two proteins have been shown to contribute to this behaviour, the TRP channel subunit OSM-9 and the G γ protein GPC-1 (Jansen *et al.*, 2002). The finding that prolonged exposure to NaCl results in avoidance suggests a mechanism that involves more than adaptation or desensitisation. We propose a model in which the behavioural response to salt involves a balance between attraction and avoidance, as has been described for the regulation of the response to benzaldehyde, aggregation behaviour and the integration of sensory signals (Nuttley *et al.*, 2001; de Bono *et al.*, 2002; Ishihara *et al.*, 2002). We call this behaviour gustatory plasticity.

In this paper, we show that four pairs of amphid sensory neurons and neurons exposed to the body fluid are involved in gustatory plasticity. Our results suggest that two signalling pathways mediate attraction to NaCl, likely in the ASE neurons. One of these pathways involves cGMP and Ca^{2+} signalling. We show that attraction to NaCl is antagonised by a G-protein-signalling pathway in the ASH neurons, which is desensitised by GRK-2. Furthermore, the response to NaCl is modulated by G-protein signalling in the ASI and ADF neurons, an additional G-protein pathway in the ASH neurons and cGMP signalling in neurons exposed to the body fluid.

Figure 1 Both chemoattraction to NaCl and gustatory plasticity require the ASE neurons. **(A)** Worms are washed in a low-salt buffer or a buffer containing 100 mM NaCl and tested for chemotaxis. The assay plates contain two quadrants with only buffered agar (C) and two quadrants with buffered agar and NaCl (A). We use 0.1, 1, 10 or 100 mM NaCl to test for chemoattraction, 1 M NaCl to test for avoidance and 25 mM NaCl in the gustatory plasticity assay. The distribution of the animals over the quadrants is determined after 10 min, and the chemotaxis index is calculated. **(B)** ASE function is required for the detection of NaCl, since chemotaxis to NaCl was abolished in *che-1* and *ceh-36* mutants ($P < 0.001$). Chemotaxis could be restored by introduction of a *che-1* transgene (*che-1* rescue, $P < 0.001$). **(C)** *che-1* and *ceh-36* animals strongly avoided 1 M NaCl. **(D)** *che-1* and *ceh-36* animals showed no significant response to NaCl after pre-exposure to 100 mM NaCl ($P < 0.001$ compared to wild-type animals). The *che-1* defect could be rescued by introduction of a *che-1* transgene (*che-1* rescue). In all figures: panels show mean $\pm$ s.e.m.; $n \geq 4$ for all assays. Open bars indicate chemotaxis to 25 mM NaCl; grey bars indicate the response to 25 mM NaCl after pre-exposure to 100 mM NaCl; black bars show the response to 1 M NaCl.

Results

Antagonistic inputs from three pairs of sensory neurons modulate gustatory behaviour

Sensation of attractive NaCl concentrations is primarily mediated by the ASE neurons (Bargmann and Horvitz,

1991). To confirm this role of the ASE cells, we tested the behaviour of *che-1* and *ceh-36* mutant animals. *che-1* mutants lack functional ASE neurons due to mutation of a zinc-finger protein similar to the *Drosophila* GLASS transcription factor (Uchida *et al*, 2003). *che-1* is predominantly expressed in the ASE neurons; only occasionally expression was observed in other neurons (Uchida *et al*, 2003). *ceh-36* encodes an Otx-related homeodomain protein expressed in the ASE and the AWC neurons, which specifies the identities of these neurons (Lanjuin *et al*, 2003). Both *che-1* and *ceh-36* mutant animals showed no significant chemotaxis to NaCl (0.1–100 mM; Figure 1B). In contrast, these animals showed wild type or even increased avoidance of 1 M NaCl (Figure 1C). Surprisingly, *che-1* and *ceh-36* animals showed no response to NaCl after pre-exposure to 100 mM NaCl (Figure 1D). The chemotaxis defects observed in *che-1* animals could be rescued by introduction of the wild-type *che-1* gene as a transgene (Figure 1B and D). Our results confirm that the ASE neurons are essential for chemotaxis to NaCl, also in our assay, and that these cells are not required for avoidance of 1 M NaCl (Bargmann and Horvitz, 1991; Uchida *et al*, 2003). Importantly, our results indicate that signalling via the ASE neurons is essential for gustatory plasticity.

To identify additional cells involved in gustatory plasticity, we used the G γ subunit, GPC-1. *gpc-1* mutants showed reduced avoidance of, or even attraction to, NaCl after pre-exposure (Jansen *et al*, 2002). Interestingly, *gpc-1* is not expressed in the ASE neurons, but it is expressed in three other pairs of neurons involved in salt perception (Jansen *et al*, 2002): the ASI cells, which have a minor function in attraction to salts (Bargmann and Horvitz, 1991), and the ADL and ASH nociceptive neurons (Bargmann *et al*, 1990). Introduction of the wild type *gpc-1* gene as a transgene in *gpc-1* animals could restore the response to NaCl after pre-exposure (Figure 2A; Jansen *et al*, 2002). However, we never obtained full rescue of the defect. Since overexpression of *gpc-1* induced a gustatory plasticity defect (Jansen *et al*, 2002), we surmise that probably the levels of GPC-1 are crucial for the behavioural response. Specific expression of GPC-1 in the ASI or ASH neurons partially restored avoidance after pre-exposure in *gpc-1* mutants (Figure 2A). Expression of GPC-1 both in the ASI and ASH neurons did not yield better rescue (results not shown). These results suggest that ASI and ASH do not have additive functions; however, since *gpc-1* expression levels seem a crucial determinant in this behaviour, these experiments are not conclusive. Expression of GPC-1 in the ADL and ASH nociceptive neurons resulted in better rescue than expression in the ASH neurons alone (Figure 2A), suggesting that GPC-1 probably also functions in ADL. Expression of *gpc-1* in the ASE neurons did not restore gustatory plasticity (Figure 2A), suggesting that GPC-1 does not function non-cell-autonomously. Taken together, our findings indicate that GPC-1 acts both in the ASI and ASH neurons, and probably also in the ADL neurons, to modulate the response to NaCl. Avoidance of 1 M NaCl is not affected in *gpc-1* animals, suggesting that separate pathways exist in the nociceptive neurons for osmotic avoidance and the modulation of the response to attractive NaCl concentrations.

To validate the importance of the ASI, ADL and ASH neurons in gustatory plasticity, we expressed a dominant mutant DEG/ENaC channel MEC-4d in specific neurons to

Figure 2 Identification of sensory neurons involved in gustatory plasticity. **(A)** Avoidance after pre-exposure could be restored by introducing the wild-type *gpc-1* gene in *gpc-1* animals (genomic), or by expressing *gpc-1* in the ASI, ASH or ASH and ADL neurons ($P < 0.01$). Expression in ASE did not rescue the plasticity defect ($P > 0.05$). **(B)** Expression of *mec-4d/mec-2* constructs in the ASI neurons, in the ASH/ADL nociceptive neurons or the ASH neurons significantly impaired gustatory plasticity ($P < 0.01$).

inactivate these cells (Harbinder *et al*, 1997). To increase the efficacy, we coexpressed *mec-2* constructs, since MEC-2 has been shown to increase MEC-4d activity (Goodman *et al*, 2002). Expression of the *mec-4d/mec-2* constructs in the ASI neurons strongly affected gustatory plasticity, while expression in the ASH or ADL cells only mildly interfered with plasticity (Figure 2B). Expression of the *mec-4d/mec-2* constructs in the ASI, ASH or ADL neurons did not affect salt detection (Figure 2B, results not shown). The *mec-4d/mec-2* data confirm that the ASI, ASH and probably ADL neurons are involved in gustatory plasticity.

Our results indicate that gustatory plasticity is the result of integration of input from three to four pairs of amphid neurons. Signalling via the ASE cells is essential for both attraction to salt and avoidance after pre-exposure. However, the ASE neurons are not involved in avoidance of 1 M NaCl. This response is mediated by the ASH neurons (Bargmann *et al*, 1990). Avoidance after pre-exposure involves cues from the ASI and ASH cells, and probably the ADL neurons.

G-protein signalling in the ADF neurons modulates gustatory plasticity

Our finding that the $G\gamma$ subunit GPC-1 plays a role in gustatory plasticity prompted us to test the behaviour of mutants of the $G\alpha$ subunits expressed in the gustatory neurons. None of the sensory $G\alpha$ mutants showed a defect in chemotaxis to NaCl (Table I). However, two $G\alpha$ mutants showed impaired gustatory plasticity: GPA-1 and ODR-3 (Figure 3A and B).

Thus far, no function has been described for GPA-1 (Jansen *et al*, 1999). We found that *gpa-1* animals have a defect in gustatory plasticity, whereas they showed wild-type avoidance of 1 M NaCl (results not shown). The behaviour of *gpa-1* animals was very similar to that of *gpc-1* animals. Moreover, like *gpc-1*, *gpa-1* is expressed in the ASI and nociceptive neurons (Jansen *et al*, 1999), suggesting that GPA-1 might function together with GPC-1 in these neurons. To test this hypothesis, we generated a *gpa-1 gpc-1* double mutant. The behaviour of the double mutant was not significantly different from the behaviour of the two single mutants (Figure 3A), indicating that *gpa-1* and *gpc-1* function in the same genetic pathway that modulates gustatory plasticity.

The $G\alpha$ subunit ODR-3 is essential for olfaction and chemoavoidance, and is expressed in the AWA, AWB and AWC olfactory neurons and in the ADF and ASH neurons (Roayaie *et al*, 1998). *odr-3* animals showed a very strong gustatory plasticity defect (Figure 3B). To test if this is caused by the osmotic avoidance defect of *odr-3* animals, we tested if expression of the *odr-3* gene in the ASH neurons could restore gustatory plasticity. Expression of ODR-3 in the ASH neurons of *odr-3* animals fully restored avoidance of 1 M NaCl (Figure 3C), confirming that ODR-3 in the ASH neurons is essential for this response (Roayaie *et al*, 1998). However, avoidance after pre-exposure could not be restored by expressing *odr-3* in the ASH neurons (Figure 3B), indicating that ODR-3-mediated signalling in the ASH neurons is not sufficient for gustatory plasticity. Next, we tested if the ADF neurons are involved in gustatory plasticity, by expressing the *odr-3* gene in these neurons. ADF specific expression of *odr-3* restored avoidance after pre-exposure (Figure 3B), but did not restore avoidance of 1 M NaCl (Figure 3C). Expression of *odr-3* in both ASH and ADF did not yield better rescue, but reproduced the ASH-specific rescue of avoidance of 1 M NaCl and ADF-specific rescue of gustatory plasticity (results not shown). Hence, ODR-3 functions in two separate processes. It mediates avoidance of 1 M NaCl in the ASH neurons, and mediates a signal in the ADF neurons that modulates gustatory plasticity.

Excessive avoidance in *grk-2* animals counterbalances chemoattraction to NaCl

Our results indicate that G proteins play an important role in gustatory plasticity. Several mechanisms that modulate G-protein signal transduction exist, providing a molecular basis for behavioural plasticity. One of these mechanisms involves G-protein-coupled receptor kinases (GRKs) and arrestins. Studies in mammals and *Drosophila* have shown that GRKs inhibit G-protein signalling by phosphorylating activated G-protein-coupled receptors, followed by arrestin binding and receptor internalisation (Ferguson, 2001). In addition to this classical function, recent studies indicate a role for GRKs and arrestins as signal transduction molecules

Table 1 Overview of strains tested for salt perception

Gene	Reference	Function	Taste	Plasticity
<i>arr-1</i> (<i>ok401</i>)	Fukuto <i>et al</i> (2004)	Arrestin	WT	$-0.12 \pm 0.17^{***}$
<i>ceh-36</i> (<i>ks86</i>)	Lanjuin <i>et al</i> (2003)	Transcription factor	m ^{***}	$0.13 \pm 0.03^{***}$
<i>che-1</i> (<i>p679</i>)	Uchida <i>et al</i> (2003)	Transcription factor	m	$0.01 \pm 0.02^{***}$
<i>cnb-1</i> (<i>jh103</i>)	Bandyopadhyay <i>et al</i> (2002)	Calcineurin B	m*	$0.74 \pm 0.04^{**}$
<i>daf-11</i> (<i>m47</i>)	Birnby <i>et al</i> (2000)	Gyanylate cyclase	WT	WT
<i>dgk-1</i> (<i>sy428</i>)	Hadju-Cronin <i>et al</i> (1999); Nurrish <i>et al</i> (1999)	Diacylglycerol kinase	WT	$0.72 \pm 0.05^{***}$
(<i>nu62</i>)			WT	$0.27 \pm 0.10^{***}$
<i>egl-4</i> (<i>ky95</i>)	Daniels <i>et al</i> (2000)	PKG	WT	$0.06 \pm 0.06^{***}$
(<i>ok1105</i>)			WT	$-0.06 \pm 0.08^{***}$
<i>fat-1</i> (<i>wa9</i>)	Watts and Browse (2002)	ω -3 fatty acyl desaturase	WT	$-0.02 \pm 0.16^{**}$
<i>fat-3</i> (<i>wa22</i>)	Watts and Browse (2002)	δ -6 fatty acid desaturase	WT	$0.20 \pm 0.06^{***}$
<i>fat-4</i> (<i>wa14</i>)	Watts and Browse (2002)	δ -5 fatty acid desaturase	WT	$0.31 \pm 0.09^{***}$
(<i>ok958</i>)			WT	$0.10 \pm 0.03^{***}$
<i>gcy-32</i> (<i>ok995</i>)	Coates and de Bono (2002)	Guanylate cyclase	WT	WT
<i>gcy-35</i> (<i>ok769</i>)	Cheung <i>et al</i> (2004)	Gyanylate cyclase	WT	$-0.10 \pm 0.10^*$
<i>gpa-1</i> (<i>pk15</i>)	Jansen <i>et al</i> (1999)	G α subunit	WT	$0.17 \pm 0.06^{**}$
<i>gpa-2</i> (<i>pk16</i>)	Zwaal <i>et al</i> (1997)	G α subunit	WT	WT
<i>gpa-3</i> (<i>pk35</i>)	Zwaal <i>et al</i> (1997)	G α subunit	WT	WT
<i>gpa-4</i> (<i>pk381</i>)	Jansen <i>et al</i> (1999)	G α subunit	WT	WT
<i>gpa-10</i> (<i>pk362</i>)	Jansen <i>et al</i> (1999)	G α subunit	WT	WT
<i>gpa-11</i> (<i>pk349</i>)	Jansen <i>et al</i> (1999)	G α subunit	WT	WT
<i>gpa-13</i> (<i>pk1270</i>)	Jansen <i>et al</i> (1999)	G α subunit	WT	WT
<i>gpa-14</i> (<i>pk347</i>)	Jansen <i>et al</i> (1999)	G α subunit	WT	WT
<i>gpa-15</i> (<i>pk477</i>)	Jansen <i>et al</i> (1999)	G α subunit	WT	WT
<i>grk-2</i> (<i>rt97</i>)	Fukuto <i>et al</i> (2004)	GRK	m ^{***}	$-0.61 \pm 0.06^{***, \#}$
<i>itr-1</i> (<i>sa73</i>)	Baylis <i>et al</i> (1999); Dal Santo <i>et al</i> (1999)	IP ₃ receptor	WT	$0.82 \pm 0.06^{***}$
<i>ncs-1</i> (<i>qa406</i>)	Gomez <i>et al</i> (2001)	Ca ²⁺ sensor	m ^{**}	$-0.13 \pm 0.14^{**}$
<i>ocr-1</i> (<i>ok132</i>)	Tobin <i>et al</i> (2002)	TRPV channel	WT	$-0.20 \pm 0.08^{***}$
(<i>ak46</i>)			WT	$-0.25 \pm 0.14^{***}$
<i>ocr-2</i> (<i>ak47</i>)	Tobin <i>et al</i> (2002)	TRPV channel	WT	$-0.18 \pm 0.12^{***}$
<i>odr-3</i> (<i>n1605</i>)	Roayaie <i>et al</i> (1998)	G α subunit	WT	$0.85 \pm 0.04^{***}$
<i>osm-9</i> (<i>ky10</i>)	Colbert <i>et al</i> (1997)	TRPV channel	WT	$0.06 \pm 0.05^{**}$
<i>pdl-1</i> (<i>gk157</i>)	Smith and Pilgrim, personal communication	Phosphodiesterase	WT	$0.13 \pm 0.09^{**}$
<i>tax-2</i> (<i>p671</i>)	Coburn and Bargmann (1996)	cGMP-gated channel	m ^{***}	$0.83 \pm 0.04^{***}$
<i>tax-4</i> (<i>p678</i>)	Komatsu <i>et al</i> (1996)	cGMP-gated channel	m ^{***}	$0.83 \pm 0.03^{***}$
<i>tax-6</i> (<i>p675</i>)	Kuhara <i>et al</i> (2002)	Calcineurin A	m ^{***}	$0.64 \pm 0.05^{***}$

In all, 32 mutant strains were tested for chemotaxis to 0.1, 1, 10 and 100 mM NaCl (taste) or chemotaxis to 25 mM NaCl after pre-exposure to 100 mM NaCl (plasticity) and their responses were compared to wild-type animals: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; unc = locomotion defect; these strains did not perform well in the assay; #pre-exposure for only 5 min, response stronger than wild types. WT = wild type.

Figure 3 G α subunits GPA-1 and ODR-3 modulate gustatory plasticity. (A) GPA-1 is involved in gustatory plasticity ($P < 0.0001$) and functions in the same genetic pathway as GPC-1, since *gpa-1 gpc-1* animals showed the same response as the single mutants ($P > 0.05$). (B) Also, ODR-3 is involved in gustatory plasticity ($P < 0.0001$). Avoidance after pre-exposure could be restored by expressing ODR-3 in the ADF neurons ($P < 0.0001$), but not by expressing it in the ASH neurons ($P > 0.05$). (C) ODR-3 signalling mediates avoidance of 1 M NaCl ($P < 0.0001$). Avoidance could be restored by expressing ODR-3 in the ASH neurons ($P < 0.0001$), but not by expressing it in the ADF neurons ($P > 0.05$).

(Perry and Lefkowitz, 2002; Kim *et al*, 2005; Ren *et al*, 2005). The *C. elegans* genome contains two GRKs, *grk-1* and *grk-2*, and one arrestin gene, *arr-1* (Fukuto *et al*, 2004). Loss of

function of *grk-2* impairs the response to attractive odorants and several repellents, including odorants, quinine and 1–4 M glycerol, but seems to have no or little involvement in

olfactory adaptation. These results suggest that GRK-2 functions as a signal transduction molecule in the nociceptive and olfactory neurons (Fukuto *et al*, 2004). The *C. elegans* arrestin, *arr-1*, is required for adaptation and recovery from adaptation to various odorants (Palmitessa *et al*, 2005). We determined the role of GRK-2 and ARR-1 in gustatory plasticity.

Loss of function of *grk-2* severely impaired chemotaxis to NaCl (Figure 4A). However, *grk-2* animals did show avoidance after pre-exposure (Figure 4B), indicating that these animals can detect NaCl. When these animals were only briefly pre-exposed to NaCl, they even showed enhanced avoidance after pre-exposure (Figure 4C). Since the ASE cells are essential for both salt taste and gustatory plasticity, we surmised that GRK-2 does not function in the ASE neurons, but in the nociceptive neurons instead. We first tested the response of *grk-2* animals to 1 M NaCl. Surprisingly, *grk-2* animals showed strong avoidance of 1 M NaCl (Figure 4D), in contrast to the previously reported defect in avoidance of 1–4 M glycerol (Fukuto *et al*, 2004). These results suggest that different signalling routes exist for avoidance of high concentrations of glycerol and NaCl.

We next tested if specific expression of *grk-2* in the nociceptive neurons ADL and ASH could restore chemotaxis of the *grk-2* mutants. Indeed, a *srb-6::grk-2* construct restored the response to 1–100 mM NaCl partially (*gJEx601* and two other strains) or fully (*gJEx602*) (Figure 4A). Probably, *gJEx601* animals did not express sufficient GRK-2 in the nociceptive neurons for full rescue, since avoidance of 1 M NaCl was not restored (Figure 4C). In contrast, *gJEx602* animals probably overexpressed GRK-2, diminishing the avoidance responses (Figure 4A and D), and restoring chemoattraction. To further confirm GRK-2 function in ASH, we also used an *osm-10::grk-2* construct to express *grk-2* in the ASH and ASI neurons. This construct could also restore the NaCl chemoattraction defect of *grk-2* animals (*gJEx639* in Figure 4A, B and D). In addition, specific expression of *grk-2* in the ASH neurons could restore avoidance of octanol and 1 M glycerol (results not shown). In contrast, expression of GRK-2 in interneurons, but not the sensory neurons, using a *glr-1::grk-2* construct did not restore chemotaxis to NaCl (results not shown). Taken together, our results suggest that GRK-2 modulates the strength of the NaCl avoidance signal mediated by the nociceptive neurons. Such a function fits with a classical function for GRK-2 in desensitisation of the NaCl avoidance-signalling pathway (Figure 7), suggesting that GRK-2 has two different functions in separate signalling pathways in the ASH cells. Moreover, these results show that signals derived from the nociceptive neurons counterbalance chemoattraction to NaCl mediated by the ASE neurons.

Mutation of *arr-1* reduced avoidance after pre-exposure, while chemoattraction or avoidance responses were not affected (Figure 4B, results not shown). These results are not in agreement with a function of ARR-1 in receptor or channel internalisation in the ASE or nociceptive neurons. *arr-1* animals rather displayed behavioural deficits comparable to those observed in *gpc-1* and *gpa-1* animals. *gpa-1 arr-1* double-mutant animals indeed showed the same response after pre-exposure as the two single mutants (Figure 4B), indicating that these genes function in the same genetic pathway. *arr-1* is however broadly expressed in the nervous system of *C. elegans* (Fukuto *et al*, 2004; Palmitessa *et al*, 2005), which makes it unclear in which cells ARR-1 functions in gustatory plasticity.

Figure 4 Excessive avoidance in *grk-2* animals counterbalances chemoattraction to NaCl. (A) Chemotaxis to 0.1–100 mM NaCl was impaired in *grk-2* animals ($P < 0.0001$). The *grk-2* defect could be restored by expressing GRK-2 in the nociceptive neurons in strains *grk-2 gJEx601* ($P < 0.05$), *gJEx602* ($P < 0.001$) and *gJEx639* ($P < 0.001$). (B) *grk-2* mutants showed no chemoattraction to 25 mM NaCl, but strong avoidance after pre-exposure to 100 mM NaCl. Expression of GRK-2 in the nociceptive neurons restored chemoattraction in *grk-2 gJEx601* animals ($P < 0.001$) and *grk-2 gJEx639* animals ($P < 0.0001$). *grk-2 gJEx602* animals showed even better rescue of chemoattraction ($P < 0.0001$), but impaired avoidance after pre-exposure ($P < 0.0001$). Mutation of *arr-1* significantly reduced avoidance after pre-exposure ($P < 0.001$), to a similar level as loss of *gpa-1*. The behaviour of the *gpa-1 arr-1* double mutant suggests that both genes function in the same pathway ($P > 0.05$). (C) *grk-2* animals showed enhanced avoidance after pre-exposure to NaCl for only 5 min ($P < 0.001$). (D) *grk-2* animals showed strong avoidance of 1 M NaCl, which was not affected in *grk-2 gJEx601* and *gJEx639* animals ($P > 0.05$), but impaired in *grk-2 gJEx602* animals ($P < 0.001$).

Gustatory plasticity requires cGMP signalling in the body cavity neurons

We next studied the role of downstream signalling molecules in gustatory plasticity. Frequently used second messengers

are cGMP and Ca^{2+} . cGMP can be generated by guanylate cyclases, and it can activate other signalling molecules such as cGMP-gated channels or protein kinases (PKG). Previous studies have shown that cGMP signalling plays an important role in chemotaxis to NaCl. Mutation of the guanylate cyclase DAF-11 and the cGMP-gated channel subunits TAX-2 and TAX-4 abolish chemotaxis to NaCl (Coburn and Bargmann, 1996; Komatsu *et al*, 1996; Birnby *et al*, 2000). Surprisingly, mutation of *daf-11* did not affect chemotaxis to NaCl in our assay (Figure 5A), whereas mutation of *tax-2* and *tax-4* strongly affected chemotaxis to 0.1–10 mM NaCl. However, these latter mutants still showed chemoattraction to 100 mM NaCl (Figure 5A). Our results confirm that cGMP signalling is involved in chemotaxis to NaCl, although it is unclear which proteins activate the TAX-2/TAX-4 channel. In addition, our results suggest that another pathway exists for the detection of NaCl, which functions in parallel to the cGMP pathway.

Mutations in *tax-2* and *tax-4* also abolish avoidance after pre-exposure (results not shown). However, since these proteins play important roles in chemotaxis to NaCl, we cannot conclude whether TAX-2 and TAX-4 function in gustatory plasticity. Analysis of other cGMP signalling molecules did indicate a role of cGMP signalling in gustatory plasticity. Mutations in the cGMP-dependent kinase EGL-4 and the phosphodiesterase delta-like protein PDL-1 affected gustatory plasticity (Figure 5C). EGL-4 is expressed in a large number of neurons (Fujiwara *et al*, 2002; Hirose *et al*, 2003) and PDL-1, responsible for cGMP breakdown, shows pan-neuronal expression (J Smith and D Pilgrim, personal communication). Hence, further cell-specific rescue and epistasis analysis are needed to determine the specific functions of these proteins.

We also identified a guanylate cyclase that plays a role in gustatory plasticity. Mutations in the soluble guanylate cyclase *gcy-35* affected this behaviour (Figure 5B), but did not affect chemoattraction or avoidance of high NaCl concentrations. *gcy-35* is expressed, among others, in the body cavity neurons AQR, PQR and URX (Cheung *et al*, 2004; Gray *et al*, 2004). These neurons are directly exposed to the pseudocoelomic body fluid (White *et al*, 1986) and regulate social feeding or aggregation behaviour (Coates and de Bono, 2002). We tested whether GCY-35 functions in these body cavity neurons during gustatory plasticity by specific expression of *gcy-35* in these neurons. Indeed, avoidance after pre-exposure was restored in *gcy-35* animals expressing a *gcy-32::gcy-35* construct (Figure 5B). We did not obtain full rescue, suggesting that *gcy-35* might also function in other cells in gustatory plasticity.

To confirm that the AQR, PQR and URX cells are involved in gustatory plasticity, we expressed a gain-of-function mutant form of the *eag*-type potassium channel EGL-2 in these neurons, using a *gcy-32::egl-2(gf)* construct. Expression of this construct has been shown to inhibit neuronal activity (Coates and de Bono, 2002). We found that expression of EGL-2(gf) in the AQR, PQR and URX neurons significantly reduced avoidance after pre-exposure to 100 mM NaCl, without affecting chemotaxis (Figure 5B, results not shown). These results show that these body cavity neurons mediate a signal that modulates the response to NaCl.

The phenotype of the *gcy-35* animals was very comparable to that of the *arr-1*, *gpa-1* and *gpc-1* animals. To test if also *gcy-35* functions in the same genetic pathway, we tested the behaviour of *gpc-1 gcy-35* double-mutant animals. The

Figure 5 Gustatory plasticity requires cGMP. (A) Salt detection was not affected in *daf-11* animals ($P > 0.05$), but significantly impaired in *tax-2* and *tax-4* animals ($P < 0.01$ at 1 and 10 mM NaCl). These mutants still responded to 100 mM NaCl. (B) Mutation of *gcy-35* affected gustatory plasticity ($P < 0.001$). This defect could be rescued by expression of *gcy-35* in the AQR, PQR and URX neurons ($P < 0.0001$). Expression of a *gcy-32::egl-2(gf)* construct to inhibit the activity of AQR, PQR and URX neurons reduced avoidance after pre-exposure ($P < 0.05$). Analysis of a *gpc-1 gcy-35* double-mutant strain indicated that these genes function in the same genetic pathway in gustatory plasticity ($P > 0.05$ compared to the single mutants). (C) Mutation of the cGMP signalling molecules *egl-4* ($P < 0.001$) and *pdl-1* ($P < 0.01$) affected gustatory plasticity, whereas mutation of *gcy-32* did not ($P > 0.05$).

double-mutant strain behaved as the two single mutants (Figure 5B). Therefore, we hypothesise that cGMP signalling via *gcy-35* in the AQR, PQR and/or URX neurons functions in the same genetic pathway as *gpc-1*, *gpa-1* and *arr-1* to modulate the response to NaCl after prolonged exposure.

Gustatory plasticity requires Ca^{2+} signalling

We also tested if Ca^{2+} signalling is involved in gustatory plasticity. Ca^{2+} levels in the cell can be increased by influx via TRP channels or cGMP-gated channels, and by depletion of intracellular stores after inositol (1,4,5) trisphosphate (IP_3)-receptor activation. Increased Ca^{2+} levels can subsequently activate various downstream effector molecules, including calcineurin and the neuronal calcium sensor NCS-1. Previous studies have shown that Ca^{2+} signalling is important for chemotaxis to NaCl, since mutation of the cGMP-gated channel TAX-2/TAX-4, which upon activation leads to Ca^{2+} influx, affects chemotaxis to NaCl (Coburn and Bargmann, 1996; Komatsu *et al*, 1996; Figure 5A). In addition, it was shown that mutation of the calcineurin A subunit TAX-6 affects chemotaxis to NaCl (Kuhara *et al*, 2002).

Calcineurin is a phosphatase consisting of two subunits A and B, encoded by *tax-6* and *cnb-1*, respectively (Bandyopadhyay *et al*, 2002; Kuhara *et al*, 2002). We tested *tax-6* and *cnb-1* mutants in our assays, as well as *ncs-1* mutants, which lack a functional neuronal calcium sensor 1 (Gomez *et al*, 2001). All three mutants showed strongly reduced chemotaxis to low NaCl concentrations (0.1–10 mM), but strong or even wild-type responses to 100 mM NaCl (Figure 6A). These mutants also showed defects in avoidance after pre-exposure; however, because of their function in chemotaxis, their role in gustatory plasticity remains unclear.

The similarities of the NaCl chemotaxis phenotypes of the *tax-2*, *tax-4* and *tax-6* animals suggested that these genes might function in the same pathway. The expression patterns of these genes overlap only in the ASE neurons (Coburn and Bargmann, 1996; Komatsu *et al*, 1996; Kuhara *et al*, 2002). Since the ASE neurons are essential for NaCl detection, it is likely that the cGMP and Ca^{2+} signalling molecules for the detection of NaCl act in the ASE neurons. However, at present, we cannot rule out a function for these proteins in other sensory neurons or interneurons. Moreover, it is

Figure 6 Gustatory plasticity requires Ca^{2+} signalling. (A) Salt detection was significantly impaired in *tax-6*, *cnb-1* and *ncs-1* animals ($P < 0.01$ at 1 and 10 mM NaCl). These mutants still responded to 100 mM NaCl. (B) TRP channel mutants *osm-9*, *ocr-1* and *ocr-2* showed defects in plasticity ($P < 0.001$). TRP-channel double mutants showed similar defects to the single mutants ($P > 0.05$). The TRP-channel triple mutant showed a very small additive effect of inactivation of all three subunits ($P < 0.05$, compared to *ocr-1* and *ocr-2* animals). The TRP channels function in the same genetic pathway as *gpc-1*, since the response of the *gpc-1 osm-9* double did not differ significantly from the single mutants ($P > 0.05$). (C) *itr-1*, *dgk-1*, *fat-1*, *fat-3* and *fat-4* showed defects in plasticity ($P < 0.001$). FAT-4 functions in the same pathway as OSM-9 and GPC-1, since no significant difference could be detected between double and single mutants ($P > 0.05$). (D) Avoidance of 1 M NaCl was not reduced in *fat-1*, *fat-3*, *fat-4* or *dgk-1* animals.

unclear which signals activate the cGMP/Ca²⁺ NaCl detection pathway.

Besides cGMP-gated channels, also TRP channels mediate Ca²⁺ influx. We have previously shown a function for the TRPV channel subunit OSM-9 in gustatory plasticity (Jansen *et al*, 2002). Here we show that also the OCR-1 and OCR-2 TRPV channel subunits are involved (Figure 6B). The responses of the three TRPV channel mutants did not differ significantly from each other, although the defect seems a little more severe in the *osm-9* animals (Figure 6B). In addition, the behaviour of doubles between these three mutants did not differ significantly from the single mutants (Figure 6B), suggesting that these three subunits function in the same genetic pathway. In the *ocr-1*, *ocr-2*, *osm-9* triple mutant, we observed a very small additive effect of inactivation of all three TRP channel subunits compared to the *ocr-1* and *ocr-2* single mutants (Figure 6B).

The plasticity defects of the TRPV channel mutants were very similar to the defects of the *gpa-1*, *gpc-1*, *arr-1* and *gcy-35* mutants. The behaviour of *gpc-1 osm-9* double-mutant animals was not significantly different from the single mutants (Figure 6B), suggesting that also the TRPV channel subunits function in this genetic gustatory plasticity pathway. The expression patterns of these TRPV genes are restricted to subsets of sensory neurons, overlapping with neurons that play a role in gustatory plasticity: ASH and ADL (Colbert *et al*, 1997; Tobin *et al*, 2002).

TRP channels can be activated in various ways, including by phospholipase C (PLC)-dependent mechanisms (Montell *et al*, 2002). Activation of PLC by G proteins leads to the production of IP₃ and diacylglycerol (DAG). IP₃ can activate IP₃ receptors, resulting in Ca²⁺ release from intracellular stores, which can activate TRP channels. TRP channels can also be activated by DAG, or its derivatives, polyunsaturated fatty acids (PUFAs; Kahn-Kirby *et al*, 2004). We tested if these signalling molecules are involved in gustatory plasticity.

Mutation of both the IP₃ receptor ITR-1 (Baylis *et al*, 1999; Dal Santo *et al*, 1999) and the DAG kinase DGK-1, essential for reduction of DAG levels (Hadju-Cronin *et al*, 1999; Nurrish *et al*, 1999), strongly reduced avoidance after pre-exposure, but did not affect chemoattraction to 0.1–100 mM NaCl or avoidance of 1 M NaCl (Figure 6C and D, results not shown). This indicates that IP₃ and DAG signalling are only involved in the plasticity response. It is possible that these second messengers activate TRP channels, but they could also affect other signalling routes such as the G_oα/G_qα network, which is involved in gustatory plasticity as well (RK Hukema and G Jansen, unpublished results).

To test if PUFAs are involved in gustatory plasticity, we tested three lipid desaturase mutants that affect PUFA synthesis, *fat-1*, *fat-3* and *fat-4* (Watts and Browse, 2002). Recently, strong avoidance defects were described for *fat-3* mutant animals, including a defect in avoidance of 1 M glycerol; mild or no defects were observed for *fat-1* or *fat-4* animals (Kahn-Kirby *et al*, 2004). In our assays, the PUFA synthesis mutants showed no defect in avoidance of 1 M NaCl (Figure 6D), again indicating that separate pathways exist for the detection of 1 M glycerol and 1 M NaCl. However, all three mutants showed aberrant behaviour in our gustatory plasticity assay (Figure 6C), indicating that PUFAs play a role in this process.

Since the PUFA synthesis mutants showed a very comparable behaviour to *gpa-1*, *gpc-1*, *arr-1*, *osm-9* and *gcy-35* animals, we analysed the behaviour of *gpc-1 fat-4* and *osm-9 fat-4* double mutants. The behaviour of these double mutants did not differ significantly from the behaviour of the single mutants, indicating that all these genes function in the same genetic pathway (Figure 6C).

Discussion

Model for gustatory plasticity in *C. elegans*

We can discriminate three responses of *C. elegans* to NaCl: first, chemotaxis to NaCl concentrations between 0.1 and 200 mM, second, avoidance of NaCl above 200 mM, and third, avoidance of otherwise attractive NaCl concentrations (25 mM) after pre-exposure to 100 mM NaCl. Data presented here and by others indicate that these responses require input from at least four chemosensory neurons and the neurons exposed to the body fluid. We propose a model to explain the different gustatory responses (Figure 7).

The ASE neurons are essential for chemotaxis to low NaCl concentrations (green arrows in Figure 7). Chemoattraction is antagonised by avoidance, mediated by the ASH neurons (red arrows in Figure 7). The ASH neurons are not activated at low NaCl concentrations, but become activated at high NaCl concentrations (Hilliard *et al*, 2004). It is unclear where the ASE and ASH derived signals are integrated and where the choice between attraction and avoidance is made. It is also not known why avoidance is preferred over attraction at NaCl concentrations above 200 mM. Perhaps ASE signalling is blocked at high salt concentrations, or alternatively, preference for avoidance might be the default state, for example,

Figure 7 Model for NaCl detection and its plasticity in *C. elegans*. NaCl is detected by the ASE sensory neurons (green arrows), probably using cGMP/Ca²⁺ signalling and an unknown signalling pathway. Attraction is antagonised by avoidance mediated by the ASH neurons (red arrows), using ODR-3, OSM-9, OCR-2 and the inhibitor GRK-2. We propose that, upon prolonged exposure to attractive NaCl concentrations, an ASE-derived signal sensitises the ASH, ADL, ASI and ADF neurons, resulting in avoidance of normally attractive NaCl concentrations. We have shown that the ADF neurons use ODR-3, and the ASI neurons, a second pathway in the ASH neurons and probably the ADL neurons use GPC-1. Integration of these signals ultimately leads to the behavioural response of the animal.

determined by the wiring of the nervous system. Our model suggests that also the ADF, ADL and ASI neurons are not or are only weakly activated at low NaCl concentrations. We propose that upon prolonged exposure to 100 mM NaCl the ASE neurons produce a signal (blue arrows in Figure 7) that sensitises the ADF, ADL, ASI and ASH neurons, resulting in avoidance of otherwise attractive NaCl concentrations (black arrows in Figure 7). We suggest that the ASE neurons signal via the body cavity neurons, AQR, PQR and URX, but it is also possible that the body cavity neurons function downstream of the ADF, ADL, ASI and ASH neurons.

At present, it is not clear which environmental signals modulate the response to NaCl. However, our results show that gustatory plasticity depends on salt concentration and exposure time (Jansen *et al*, 2002). In addition, the involvement of the guanylate cyclase GCY-35 suggests that perhaps oxygen levels might play a role (Gray *et al*, 2004). Finally, our preliminary data suggest that also food signals modulate gustatory plasticity (RK Hukema and G Jansen, unpublished data). In line with these results, Saeki *et al* (2001) have reported that chemotaxis to NaCl is drastically decreased when *C. elegans* are starved on plates that contain NaCl.

Based on the cellular circuit described above, expression patterns and our cell-specific rescue experiments, we can place several molecules in our model (Figure 7). We propose that stimulation of the ASE neurons by low NaCl concentrations activates a cGMP and Ca^{2+} signalling pathway and another unknown pathway. Avoidance of 1 M NaCl, mediated by the ASH neurons, requires the $\text{G}\alpha$ subunit ODR-3 and the TRP channel subunits OSM-9 and OCR-2, and is inhibited by GRK-2. ODR-3 also functions in the ADF neurons in gustatory plasticity, where it might either transduce the avoidance signal, or alternatively transduce the ASE-derived sensitising signal.

In addition, we have identified a genetic pathway that mediates gustatory plasticity. This pathway involves the G γ GPC-1, the $\text{G}\alpha$ GPA-1, the arrestin ARR-1, the TRPV channel subunits OSM-9, OCR-1 and OCR-2, PUFA signalling (FAT-4) and the guanylate cyclase GCY-35. It is unclear if these proteins function in the same cells, but our and previously published data are consistent with a function for GPC-1 in the ASI, ASH and perhaps ADL neurons (based on our rescue experiments), GPA-1 and OSM-9 in these same neurons (based on expression patterns; Colbert *et al*, 1997; Jansen *et al*, 1999), OCR-1 in ADL and OCR-2 in ADL, ASH and ADF (Tobin *et al*, 2002) and GCY-35 in the AQR, PQR and URX body cavity neurons (based on rescue experiments). We cannot discriminate if these proteins transduce the avoidance response or function in a pathway that mediates the ASE-derived sensitising signal. As indicated, part of this model is based on previously described expres-

sion patterns. Most of these expression patterns have been derived using GFP reporter constructs. These constructs may not fully represent the gene's expression pattern. Therefore, additional cell-specific rescue experiments will be needed to confirm our model.

Materials and methods

Strains, genetics and germline transformation

Strains used in this work are listed in Table I. Wild-type *C. elegans* were strain Bristol N2. Germline transformation and transgene integration were performed as described (Mello *et al*, 1991). We used an *elt-2::GFP* construct (30 ng/ μl) as coinjection marker (Fukushige *et al*, 1999). Rescue of *che-1*, *gpc-1*, *odr-3* and *grk-2* and cell inactivation using *mec-4d* and *mec-2* or *egl-2(gf)* were tested using five or more transgenic strains for each clone injected at various concentrations (1–100 ng/ μl). Although cell-specific expression of *mec-2/mec-4d* or *egl-2gf* did affect gustatory plasticity, we did not observe neuronal degeneration.

Molecular biology

Details of plasmid construction are available on request. Promoters used for cell-specific rescue or cell inactivation were: *flp-5* (ASE; Li *et al*, 1999), *gpa-4* (ASI; Jansen *et al*, 1999), *gpa-11* (ADL, ASH; Jansen *et al*, 1999), *gpa-13* (ADF, ASH, AWC; Jansen *et al*, 1999), *sra-6* (ASH, ASI faint; Troemel *et al*, 1995), *srb-6* (ADL, ASH; Troemel *et al*, 1995), *glr-1* (17 classes of neurons; Hart *et al*, 1995; Maricq *et al*, 1995) and *srh-142* (ADF; Sagasti *et al*, 1999). Expression patterns were confirmed using GFP-fusion constructs driven by the same promoter.

Behavioural assays

Chemotaxis towards NaCl and gustatory plasticity were assessed as described before (Wicks *et al*, 2000; Jansen *et al*, 2002), using 0.1, 1, 10 or 100 mM or 1 M NaCl; after pre-exposure to 100 mM NaCl, animals were tested for chemotaxis to 25 mM NaCl. Gustatory plasticity was assayed after 15 min pre-exposure; enhanced plasticity was tested by pre-exposing only for 5 min. All newly identified gustatory plasticity mutants were tested for recovery, by using a 5-min wash in CTX buffer, after the 15-min pre-exposure in CTX buffer containing 100 mM NaCl. All mutants showed wild-type levels of recovery (results not shown). A chemotaxis index was calculated: $(A-C)/(A+C)$, where *A* is the number of worms at the quadrants with NaCl and *C* is the number of worms at the quadrants without attractant. Statistical significance was determined using the two-tailed *t*-test. Error bars represent the s.e.m.

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