An enteric neuron-expressed variant ionotropic receptor detects ingested salts to regulate salt stress resistance

Jihye Yeon[1,3], Laurie Chen[1], Nikhila Krishnan[1,2], Sam Bates[1], Charmi Porwal[1], and Piali Sengupta[1,3]

[1]Department of Biology, Brandeis University, Waltham, MA 02454

[2]Current address: Leica Microsystems Inc., Deerfield, IL 60015

[3]Corresponding authors: P.S.: sengupta@brandeis.edu; J.Y.: jihyeyeon@brandeis.edu

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ABSTRACT

The detection of internal chemicals by interoceptive chemosensory pathways is critical for

regulating metabolism and physiology. The molecular identities of interoceptors, and the

functional consequences of chemosensation by specific interoceptive neurons remain to be fully

described. The *C. elegans* pharyngeal neuronal network is anatomically and functionally

homologous to the mammalian enteric nervous system. Here, we show that the I3 pharyngeal

enteric neuron responds to cations via an I3-specific variant ionotropic receptor (IR) to regulate

salt stress tolerance. The GLR-9 IR, located at the gut lumen-exposed sensory end of I3, is

necessary and sufficient for salt sensation, establishing a chemosensory function for IRs beyond

insects. Salt detection by I3 protects specifically against high salt stress, as *glr-9* mutants show

reduced tolerance of hypertonic salt but not sugar solutions, with or without prior acclimation.

While cholinergic signaling from I3 promotes tolerance to acute high salt stress, peptidergic

signaling from I3 during acclimation is essential for resistance to a subsequent high salt

challenge. Transcriptomic analyses show that I3 regulates salt tolerance in part via regulating the

expression of osmotic stress response genes in distal tissues. Our results describe the

mechanisms by which chemosensation mediated by a defined enteric neuron regulates

physiological homeostasis in response to a specific abiotic stress.

INTRODUCTION

While foraging for food, animals often encounter toxic substances that adversely affect health and longevity. Consequently, animals have evolved multiple mechanisms to identify and cope with harmful chemicals. A first line of defense involves behavioral avoidance mediated by exteroceptive chemosensory neurons present in olfactory and taste organs (Ferkey et al., 2021; Taruno et al., 2021). If ingested, chemicals in the gastrointestinal tract are detected directly or indirectly by non-neuronal cells, as well as gut-intrinsic enteric neurons and extrinsic sensory afferents, to regulate and maintain metabolic homeostasis (Furness et al., 1999; Miguel-Aliaga et al., 2018; Prescott and Liberles, 2022; Spencer and Hu, 2020). In contrast to the well-described exteroceptive chemotransduction pathways, the molecular mechanisms and functional roles of interoceptive chemosensation remain to be fully described.

Inorganic cations such as Na^+ and Ca^{2+} and related salts are essential for the regulation of a range of physiological processes and are typically attractive at low concentrations (Berridge et al., 2003; Taruno and Gordon, 2023). However, high salt concentrations are harmful (Hunter et al., 2022; Rucker et al., 2018), and are detected by gustatory neurons to drive rejection and avoidance, as well as by interoceptive neurons in multiple organs including the gut and brain to modulate salt and fluid intake (Stocker et al., 2024; Taruno and Gordon, 2023). Although the toxic effects of high salt are generally attributed to osmotic stress, comparisons with cellular responses elicited by equiosmolar sugar solutions suggest that individual hypertonic solutes can be discriminated and their concentrations monitored to regulate different physiological responses (Kinsman et al., 2017; Lamitina et al., 2004; Stocker et al., 2024).

Salt sensation is mediated by a diverse range of molecules across species. These include amiloride-sensitive Na^+ channels in mammals, and an expanded family of variant ionotropic

receptors (IRs) in *Drosophila* (Stocker et al., 2024; Taruno and Gordon, 2023). In *Drosophila*,

distinct IR protein complexes expressed in different external and internal taste sensilla have been

implicated in detecting a range of salt concentrations (Kim et al., 2024; Sang et al., 2024; Taruno

and Gordon, 2023). Although IR proteins are encoded by the genomes of all protostomes (Croset

et al., 2010), chemosensory roles for these proteins in species beyond insects remain to be

definitively established.

The nematode *C. elegans* is also attracted to low salt but avoids high salt using a small set

of ciliated chemosensory neurons present in sense organs of the head and tail (Ferkey et al.,

2021). Exposure to high NaCl rapidly paralyzes and eventually kills worms but prolonged

exposure to sub-lethal NaCl concentrations increases tolerance upon subsequent high NaCl

exposure (acclimation) (Lamitina et al., 2004). Worms are also paralyzed rapidly in high sucrose

concentrations (Lamitina et al., 2004), implying that the effects of high salt are due to hypertonic

stress. However, worms survive to different extents on equiosmolar NaCl and sugar suggesting

that as in mammals, unidentified salt-specific sensors and pathways may regulate salt stress

(Lamitina et al., 2004).

Food ingestion in *C. elegans* is primarily driven by the foregut or pharynx (Albertson and

Thomson, 1976; Avery and You, 2012). This neuromuscular organ is innervated by a densely

interconnected, autonomously functioning network of 20 pharyngeal neurons that exhibit

developmental, anatomical, and functional homology to the mammalian enteric nervous system

(Albertson and Thomson, 1976; Cook et al., 2020; Vidal et al., 2022). 15/20 of these pharyngeal

neurons (henceforth referred to as pharyngeal enteric neurons; PENs) exhibit sensory features,

although to date, only two PENs have been shown to respond to bacterial compounds to regulate

feeding and foraging (Bhatla and Horvitz, 2015; Rhoades et al., 2019).

Here we show that the single I3 PEN in *C. elegans* responds to a range of monovalent

and divalent cations and regulates salt stress resistance. Salt detection is mediated by the I3-

specific GLR-9 IR-related protein which is both necessary and sufficient for salt responses. Salt

sensation by I3 is transmitted via cholinergic and peptidergic signaling to increase tolerance to

high salt but not high sugars in unacclimated and acclimated animals, respectively. Salt acts via

both GLR-9-dependent and -independent pathways to alter the expression of genes in distal

tissues including the epidermis and gut that enable salt stress resistance; misregulation of this

gene expression program in *glr-9* mutants likely underlies their salt-sensitivity phenotype.

Together, our results identify a chemosensory function of an IR in nematodes, and elucidate how

a single enteric neuron detects and relays the presence of ingested cations to counter salt toxicity.

RESULTS

The GLR-9 tuning and GLR-7 co-receptor IRs are co-localized to the gut lumen-exposed

sensory ending of the I3 pharyngeal enteric neuron

The *C. elegans* genome encodes 15 predicted ionotropic receptors related to glutamate-

gated cation channels (Brockie et al., 2001; Hobert, 2013) (Fig. 1a). Of these, seven exhibit

features characteristic of variant sensory IRs in insects including amino terminal domains of

varying lengths, the lack of canonical glutamate-interacting residues in their ligand binding

domains, and loss of highly conserved sequences essential for regulating ion permeability in their

pore-forming domains (Benton et al., 2009; Brockie et al., 2001; Croset et al., 2010) (Fig. 1a,

Fig. S1a). Examination of the neuronal transcriptome database (Taylor et al., 2021) showed that

six of these IR-like genes are exclusively or highly expressed in subsets of PENs (Brockie et al.,

2001) (Fig. S1b). In particular, the *W02A2.5* IR gene (henceforth referred to as *glr-9*) is

expressed only in the single I3 PEN (Fig. S1b), suggesting that *glr-9* may play a specialized

sensory role in this neuron.

Consistent with transcriptomics data, an endogenous GFP-tagged GLR-9 fusion protein

was expressed only in I3 (Fig. 1b). The GLR-9 fusion protein localized to a structure that is

present asymmetrically at the I3 ending and protrudes from the cell surface towards the interior

of the animal (Fig. 1b, Movie S1). Reconstruction of the sensory ending of I3 from serial section

electron micrographs also showed a structure that is expanded and curved (Fig. 1c, Movie S2)

(Albertson and Thomson, 1976; Cook et al., 2020), and that may represent the domain occupied

by GLR-9. The internal side of this ending is directly adjacent to and is likely exposed to the

pharyngeal lumen (Fig. 1c, Movie S2). However, I3 is not ciliated and does not express ciliary

genes (Taylor et al., 2021), leaving open the question of the identity of this structure. We infer

that GLR-9 may be positioned in I3 to directly sample ingested molecules present in the

pharyngeal lumen.

Sensory IRs in insects typically function as heterotetramers comprised of a stimulus-

specific tuning receptor and one or more co-receptor(s) that traffic the IR complex to the ciliated

endings of sensory neurons (Abuin et al., 2011; Abuin et al., 2019; Ai et al., 2013). Similar to

other tuning IRs, GLR-9 does not contain an extended amino-terminal domain (Fig. S1a),

suggesting that this IR may also act as a tuning receptor. Among the characterized IR co-

receptors, IR25a is deeply conserved in all protostomes and is considered the ancestral

chemosensory IR (Croset et al., 2010). The *glr-7* IR25a ortholog in *C. elegans* (Croset et al.,

2010) (Fig. 1a) is expressed in a small number of pharyngeal and non-pharyngeal neurons with

the highest expression in I3 (Fig. S1b) (Taylor et al., 2021). A GFP-tagged GLR-7 fusion protein

expressed in I3 was also localized to the sensory ending of I3 (Fig. 1d). Localization of GLR-9

was abolished in *glr-7* mutants and *vice versa* (Fig. 1e). Together, these results suggest that

GLR-9 and GLR-7 may comprise a sensory receptor complex in the I3 PEN.

GLR-9/GLR-7 are necessary and sufficient to mediate I3 responses to high concentrations

of monovalent and divalent cations

To determine whether the GLR-9/GLR-7 complex detects chemicals present in the

pharyngeal lumen, we surveyed chemosensory stimuli-evoked intracellular calcium dynamics in

I3 neurons expressing the GCaMP6s calcium sensor. I3 responded robustly to both bacterial

supernatant as well as bacterial growth media (LB: Lysogeny or Luria Broth) alone (Fig. S2a)

suggesting that I3 responds to one or more chemical components in LB. Further analyses showed

that I3 responded to a range of monovalent and divalent salts including NaCl, NaAc, KPO_4,

NH_4Cl, $MgSO_4$, NH₄Ac, KCl, $NaPO_4$, $(NH_4)_2SO_4$, and $CaCl_2$ (Fig. 2a, Fig. S2b). Replacement of

Na^+ with N-methyl-D-glucamine (NMDG) in NMDG-Cl^- failed to elicit a response, whereas

replacing Cl^- with gluconate in Na^+-gluconate resulted in increased intracellular calcium levels

(Fig. 2b, Fig. S2c), suggesting that I3 responds non-selectively to cations. No responses were

observed to equiosmolar concentrations of fructose, sorbitol, or glycerol (Fig. 2c, Fig. S2d),

indicating that I3 does not respond to high osmolarity.

I3 responded largely in a dose-dependent manner to NaCl, NaAc, KPO_4 and NH_4Cl (Fig.

2d-g). Responses at higher salt concentrations appeared to be tonic with little to no

desensitization, and returned to baseline upon removal of the stimulus (Fig. 2d-h, Fig. S2e). Salt

responses were maintained in *unc-13* and *unc-31* mutants which abolish synaptic or

neuropeptidergic transmission, respectively (Sieburth et al., 2007; Speese et al., 2007) (Fig. S2f),

suggesting that I3 responds to salts cell-autonomously, although we are unable to exclude

communication via gap junctions. Salt responses in I3 were also unaltered in animals mutant for

the *tax-4* cyclic nucleotide-gated and *osm-9* TRPV channel genes required broadly for

chemosensation in the ciliated chemosensory neurons of the head amphid sense organs, as well

as in animals mutant for the *che-1* terminal selector transcription factor that specifies the fate of

the salt-sensing ASE amphid chemosensory neurons (Fig. S2g) (Etchberger et al., 2007; Ferkey

et al., 2021; Uchida et al., 2003). All examined salt responses in I3 were abolished in *glr-9*

mutants; responses were restored in *glr-9* mutants upon I3-specific expression of wild-type *glr-9*

sequences (Fig. 2h, Fig. S2h). We conclude that GLR-9/GLR-7 mediate I3 responses to a broad

range of salts.

We next tested whether GLR-9/GLR-7 are also sufficient to confer salt responses onto a

non salt-sensing neuron. Misexpression of GFP-tagged GLR-9 with or without GLR-7 in the

salt-sensing ASE chemosensory neurons resulted in protein accumulation in the soma and failure

to traffic into sensory cilia (Fig. S2i). Reasoning that pathways required for trafficking and

localization of sensory IRs may be conserved in PENs, we misexpressed GLR-9 and GLR-7 in

the I1 PENs whose distal non-ciliated sensory endings are also apposed to the gut lumen

(Albertson and Thomson, 1976; Cook et al., 2020). Co-expression of both proteins but not each

protein alone led to localization to the I1 sensory endings similar to the pattern in I3 (Fig. S2j).

Although I1 did not respond to NaCl, misexpression of both GLR-9 and GLR-7 in I1 was

sufficient to confer responses to NaCl (Fig. 2i). These observations indicate that GLR-9 and

GLR-7 are both necessary and sufficient to confer salt responses in PENs.

GLR-9-mediated salt sensation promotes salt stress resistance

To examine the functional consequences of I3-mediated salt sensation, we assessed

multiple salt-regulated behaviors in *glr-9* mutants. Salt chemotaxis behaviors were examined in

microfluidics behavioral arenas which allow analyses of behavioral responses to the same salt

concentrations as those used to examine neuronal calcium responses (Albrecht and Bargmann,

2011; Khan et al., 2022). Wild-type animals were robustly attracted to a central stripe of 75 mM

NaCl but avoided 250 mM NaCl (Fig. S3a). *glr-9* mutants exhibited no defects in either salt

attraction or avoidance (Fig. S3a), and also exhibited robust avoidance of high glycerol

concentrations (Fig. S3b). *glr-9* mutants also exhibited no defects in pharyngeal pumping

frequency in low and high salt concentrations in the presence of bacterial food (Fig. S3c). These

observations suggest that salt-sensing by I3 may contribute to organismal responses other than

taxis or feeding.

C. elegans is typically grown on media containing 50 mM NaCl which is considered

isotonic. When moved to media containing >500 mM NaCl, animals rapidly lose cell volume,

become immotile due to loss of turgor pressure, and eventually die (Choe and Strange, 2007a;

Lamitina et al., 2004). Since salt-specific pathways independent of hypertonicity have been

proposed to also regulate salt toxicity (Lamitina et al., 2004), we tested the notion that I3-

mediated salt sensation regulates high salt tolerance. Wild-type animals became rapidly immotile

when transferred from 50 mM NaCl to 500 mM NaCl or high concentrations of other I3-sensed

salts including NaAc, NH_4Cl, or KPO_4 (Fig. 3a). *glr-9* mutants were consistently paralyzed more

rapidly than wild-type animals when moved from low to high NaCl (Fig. 3b), suggesting

decreased salt tolerance. However, *glr-9* mutants did not exhibit enhanced sensitivity to

equiosmolar sucrose solutions, (Fig. 3c, Fig. S3d) further highlighting the salt-specificity of I3

responses. We conclude that I3 activation specifically by salt is partly protective against acute

high salt stress.

While animals are highly sensitive to acute salt stress, pre-adaptation to 200–250 mM

NaCl for 24 hrs allows *C. elegans* to tolerate subsequent exposure to >500 mM NaCl (Lamitina

et al., 2004; Urso et al., 2020). This acclimation is thought to be mediated in part via increased

expression of enzymes required for the production of intracellular osmolytes which enable

osmotolerance (Choe, 2013; Lamitina et al., 2004; Urso and Lamitina, 2021). We examined

whether *glr-9* mutants exhibit reduced salt tolerance even upon acclimation. Wild-type animals

pre-exposed to 250 mM NaCl or 250 mM KPO_4 for as little as 3 hrs continued to exhibit normal

locomotion and pharyngeal pumping for >30 mins when placed on 500 mM NaCl or 500 mM

KPO_4, respectively (Fig. 3d-e). However, a significant fraction of *glr-9* mutants again became

immotile within 5-10 mins when placed on high concentrations of either NaCl or KPO_4 (Fig. 3d-

e). Acclimation was unaffected in animals mutant for signaling genes required for sensory

transduction in ciliated chemosensory neurons (Fig. S3e), indicating that these neurons do not

contribute to high salt resistance. Since I3 exhibits responses to multiple salts, we next tested

whether acclimation to sub-lethal concentrations of one salt enables tolerance of high

concentrations of a different salt. Pre-exposure to 250 mM NaCl was sufficient to partly increase

tolerance of wild-type but not *glr-9* mutant animals to 500 mM KPO_4 (Fig. 3f). Taken together,

we conclude that GLR-9-dependent salt responses in I3 are necessary for tolerance of high salts

in both unacclimated and acclimated conditions.

Cholinergic and FLP-6 neuropeptide-mediated signaling from I3 are necessary for high

salt tolerance under different conditions

Osmoregulatory mechanisms operate in multiple tissues and organs to regulate

osmotolerance in *C. elegans* (Choe, 2013; Urso and Lamitina, 2021), suggesting that I3 may

signal to one or more of these tissues to regulate salt stress resistance. I3 is a cholinergic neuron

and also expresses multiple neuropeptides (Cook et al., 2020; Taylor et al., 2021). Knocking out

the *unc-17* vesicular acetylcholine transporter specifically in I3 using Cre-Lox mediated

recombination (Huang et al., 2023) resulted in rapid paralysis upon exposure to high salt similar

to *glr-9* mutants (Fig. 4a), indicating that cholinergic signaling from I3 is necessary for acute salt

tolerance.

We next addressed the mechanisms by which GLR-9-mediated signaling contributes to

salt acclimation. I3 responses to 500 mM NaCl were similar in animals with or without prior

acclimation (Fig. S4a), indicating that acclimation does not alter I3 responses to high salt. Loss

of *unc-17* specifically in I3 also did not affect acclimation (Fig. S4b), suggesting that cholinergic

signaling from I3 regulates salt tolerance only under unacclimated conditions. Since I3 expresses

multiple neuropeptides (Taylor et al., 2021), we next knocked out the *egl-21* carboxypeptidase

required for processing of a subset of neuropeptides (Jacob and Kaplan, 2003) specifically in I3

using Cre-Lox mediated recombination (Huang et al., 2023) and assessed acclimation. Animals

lacking *egl-21* in I3 became immotile more rapidly in high salt similar to *glr-9* mutants following

acclimation (Fig. 4b), but responded similarly to wild-type animals upon acute salt challenge

(Fig. S4c). We conclude that peptidergic signaling from I3 is required for acclimation and

subsequent tolerance of high salt but is not required under conditions of acute salt stress.

Neuronal transcriptomics analyses indicate that I3 expresses at least 24 neuropeptide

genes at different levels (Taylor et al., 2021). Analyses of salt acclimation phenotypes of a subset

of I3-expressed neuropeptides showed that animals mutant for the *flp-6* FMRFamide-like

neuropeptide failed to tolerate high salt following acclimation (Fig. S4d-e). The salt tolerance

phenotype of *glr-9; flp-6* double mutants was similar to that of each single mutant alone

suggesting that these molecules act in a linear pathway to regulate acclimation (Fig. S4e).

However, *flp-6* mutants behaved similarly to wild-type animals upon acute high salt exposure

(Fig. S4f). Since *flp-6* is expressed in multiple neuron types in addition to I3 (Kim and Li, 2004;

Taylor et al., 2021), we confirmed that *flp-6* acts in I3 by knocking out *flp-6* specifically in I3 via

Cre-Lox-mediated recombination. Loss of *flp-6* expression specifically in I3 was again sufficient

to reduce tolerance of high salt following acclimation similar to the phenotype of *flp-6* global

mutants, but did not affect tolerance in an acute high salt challenge (Fig. 4c, Fig. S4f). FLP-6 has

been shown to interact with multiple candidate neuropeptide receptors that are expressed broadly

in neuronal and non-neuronal cells (Beets et al., 2023). However, mutations in single candidate

receptor genes had minor or no effects on salt acclimation (Fig. S4g) suggesting that FLP-6

receptors may act redundantly within a single cell or across multiple cell/tissue types to regulate

salt tolerance. Together, these results indicate that FLP-6-mediated signaling from I3 is

necessary to promote tolerance of high salt following acclimation.

We next examined the temporal requirement for neuropeptide signaling from I3 to

regulate salt tolerance following acclimation. To temporally modulate neuropeptide release from

I3, we expressed the auxin receptor TIR1 specifically in I3 in a strain in which the *unc-31* CAPS-

related protein required for dense core vesicle fusion (Speese et al., 2007) is endogenously

tagged with degron sequences (Cornell et al., 2022; Nishimura et al., 2009; Zhang et al., 2015).

Addition of exogenous auxin to the growth plates during acclimation and exposure to high salt

resulted in rapid paralysis further supporting the necessity of peptidergic signaling from I3 for

tolerance of high salt (Fig. 4d). Addition of auxin only during the 3 hr acclimation period but not

during high salt exposure was sufficient to induce rapid paralysis (Fig. 4d), indicating that

peptidergic signaling from I3 is necessary only during the acclimation period to promote high

salt tolerance. Taken together, our results indicate that cholinergic signaling from I3 increases

tolerance of acute salt stress (Fig. 4e). In contrast, FLP-6 neuropeptide signaling from I3 during

acclimation is protective in response to subsequent high salt exposure (Fig. 4e).

Salt acclimation alters gene expression via both GLR-9-dependent and -independent

pathways

The rapidity with which *C. elegans* loses fluid and paralyzes in hypertonic solutions is in

part determined by the permeability of the animal's cuticle as well as the concentration of

internal osmolytes. Consistently, increased salt resistance following acclimation occurs as a

consequence of altered expression of molecules such as cuticular collagens and osmolyte

biosynthetic enzymes predicted to be protective against hypertonicity (Burkewitz et al., 2012;

Dodd et al., 2018; Lamitina et al., 2004; Lamitina et al., 2006; Rohlfing et al., 2010). We

hypothesized that I3 signaling regulates the expression of a subset of genes in distal tissues such

as the cuticle, epidermis and gut that contribute specifically to salt tolerance, and that failure to

correctly regulate these genes in *glr-9* mutants contributes to their decreased salt resistance. To

test this notion, we compared the transcriptional profiles of wild-type and *glr-9* mutants prior to

and following 3 hrs of salt acclimation (Fig. 5a-b).

Principal component analyses indicated that transcriptional profiles clustered based on

both genotype and condition (Fig. S5a). Comparison of the transcriptional profiles of wild-type

animals prior to and following acclimation identified 92 and 33 significantly up- and down-

regulated genes, respectively (Fig. 5a, Data Table 1). A small subset of these differentially

expressed genes overlapped with those identified previously using partly distinct experimental

conditions (Dodd et al., 2018; Rohlfing et al., 2011) (Fig. S5b). Consistent with previous

observations, the *gpdh-1* and *gpdh-2* glycerol-3-phosphate dehydrogenase, *pgph-2*

phosphoglycolate phosphatase, and *tre-5* trehalase encoding genes were upregulated following

acclimation (Fig. 5a,c, Data Table 1). Transcriptional upregulation of these enzymes upon

hypertonic stress contributes to the production of the osmolyte glycerol which is protective for

hypertonic stress (Dodd et al., 2018; Lamitina et al., 2004; Lamitina and Strange, 2005; Possik et

al., 2022; Rohlfing et al., 2011). The expression of a subset of genes encoding collagen was also

altered consistent with a role for cuticular components in regulating hypertonic stress responses

(Fig. 5a,d, Data Table 1) (Choe, 2013; Dodd et al., 2018; Lamitina et al., 2006; Rohlfing et al.,

2011; Solomon et al., 2004; Urso and Lamitina, 2021; Wheeler and Thomas, 2006; Wimberly

and Choe, 2022).

Acclimation resulted in altered expression of a larger set of genes in *glr-9* mutants (Fig.

5b, Data Table 1). However, the expression of *gpdh-1*, *pgph-2* and *tre-5* was no longer

significantly altered in *glr-9* mutants (Fig. 5b-c, Data Table 1), indicating that I3-mediated

signaling in response to salt is necessary for the upregulation of these enzymes. Although a

subset of genes including genes encoding cuticular collagens was regulated similarly in both

wild-type and *glr-9* mutants following acclimation (Fig. 5e, Data Table 1), the expression of

distinct sets of genes implicated in stress responses and in the regulation of metabolism, as well

as genes are predicted to encode components of the cuticle or hypodermis, was altered in

acclimated *glr-9* mutants (Fig. 5b,d, Fig. S5c, Data Table 1). We conclude that altered gene

expression upon salt acclimation is mediated via both I3-dependent pathways that may be salt-

specific, and I3-independent pathways that may respond to general hypertonic stress.

We noted that the transcriptional profile of *glr-9* mutants was distinct from those of wild-

type animals even at 50 mM NaCl (Fig. 5c-d, Fig. S5a,d, Data Table1) suggesting that chronic

I3-mediated signaling may be necessary for regulating physiology even at isotonic salt

concentrations. Significantly upregulated genes in *glr-9* mutants included genes implicated in the

regulation of metabolism and stress, multiple components of the cuticle and hypodermis

including collagen and chitinase-like genes, as well as nematode-specific groundhog-like (GRL)

and ground-domain (GRD) secreted proteins that are apical extracellular matrix components of

the cuticle and precuticle (Hao et al., 2006; Serra et al., 2024) (Fig. 5b,d, Fig. S5d, Data Table 1).

These observations suggest that chronic *glr-9* signaling under standard growth conditions may

signal the presence of salt, and that the cuticular barrier may be structurally remodeled in the

absence of this signaling, potentially contributing to the decreased resistance of these animals to

acute salt stress (see Discussion).

To assess the functional significance of these expression changes, we tested two

predictions arising from the transcriptional profiling data. Animals moved from 50 mM NaCl to

either 400 mM or 500 mM NaCl exhibit similar initial responses including rapid shrinkage and

paralysis (Choe, 2013; Choe and Strange, 2007a). However, while prolonged exposure to 500

mM NaCl leads to death (Lamitina et al., 2004; Solomon et al., 2004), animals regain body

volume and motility after 2-4 hrs on 400 mM NaCl via modulation of ion transport and

upregulation of osmotolerance genes (Choe and Strange, 2007a, b). The failure of *glr-9* mutants

to upregulate osmotolerance genes and their extensively remodeled cuticle even under isotonic

conditions predicts that these animals will be unable to recover, or will recover more slowly, on

400 mM NaCl. Consistent with this prediction, we found that *glr-9* mutants regained motility

significantly more slowly than wild-type animals under these conditions (Fig. S6a). These results

315 further support the notion that I3 signaling-regulated gene expression changes play a critical role

316 in regulating salt tolerance.

317   The finding that salt acclimation results in gene expression changes that are partly I3-

318 dependent and are thus salt-specific, also predicts that different hypertonic solutions may alter

319 the expression of different gene subsets, with only a shared subset being regulated by general

320 osmotic stress. This hypothesis predicts that acclimation to high salt would not be protective in

321 response to high sugar challenge and *vice versa*. We found that acclimation to 250 mM NaCl did

322 not increase tolerance of either wild-type or *glr-9* mutants to 500 mM sucrose, and conversely,

323 acclimation to 250 mM sucrose had no effect on resistance to 500 mM NaCl under our

324 experimental conditions (Fig. S6b). We infer that partly distinct pathways contribute to the

325 detection of individual hypertonic solutes to regulate specific organismal stress responses.

327 **DISCUSSION**

328   Here we show that salt-sensing by the single I3 enteric neuron specifically promotes salt

329 stress resistance in *C. elegans.* I3 salt responses are dependent on the GLR-9/GLR-7 IR proteins

330 which are both necessary and sufficient for salt detection. I3 signals via cholinergic and

331 peptidergic signaling to increase salt stress resistance in unacclimated and salt-acclimated

332 animals, respectively. We find that signaling from I3 modulates the expression of genes that

333 regulate cuticle composition and internal osmolyte concentration, resulting in tolerance of high

334 salts but not high sugars (summarized in Fig. 5f). Our observations describe the molecular and

335 neuronal mechanisms by which chemosensation by an enteric neuron regulates physiological

336 homeostasis in response to a specific abiotic stress.

Chemoreceptors are among the fastest evolving gene families in multicellular animals,

with some gene families being entirely lost or gained within specific lineages (Policarpo et al.,

2024; Sanchez-Gracia et al., 2009; Valencia-Montoya et al., 2024). For example, while variant

IR sensory proteins are found in all protostomes, they are absent from deuterostomes (Croset et

al., 2010; Valencia-Montoya et al., 2024). However, to date, chemosensory roles for these IR

proteins have been described and/or suggested primarily in arthropods (Benton et al., 2009; Eyun

et al., 2017; Graeve et al., 2022; Groh-Lunow et al., 2014; Ni, 2020). Our identification of a

chemosensory function of an IR in *C. elegans* implies that IRs may have retained chemosensory

functions in the protostome lineage through evolution. It remains to be determined whether the

remaining PEN-expressed IRs in *C. elegans* also function as chemosensors, or whether similar to

some *Drosophila* and mosquito IRs (Ni, 2020; van Giesen and Garrity, 2017), subsets of these

proteins also detect other sensory cues.

GLR-9-mediated salt sensation is transmitted via distinct signaling pathways from I3 to

modulate gene expression in tissues and organs such as the gut and cuticle to enhance salt

tolerance. We find that even under isotonic conditions, *glr-9* mutants exhibit broad changes in

the expression of genes that may impact hypertonic salt resistance. Since I3 responds tonically to

a range of salt concentrations, we propose that chronic activation of GLR-9 and cholinergic

signaling from I3 upon growth on low salt enhances resistance to acute high salt. Remodeling of

the cuticle in the absence of I3 signaling may thus sensitize animals to high salt stress, resulting

in more rapid paralysis upon an acute high salt challenge (Fig. 5f). During acclimation, FLP-6-

mediated signaling from I3, as well as from other neuronal and non-neuronal osmosensory

pathways, drive broad gene expression changes that promote salt tolerance. The failure to alter

the expression of a subset of these genes, including osmolyte biosynthesis genes, in acclimated

glr-9 mutants, along with their dysregulated gene expression under isotonic conditions, may

underlie their decreased salt tolerance phenotype (Fig. 5f). Additional pathways likely also

contribute to osmotolerance under different conditions and timepoints following stress exposure

(Huang et al., 2007; Igual Gil et al., 2017; Lee et al., 2016).

While exposure to hypertonic solutions can cause general effects such as altered protein

quality control and tissue deformation (Burkewitz et al., 2011; Choe and Strange, 2008; Lamitina

et al., 2006; Urso and Lamitina, 2021), several lines of evidence including our findings suggest

that *C. elegans* and other animals can discriminate among different hypertonic solutes. First,

equiosmolar concentrations of salt and sucrose result in distinct survival rates in *C. elegans*

(Chandler-Brown et al., 2015; Choe and Strange, 2007a; Lamitina et al., 2004), implying that

individual osmotic stressors can elicit partly different organismal responses. Second, *glr-9*

mutants exhibit decreased tolerance of high salts but not equiosmolar sugar, highlighting the salt-

specificity of the I3 pathway. Third, acclimation to NaCl does not confer resistance to sucrose

and *vice versa* under our experimental conditions (see (Chandler-Brown et al., 2015)), indicating

that the adaptive response to one osmotic stressor is not fully sufficient to protect against others.

Fourth, hypertonic salt regulates the expression of a subset of osmotolerance genes in a GLR-9-

dependent manner, further suggesting the presence of solute-specific pathways. Similarly, in

rodents, while a number of physiological responses are independent of hyperosmotic solute

identity, arterial blood pressure regulation and sympathetic nerve activity are more sensitive to

central NaCl infusion than to equiosmolar sugars (Kinsman et al., 2017). Moreover, subsets of

OVLT neurons in the circumventricular organs of the brain preferentially respond to hypertonic

NaCl over equiosmolar sugars (Kinsman et al., 2017; Stocker et al., 2024), indicating the

existence of NaCl-specific, non-osmosensory pathways. Given the critical role of salts in

regulating physiological processes, we suggest that dedicated neuronal pathways for high salt

detection provides additional flexibility in coordinating responses based on internal state.

Enteric sensory neurons in *C. elegans* appear to utilize partly distinct sensory

mechanisms from those of somatic exteroceptive sensory neurons. For instance, the GUR-3

gustatory receptor-like protein required for hydrogen peroxide detection by the I2 PEN in *C.*

elegans, and the DEL-3 and DEL-7 ASIC channels that detect ingested bacteria in the NSM PEN

are expressed exclusively or primarily in PENs (Bhatla and Horvitz, 2015; Rhoades et al., 2019;

Taylor et al., 2021). Similarly, 6 of the 7 IRs including GLR-7 and GLR-9 are expressed

primarily or highly in PENs. Moreover, PENs are non-ciliated unlike exteroceptive sensory

neurons, suggesting novel mechanisms for receptor trafficking and localization at their sensory

endings. The intrinsic enteric neuronal network has ancient evolutionary origins and is thought to

have arisen prior to and independently of the central nervous system (Furness and Stebbing,

2018; Watanabe et al., 2009). Investigating the mechanisms underlying enteric neuron

development and function in diverse organisms may provide insights into ancestral neuronal

strategies, and expand our understanding of interoceptive sensory functions across species.

METHODS

Growth of *C. elegans* strains

C. elegans strains were grown on NGM plates seeded with *E. coli* OP50 at 20˚C. *unc-*

*122*p*::dsRed* or *gfp* were used as co-injection markers (at 40 ng/μl) to generate transgenic strains.

Strains were generated using standard genetic crosses. The genotypes of all strains were verified

by sequencing or phenotypic examination. All experiments were performed using young adult

hermaphrodites grown under well-fed conditions for at least three generations. A list of all

strains used in this work is provided in Table S1.

Molecular biology

1.1 kb of sequences upstream of and including the *glr-9* START codon were amplified

from wild-type animal lysate and used to drive expression specifically in I3. Expression in I1

was driven using 1.1 kb of sequences upstream of the *lgc-8* START codon. GLR-9 cDNA was

amplified from wild-type animal lysate and tagged with *gfp* for localization and for functional

rescue experiments. Since *glr-7* has *a* and *b* isoforms, the genomic region of *glr-7* from the

START codon of the *b* isoform (X: 2,419,477) to the STOP codon of both *a* and *b* isoforms

(X:2,414,064) was amplified from wild-type animal lysate. These sequences were tagged with

TagRFP to examine localization of *glr-7*. All plasmids were generated using standard cloning

methods and verified by Sanger sequencing. A list of plasmids used in this work is provided in

Table S2.

CRISPR/Cas9-mediated genome editing

Deletion alleles of *glr-9* and *osm-9* were generated using an injection mix containing 0.5

μl of Cas9 (IDT), 1.5 μl of 100 μM tracrRNA, 1 μl of 50 μM 5'-crRNA, 1 μl of 50 μM 3'-

crRNA, 1 μl of 17 μM *dpy-10* crRNA as a co-CRISPR injection marker, and nuclease-free water,

for a total volume of 10 μl. No DNA donor was used. Worms were maintained at 25°C after

injection. Three days following injection, dumpy or roller F1 progeny were segregated onto

individual plates. After the singled F1 animals laid eggs, parents were lysed and genotyped using

PCR.

To generate the *flp-6::loxP* insertion strain (PY12288, Table S1), the injection mix

included 0.5 µl of Cas9 (IDT), 2.8 µl of 34 µM crRNA, 0.2 µl of 100 µM tracrRNA, 2 µl of 26

µM donor DNA, 50 ng/µl of column-purified *unc-122*p*::gfp*, and nuclease-free water for a total

volume of 20 µl. Following injection, worms were kept at 25°C. F1 progeny carrying the *unc-*

*122*p*::gfp* co-injection marker were singled onto individual plates and genotyped. The C-

terminal *loxP* site was inserted first, followed by insertion of the N-terminal *loxP* site. All

tracrRNA, crRNAs, and donor DNAs were obtained from IDT. The sequences of the crRNAs

and donor DNA are listed below.

glr-9	crRNA #1	5'- atatgcactttcattcaat -3'
(oy180)	crRNA #2	5'-ccacattgtgaaatctttac-3'
osm-9	crRNA #1	5'- tttttcagaaaccaaacat-3'
(oy202)	crRNA #2	5'–acactttgtgtaccatactt-3'
flp-6::loxP (oy203)	crRNA #1 (N-terminal *loxP*)	5'-cttattgaacaccatggcaa-3'
	crRNA #2 (C-terminal *loxP*)	5'-cttcgtttccttcgattgat-3'
	Donor #1 (N-terminal *loxP*)	5' - ccgtaatttttgtaagtaaaaagagagacattcgtttttcaataacttcgtatagcatacattata cgaagttatcttattgaacaccatggcaatgagatactagtttccgctgatttgtgatattttc cctttgattgaaaac - 3'

	Donor #2 (C-terminal *loxP*)	5' - gtaaacctacactttaatccctactttttacaattctactagtcccatataacttcgtatagc atacattatacgaagttatactcttcgtttccttcgattgataaagttttgcaccgtgtttttca cacagaatcacctcttttatc -3'

Phylogenetic analysis

iGluR sequences from *Mus musculus* and *Drosophila melanogaster* were obtained from

Croset et al (Croset et al., 2010). iGluR and IR sequences from *C. elegans* were obtained from

Wormbase (www.wormbase.org). Sequences were aligned using MUSCLE (Edgar, 2004), and a

phylogenetic tree was constructed using the Maximum Likelihood method with adaptive

bootstrapping (116 replicates) in MEGA 12.0.9. The resulting tree was visualized and modified

using FigTree v1.4.4.

Fluorescent reporter imaging

1 day-old adult animals were anesthetized with 10 mM (-)- tetramisole hydrochloride

(Sigma-Aldrich L9756) and mounted on 10% agarose pads on microscope slides. Images in

Figure 1b,d, Fig. S2i were acquired at 0.2 μm *z*-intervals using a 100× oil immersion objective

on an inverted two- camera spinning disk confocal microscope (Leica DMI6000B with a CSU-

W1 spinning disk head and two Andor Neo sCMOS cameras) and Andor IQ3.5 Software. Images

in Figure 1e and Fig. S2j were acquired using a 64× oil immersion objective on an inverted

spinning disk confocal microscope (Zeiss AxioObserver with Yokogawa CSU-X1 confocal head

system and QuantEM SC512 camera) and SlideBook™ software. All images were processed

using FIJI/Image J.

3D reconstruction of I3 sensory ending

Serial section electron microscope (EM) images of I3 were obtained from Wormimage (www.wormimage.org). I3 was identified as "blue 14" on the EM images (with the assistance of David Hall), specifically from the sections N2T_114522 to N2T_114583. The TrakEM2 plugin in Fiji was used for manually aligning I3 images. Following the alignment, manual segmentation was performed using 3dmod v4.11 from the IMOD suite (https://bio3d.colorado.edu/imod/) to generate the 3D reconstruction of the I3 sensory ending.

Acute salt stress assay

Acute responses to high salt were examined using a protocol adapted from (Rohlfing et al., 2010). 6 cm unseeded NGM plates supplemented with the indicated salt concentrations were divided into two sections to assess the behaviors of wild-type and *glr-9* mutant in parallel. Growth-synchronized 1-day old adult hermaphrodites were transferred from NGM plates containing 50 mM NaCl to high salt-containing plates to initiate the assay. Each assay included 10 animals of each genotype, and the time at which all 10 animals were unresponsive to touch with a platinum wire was recorded. Unresponsive was defined as failure to move upon prodding with a platinum wire at the nose and tail, and cessation of pharyngeal pumping. At least three independent assays were conducted over multiple days for each data point.

Salt acclimation assay

Acclimation experiments were performed using an adaptation of a previously published protocol (Rohlfing et al., 2010). 35 growth-synchronized 1-day adult hermaphrodites were

transferred from NGM growth plates (50 mM NaCl) to NGM plates containing 250 mM NaCl,

250 mM KPO_4, or 250 mM sucrose and seeded with 200 µl *E. coli* OP50. Animals were

maintained at 20˚C for 3 hrs. Following 3 hrs of acclimation, animals were transferred to

unseeded plates containing 500 mM NaCl, 500 mM KPO_4, or 500 mM or 1 M sucrose, and

tested for responses upon prodding with a platinum wire after 5, 10, 15 and 30 mins.

Unresponsive was defined as failure to move upon prodding with a platinum wire at the nose and

tail, and cessation of pharyngeal pumping. At least three independent assays were conducted

over multiple days for each data point.

Salt recovery assay

Recovery from paralysis on 400 mM NaCl was examined using an adaptation of

previously published protocols (Choe and Strange, 2007a; Lamitina et al., 2004). Bacteria-

seeded growth plates were supplemented with 400 mM NaCl and divided into two sections to

assess wild-type and *glr-9* animals in parallel. Growth-synchronized 1-day old adult

hermaphrodites were transferred from growth plates containing 50 mM NaCl to the assay plates

and were examined every 15 mins up to 4 hrs. Each assay included 10 animals of each genotype.

Unresponsive was defined as failure to move upon prodding with a platinum wire at the nose and

tail, and cessation of pharyngeal pumping. At least three independent assays were conducted

over multiple days for each data point.

Glycerol avoidance assay

The avoidance assay was performed as previously described (Culotti and Russell, 1978).

Briefly, a ring of 8M glycerol with xylene cyanol (Sigma-Aldrich X4126) was pipetted onto an

unseeded NGM plate dried overnight. For each assay, 10 one-day-old adult hermaphrodites were

transferred to the center of the glycerol ring. After 10 mins, the number of animals inside and

outside the ring was counted. Wild-type and *glr-9(oy180)* mutants were examined in parallel.

Auxin-induced degradation

400 mM Auxin [1-naphthaleneacetic acid (NAA), Sigma 317918)] was dissolved in

EtOH and added to NGM containing the indicated salt concentrations to make 4 mM auxin-

containing plates. Control plates contained equal concentrations of EtOH alone. Plates were

seeded with 100 μl *E. coli* OP50, dried overnight, and stored at 4˚C wrapped in aluminum foil to

prevent light exposure.

At least 70 young adult hermaphrodites were transferred from NGM growth plates to

plates containing 250 mM NaCl supplemented with either auxin or ethanol. After 3 hrs

acclimation, animals were tested for movement on plates containing 500 mM NaCl

supplemented with either auxin or ethanol. Animals were examined at 5, 10, 15, and 30 mins for

responses to touch with a platinum wire. Each assay included 30 animals per condition and per

genotype and was conducted over multiple days. At least 2 sets of experiments were performed

for each genotype on the same day. Experimental and control strains were examined together in

the same assays in each experiment.

Quantification of pumping frequency

Pumping frequency was measured manually using 1-day old adult animals on OP50-

seeded NGM plates containing 50 mM or 250 mM NaCl. For each data point, the pumping

frequency of individual animals was measured three times for 20 sec each to obtain the average

pumping frequency. Wild-type and *glr-9* mutants were examined together in the same assays for

each experiment. At least three independent assays were conducted over multiple days for each

data point.

Microfluidics behavioral assay

Behavioral assays using microfluidics devices were performed essentially as previously

described (Khan et al., 2022). After degassing the assembled microfluidic devices, the outlet port

was loaded with 5% v/v poloxamer surfactant (Sigma P5556) containing 2 mg/ml xylene cyanol

to eliminate bubbles. 20 to 30 1-day old adult hermaphrodites were transferred to unseeded

NGM plates, washed with water, and loaded into two separate arenas within a single device. *glr-*

9 mutants were assessed in the same device, with wild-type controls placed in the adjacent arena.

After allowing the worms to disperse (<5 min), the stimulus flow containing either 75 mM NaCl

or 250 mM NaCl and 2 mg/ml xylene cyanol for visualization was initiated. 20 min videos were

recorded at 2 Hz using a PixelLink camera. Microfluidic devices were cleaned after each

experiment by washing with water and 100% ethanol before reuse. Custom MATLAB software

was used to process and analyze all recordings, and data were visualized using custom R scripts

(https://doi.org/10.5281/zenodo.13748735). Mean residency histograms and chemotaxis indices

were analyzed as previously described (Khan et al., 2022). Three independent assays were

performed over multiple days.

Calcium imaging

Custom microfluidic devices for calcium imaging were fabricated as described previously

(Khan et al., 2022). Images were acquired using an Olympus BX52WI microscope with a 40× oil

objective and a Hamamatsu Orca CCD camera at 4 Hz with 4×4 binning. All stimuli were

diluted in filtered Milli-Q water, and animals were paralyzed with 10 mM (-)-tetramisole

hydrochloride (Sigma L9756) dissolved in water prior to loading on the unseeded NGM. Stimuli

dilutions were prepared fresh in water on the day of each imaging session. Responses to 30 sec

of water, 30 sec of stimuli, and 30 sec of water were recorded in I3. Data were collected from

biologically independent experiments conducted over at least two days. For GCaMP

fluorescence analysis, recorded videos were processed using Fiji as previously described (Khan

et al., 2022). Briefly, images were aligned using the Template Matching plugin, and cell body

and background fluorescence were calculated using manually drawn ROIs. Background-

subtracted fluorescence intensity values were used for analysis in R Studio. The baseline

fluorescence intensity (F_0) was calculated as the average $\Delta F/F_0$ value over 5 sec prior to stimulus

onset. R Studio was used to compute the response mean and SEM

(https://doi.org/10.5281/zenodo.13748735). Peak $\Delta F/F_0$ values were calculated as the maximum

change in fluorescence relative to F_0 within 10 sec following stimulus addition.

RNA-Seq

Animals were synchronized by bleaching adults, collecting eggs, and then arresting

hatched L1s in the absence of food for ∼16 hours. ~1,000 animals were cultured on *E. coli* OP50

seeded at 1 ml per 10 cm plate at 20˚C until the 1 day-old adult stage. Animals from a total of

four 10 cm plates were used per sample. Animals were washed with M9 containing 0.01% Triton

(Sigma T8787) and placed onto either 10 cm OP50 seeded NGM plates or NGM plates

supplemented with 250 mM NaCl for 3 hrs at 20˚C. Animals were then washed off the plates

with M9 containing 0.01% Triton. Total RNA extraction was performed using TRIzol

(Invitrogen 15596026) and chloroform (Sigma 288306), followed by RNA Clean &

Concentrator™-5 (Zymo Research R1014) to extract total RNA. The quality and concentration

of total RNA was assessed using Agilent High Sensitive RNA ScreenTape analysis (Agilent

5067-5579).

100 ng/µl of total RNA per sample was used as input to generate a sequencing library

using the xGen™ Broad Range RNA library preparation Kit (IDT 10009865) with AMPure XP

Beads (Beckman Coulter A63880). Agilent DNA ScreenTape (D1000 5067-5582) assays were

used to assess the quality of the library prior to sequencing. Libraries were paired-end sequenced

at 75x75 bases on a NextSeq 1000 system (Illumina). Sequencing reads were adapter trimmed

using Trim Galore (https://doi.org/10.5281/zenodo.7598955), mapped to the WBcel235 *C.*

elegans genome, and counted using STAR (Dobin et al., 2013) with alignMatesGapMax 2500.

Differential expression analysis was performed in R studio with DESEQ2 (Love et al., 2014)

(BioProject accession number PRJNA1227491). The PCA, heatmaps, and volcano plots were

generated using the ggplot package in R. Venn diagrams were created using VennDiagram in R.

GO terms, Wormbase, WomCat, Uniprot, and PANTHER were used to categorize individual

genes listed in Data Table 1.

Statistical analyses

Statistical analyses were performed using Prism v10.4.1. Comparisons to wild-type were

performed with t-test or one-way ANOVA across the genotype and condition unless indicated

otherwise. Corrections for multiple comparisons were performed using Dunnett's tests. Scatter

plots and bar graphs were generated and analyzed using Prism 10. The statistical tests used,

significance values, and the number of animals and assays are indicated in the legend to each

Figure.

Data availability

Underlying data for all behavioral and calcium imaging experiments have been deposited

to Figshare. All other data necessary for confirming the conclusions of this article are

represented fully within the article and its tables and figures.

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819

820
821

a

b
GLR-9::GFP
I3p::myrtagRFP

GLR-9
I3

c
Pharyngeal lumen
I3
Vacuole

Pharyngeal lumen
Vacuole
9.3 μm

d
*glr-7*p::GLR-7::GFP
I3p::myrtagRFP

GLR-7
I3

e
GLR-9::GFP
I3p::myrtagRFP
glr-7

*glr-7*p::GLR-7::GFP
I3p::myrtagRFP
glr-9

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Figure 1

Fig. 1. GLR-9 and GLR-7 are localized at the pharyngeal lumen-exposed sensory ending of

I3.

a) A rooted polar phylogenetic tree of ionotropic glutamate receptor sequences from *Drosophila*

(Dmel), *C. elegans* (Cele; bolded) and mouse (Mmus). Variant *C. elegans* IRs are indicated in

bold red; canonical ionotropic glutamate receptors are in bold black. The scale bar represents the

branch length, with each unit equivalent to 0.2 nucleotide substitutions per site.

b,d) Representative images of GLR-9 (b) or GLR-7 (d) fusion protein localization in I3. The I3

sensory ending is boxed and magnified in images on the right. GLR-9 is tagged with GFP at its

endogenous locus; *glr-7*p::GLR-7::GFP is expressed from an extrachromosomal array. I3 is

marked via the expression of *glr-9*p::myrtagRFP. Anterior is at top in all images. Scale bars: 10

μm (images at left); 0.5 μm (images at center and right).

c) (Left) Cross-section electron microscope image approximately 20 μm from the worm nose

showing the pharyngeal lumen, a vacuole, and I3 (shaded). (Right) 3D reconstruction of the I3

distal ending from electron microscope serial sections. The location of the pharyngeal lumen is

shown. The length of the reconstructed I3 ending is indicated at right. A dashed box indicates the

approximate position of the electron microscope cross-section image shown at left. Arrowhead

indicates the sensory ending. Scale bar: 0.25 μm.

e) Localization of GLR-9::GFP or GLR-7::GFP in I3 in *glr-7(tm2877)* or *glr-9(oy180)* mutant

backgrounds, respectively. Anterior is at top. The I3 sensory ending is boxed and magnified in

the insets. Scale bars: 10 μm; 2.5 μm (insets).

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Figure 2

Fig. 2. I3 responds to multiple monovalent and divalent cations in a GLR-9-dependent manner.

a-c) Average changes in GCaMP6s fluorescence in I3 in response to a 30 sec pulse of the indicated salts (a,b) or sugars (c) at the shown concentrations. Quantification of peak fluorescence intensities are shown in Fig. S2b-d.

d-g) Average (top) and peak intensity changes (bottom) of GCaMP6s fluorescence in I3 in response to a 30 sec pulse of different concentrations of NaCl, NaAc, KPO_4, and NH_4Cl. * and ** indicate different from corresponding 50 mM values at $P< 0.05$ and 0.01, respectively (one-way ANOVA and Dunnett's test).

h) Average (left) and peak intensity changes (right) of GCaMP6s fluorescence in I3 in response to a 30 sec pulse of 250 mM NaCl in animals of the indicated genotypes. The *glr-9(oy180)* allele was used. Wild-type *glr-9* sequences were expressed in I3 under the *glr-9* promoter. Rescue data shown are combined values from two independent transgenic lines; responses of each line were similar. *** indicates different from wild-type at $P<0.001$ (one-way ANOVA and Dunnett's test).

i) Average (left) and peak intensity changes (right) of GCaMP6s fluorescence in wild-type I1 neurons or in I1 misexpressing GLR-7 and GLR-9 to a 30 sec pulse of 250 mM NaCl. Expression in I1 was driven under the *lgc-8* promoter. Responses from two independent misexpressing lines are shown. * and *** indicate different from wild-type I1 at $P<0.01$ and 0.001, respectively (one-way ANOVA and Dunnett's test).

Shaded regions in all traces indicate SEM. Each dot in the scatter plots is the value from a single neuron. Horizontal lines in scatter plots indicate the mean; errors are SEM. Data shown are from 2-3 independent experiments each. n.s. – not significant.

Fig. 3. *glr-9* mutants exhibit reduced tolerance of high salt.

a) (Left) Cartoon of experimental conditions. (Right) Quantification of time to paralysis upon

shifting animals to each salt at concentrations indicated in the cartoon. Each dot is the time at

which all ten animals in a single assay are immotile following the shift to high salt.

b,c) Quantification of time to paralysis upon shifting animals of the indicated genotypes from 50

to 500 mM NaCl or equiosmolar 1 M sucrose (cartoon at left). Each dot is the time at which all

ten animals in a single assay are immotile. Wild-type and *glr-9* mutants were examined in

parallel in the same assay. *** indicates different from wild-type at $P<0.001$ (t-test).

d-f) Percentage of animals of the indicated genotypes that are motile at each time point following

a shift from the acclimation plate (250 mM salt for 3 hrs) to 500 mM salt (cartoons at left). Each

dot is the value from a single assay; n=30 animals per assay. *, **, and *** indicate different

from wild-type at the corresponding time at $P<0.05, 0.01,$ and 0.001 (one-day ANOVA and

Dunnett's test).

The *glr-9(oy180)* allele was used in all experiments. Horizontal lines in scatter plots indicate the

mean; errors are SEM. Data shown are from 2-3 independent experiments each. n.s. – not

significant.

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Figure 4

Fig. 4. Cholinergic and FLP-6-mediated peptidergic signaling from I3 are necessary for

high salt tolerance in unacclimated and acclimated animals, respectively.

a) Quantification of time to paralysis upon shifting animals of the indicated genotypes from 50 to

500 mM NaCl (cartoon at top). Each dot is the time at which all ten animals in a single assay are

immotile. Cre was expressed in I3 under the *glr-9* promoter. Control and mutant animals were

examined in parallel in the same assay. *** indicates different from wild-type at $P<0.001$ (t-test).

b,c) Percentage of animals of the indicated genotypes that are motile at each time point following

a shift from the acclimation plate (250 mM NaCl for 3 hrs) to 500 mM NaCl. Each dot is the

value from a single assay; n=30 animals per assay. Cre was expressed in I3 under the *glr-9*

promoter. *, **, and *** indicate different from wild-type at the corresponding time at $P<0.05$,

0.01, and 0.001 (one-way ANOVA and Dunnett's test).

d) (Left in all panels) Cartoon of the experimental protocol. (Right in all panels) Percentage of

animals of the indicated genotypes that are motile at each time point following a shift from the

acclimation plate to the assay plate in the presence or absence of added exogenous auxin as

shown. TIR1 was expressed in I3 under the *glr-9* promoter. Each dot is the value from a single

assay; n=30 animals per assay. *, **, and *** indicate different from wild-type at the

corresponding time at $P<0.05$, 0.01, and 0.001 (one-way ANOVA and Dunnett's test).

e) Cholinergic signaling from I3 promotes high salt tolerance under conditions of acute salt

stress, whereas FLP-6 signaling from I3 during acclimation enables tolerance upon subsequent

exposure to high salt.

The *glr-9(oy180)* allele was used in all experiments. Horizontal lines in scatter plots indicate the

mean; errors are SEM. Data shown are from 2-3 independent experiments each. n.s-- not

significant.

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Figure 5

Fig. 5. GLR-9-mediated signaling is required for altered expression of a subset of

osmotolerance genes.

a,b) Volcano plots of differential gene expression in unacclimated (50 mM NaCl) and salt

acclimated (250 mM NaCl for 3 hrs) wild-type and *glr-9(oy180)* animals. Horizontal and vertical

dashed lines indicate significant at adjusted p-value <0.05 and $\log_2$ fold change >2 or <−2,

respectively. A subset of osmolyte biosynthetic enzymes previously implicated in osmotolerance

is indicated. Differentially expressed genes predicted to encode molecules involved in stress

responses or metabolism, and cuticular components, are indicated in yellow and purple,

respectively.

c) Dot plot of average expression changes of the indicated genes in the shown genetic

backgrounds and conditions. Color indicates $\log_2$ fold change and size indicates adjusted p-value.

Blank indicates no expression change detected.

d) Hierarchically clustered heatmaps of differentially expressed genes categorized as being

involved in extracellular matrix remodeling (top) or in stress and metabolic responses (bottom)

in the indicated conditions and genetic backgrounds.

e) Venn diagram indicating unique and common differentially expressed genes in unacclimated

and acclimated wild-type and *glr-9(oy180)* mutants.

f) Working model of the contribution of GLR-9-mediated signaling to high salt tolerance. In the

absence of *glr-9* or cholinergic signaling from I3, remodeling of the cuticle even under isotonic

conditions may contribute to decreased resistance to acute high salt stress. Upon acclimation,

both GLR-9-dependent and -independent pathways contribute to cuticular remodeling and

altered expression of osmotolerance genes, thereby enabling prolonged tolerance of high salt.

The failure to alter expression of a subset of osmotolerance genes in *glr-9* mutants or upon loss

of FLP-6 signaling from I3, and/or the dysregulated gene expression program under isotonic and

acclimated conditions, reduces high salt tolerance following acclimation.

Movie S1. (Right) 3D-rendered image of GLR-9::GFP localization at the I3 sensory ending

(Left) generated using Imaris Analysis Software (version 10.2.0).

Movie S2. 3D reconstruction of the I3 sensory ending from electron microscope serial sections.

Data Table 1. List of upregulated and downregulated genes and gene categories across each

condition/genotype from RNA-Seq analysis. The category list is ordered from highest to lowest

gene count.

Table S1. List of strains used in this work.

Strain name	Genotype	Source/ Parent strain
PY12290	*glr-9::gfp*(*sy5669*); *oyEx761* [*glr-9*p::*myr-TagRfp*; *unc-122*p::*dsRed*]	Suny Biotech, This paper
PY12291	*oyEx762* [*glr-7*p::*glr-7::gfp*; *glr-9*p:: *myr-TagRfp; unc-122*p::*dsRed*]	This paper
PY12292	*glr-7(tm2877)*; *glr-9::gfp*(*sy5669*); *oyEx761* [*glr-9*p::*myr-TagRfp*; *unc-122*p::*dsRed*]	CGC, This paper
PY12293	*glr-9(oy180)*; *oyEx762* [*glr-7*p::*glr-7::gfp*; *glr-9*p:: *myr-TagRfp; unc-122*p::*dsRed*]	This paper
PY12286	*oyIs98* [*glr-9*p::GCaMP6s; *unc-122*p::*dsRed*]	This paper
PY12294	*glr-9(oy180)*; *oyIs98* [*glr-9*p::GCaMP6s; *unc-122*p::*dsRed*]	This paper
PY12295	*glr-9(oy180)*; *oyIs98* [*glr-9*p::GCaMP6s; *unc-122*p::*dsRed*]; *oyEx763* [*glr-9*p::*glr-9::TagRfp*; *unc-122*p::*gfp*] Line-1	This paper
PY12296	*glr-9(oy180)*; *oyIs98* [*glr-9*p::GCaMP6s; *unc-122*p::*dsRed*]; *oyEx764* [*glr-9*p::*glr-9::TagRfp*; *unc-122*p::*gfp*] Line-2	This paper
PY12297	*unc-119(ed3)*; *otIs898* [*pha-4*prom2::3xNLS::GCaMP6s::*unc-54* 3' UTR]; *oyEx765* [*lgc-8*p::*mCherry*; *unc-122*p::*dsRed*]	OH18562, This paper
PY12298	*unc-119(ed3)*; *otIs898* [*pha-4*prom2::3xNLS::GCaMP6s::*unc-54* 3' UTR]; *oyEx765* [*lgc-5*p::*mCherry*; *unc-122*p::*dsRed*]; *oyEx766* [*lgc-8*p::*glr-7::TagRfp*; *lgc-8*p::*glr-9::TagRfp*; *unc-122*p::*gfp*] Line-1	OH18562, This paper
PY12299	*unc-119(ed3)*; *otIs898*[*pha-4*prom2::3xNLS::GCaMP6s::*unc-54* 3' UTR]; *oyEx765* [*lgc-5*p::*mCherry*; *unc-122*p::*dsRed*]; *oyEx767* [*lgc-8*p::*glr-7::TagRfp*; *lgc-8*p::*glr-9::TagRfp*; *unc-122*p:: *gfp*] Line-2	OH18562, This paper
PY12230	*glr-9(oy180)*	This paper
PY12228	*oyEx717* [*glr-9*p::*Chrimson::*T2A*::mCherry*; *unc-122*p::*gfp*] Line-1	This paper
PY12289	*glr-9(oy180)*; *oyEx717* [*glr-9*p::*Chrimson::*T2A*::mCherry*; *unc-122*p::*gfp*]	This paper
PY12800	*loxP::unc-17::loxP* (*syb5779 syb5987*)	SWF1012, This paper
PY12801	*loxP::unc-17::loxP* (*syb5779 syb5987*); *oyEx768* [*glr-9*p::*Cre*::SL2::*gfp*; *unc-122*p::*gfp*]	SWF1012, This paper
PY12802	*egl-21(n476)*; *kySi61* [*loxP::egl-21genomic::loxP*]	SWF717, This paper

PY12803	*egl-21(n476); kySi61* [*loxP::egl-21genomic::loxP*]; *oyEx768* [*glr-9*p::*Cre*::SL2::*gfp*; *unc-122*p::*gfp*]	SWF717, This paper
VC2324	*flp-6(ok3056)*	CGC
PY12804	*glr-9(oy180)*; *flp-6(ok3056)*	This paper
PY12288	*loxP::flp-6::loxP(oy203)*	This paper
PY12805	*loxP::flp-6::loxP(oy203); oyEx768* [*glr-9*p::*Cre*::SL2::*gfp*; *unc-122*p::*gfp*]	This paper
RJP5269	*unc-31 (rp166*[GFP::TEV::AID::FLAG::*unc-31*])	CGC
PY12806	*unc-31 (rp166*[GFP::TEV::AID::FLAG::*unc-31*]); *oyEx769* [*glr-9*p::TIR1::*mScarlet*; *unc-122*p::*gfp*] Line-1	This paper
PY12807	*unc-31 (rp166*[GFP::TEV::AID::FLAG::*unc-31 oyEx769* [*glr-9*p::TIR1::SL2::*mScarlet*; *unc-122*p::*gfp*] Line-2	This paper
PY12808	*unc-13(e51); oyIs98* [*glr-9*p::GCaMP6s; *unc-122*p::*dsRed*]	This paper
PY12809	*unc-31(e169); oyIs98* [*glr-9*p::GCaMP6s; *unc-122*p::*dsRed*]	This paper
PY12810	*tax-4(p678); oyIs98* [*glr-9*p::GCaMP6s; *unc-122*p::*dsRed*]	This paper
PY12811	*osm-9(oy202); oyIs98* [*glr-9*p::GCaMP6s; *unc-122*p::*dsRed*]	This paper
PY12812	*che-1(p674); oyIs98* [*glr-9*p::GCaMP6s; *unc-122*p::*dsRed*]	This paper
PY12813	*oyIs97* [*flp-6*p::GCaMP; *unc-122*p::*gfp*]; *oyEx770* [*che-1*p::*glr-9*::TagRfp; *che-1*p::*glr-7*::TagRfp; *unc-122*p::*dsRed*]	This paper
PR678	*tax-4(p678)*	CGC
CX10	*osm-9(ky10)*	CGC
CX4544	*ocr-2(ak47)*	CGC
FX30280	*flp-5(tm10075)*	NBRP
FX02427	*flp-13(tm2427)*	NBRP
AX1410	*flp-18(db99)*	CGC
FX02179	*flp-18(tm2179)*	NBRP
RB2269	*flp-34(ok3071)*	CGC
FX03023	*nlp-3(tm3023)*	NBRP
TM2569	*nlp-7(tm2984)*	NBRP
FX06232	*nlp-10(tm6232)*	NBRP
PHX530	*nlp-11(syb530)*	CGC
FX02569	*nlp-21(tm2569)*	NBRP
PS8789	*dmsr-1(sy1522)*	CGC
PS8854	*dmsr-7(sy1539)*	CGC
PS8483	*frpr-8(sy1362)*	CGC
MT14666	*egl-6(n4537)*	CGC

13   **Table S2.** List of plasmids used in this work.

Plasmid name	Description	Source
PSAB1374	*glr-9*p::*myr-TagRfp*	This paper
PSAB1373	*glr-7*p::*glr-7*::*gfp*	This paper
PSAB1383	*glr-9*p::GCaMP6s	This paper
PSAB1375	*glr-9*p::*glr-9*::*TagRfp*	This paper
PSAB1376	*lgc-8*p::*mCherry*	This paper
PSAB1378	*lgc-8*p::*glr-7*::*TagRfp*	This paper
PSAB1377	*lgc-8*p::*glr-9*::*TagRfp*	This paper
PSAB1371	*glr-9p::Chrimson::T2A::mCherry*	This paper
PSAB1379	*glr-9p::Cre::SL2::gfp*	This paper
PSAB1380	*glr-9*p::TIR1::*mScarlet*	This paper
PSAB1381	*che-1*p::*glr-7*::*TagRfp*	This paper
PSAB1382	*che-1*p::*glr-9*::*TagRfp*	This paper

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a

IR-like protein	TM #	Extended amino-terminal domain	Ala in TMIII	Arg in S1 domain	Thr in S2 domain	Asp or Glu in S2 domain
GLR-7	3	Yes	Yes	Yes	No	Yes
GLR-8	3	No	Yes	Yes	No	No
GLR-9	3	No	No	Yes	No	Yes
GLR-10	3	No	No	Yes	No	No
ZK867.2	3	No	Yes	Yes	No	No
F59E12.8	3	No	No	Yes	No	No
T25E4.2	3	No	No	Yes	No	No

b

IR-like gene	Expression In:	
	Pharyngeal Enteric Neurons	**Somatic Neurons**
glr-7	I3, I6, M3, NSM	PLM, ALM, PVM
glr-8	I6, NSM, I1, MI, MC, M3, I3, M5, I2	PVD, PHB, PHA, IL2, AVL, URB, PLM, ALM, ADL, AVM
glr-9	I3	none
glr-10	I6, NSM	none
ZK867.2	I6, NSM	PQR, PHA, PVD, AQR, ADL, AUA, AVH, URY, PHC, ASK, PHB, OLQ, URX, AVF, PVT, RIH
F59E12.8	none	none
T25E4.2	NSM, I6, MI	none

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Figure S1

Fig. S1. *C. elegans* IR-like genes are expressed highly or exclusively in PENs.

a) Features of IR-like proteins in *C. elegans*. TM: transmembrane domain. Mutating the

conserved Ala in TMIII in ionotropic glutamate receptors results in a constitutively open channel

(Zheng et al., 1999; Zuo et al., 1997). The Arg, and Thr and Asp/Glu residues, in the S1 and S2

ligand-binding domains, respectively, directly interact with glutamate or synthesized agonists in

ionotropic glutamate receptors (Benton et al., 2009).

b) Predicted expression of IR-like genes adapted from the *C. elegans* neuronal gene expression

map and network (CeNGEN) (Taylor et al., 2021).

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Figure S2

Fig. S2. I3 responds cell-autonomously to multiple cations via GLR-9.

a) Average (left) and peak intensity changes (right) of GCaMP6s fluorescence in wild-type I3 neurons in response to a 30 sec pulse of LB alone or supernatant of an OP50 bacterial culture grown in LB. *** indicates different at P<0.001 (t-test).

b-d) Peak fluorescence intensity changes in I3 in response to the indicated chemical concentrations. Average traces are shown in Fig. 2a-c. *** indicates different at P<0.001 (c: t-test; d: one-way ANOVA and Dunnett's test).

e) Mean GCaMP6s fluorescence change in I3 in response to a 3 min pulse of 250 mM NaCl.

f,g) Average (left) and peak intensity changes (right) of GCaMP6s fluorescence in I3 in response to a 30 sec pulse of 250 mM NaCl in animals of the indicated genotypes. Alleles used were *unc-13(e51), unc-31(e169)*, *tax-4(p678), osm-9(oy202),* and *che-1(p674).*

h) Mean GCaMP6s fluorescence changes in response to a 30 sec pulse of the indicated salts at 250 mM in wild-type and *glr-9(oy180)* mutants. n>17 each. Wild-type data were interleaved with data in Fig. 2d-g, and are repeated.

i,j) Representative images of GLR-9/GLR-7::tagRFP localization in ASE soma (i), and I1 (j). Expression in ASE and I1 was driven under the *che-1* and *lgc-8* promoters, respectively. Arrowhead indicates the non-ciliated sensory ending of I1 in j. Anterior is at left in all images. Scale bar: 5 μm.

Shaded regions in all traces indicate SEM. Each dot in the scatter plots is the value from a single neuron. Horizontal lines in scatter plots indicate the mean; errors are SEM. Data shown are from 2-3 independent experiments each. n.s.- not significant.

Fig. S3. *glr-9* mutants do not exhibit altered behavioral responses to salt.

a) (Left) Histograms showing average relative residence of wild-type and *glr-9(oy180)* animals

in a microfluidics behavioral arena (Albrecht and Bargmann, 2011; Khan et al., 2022) with a

central stripe of the indicated salt concentrations. (Right) Chemotaxis indices quantified from the

microfluidics behavioral assays. Each dot is the index from a single assay of ~15 animals each.

b) Percentage of animals of the indicated genotypes remaining within the glycerol ring after 10

mins. Each dot is the value from a single assay of 10 animals each.

c) Pumping rate on bacteria-seeded plates containing the indicated salt concentrations. Each dot

is the measurement from a single animal.

d) Quantification of time to paralysis of wild-type or *glr-9* mutants in the shown assay conditions

(cartoons at left). Each dot is the time at which all ten animals in a single assay are immotile.

Wild-type and *glr-9* mutants were examined in parallel in the same assay.

e) Percentage of animals of the indicated genotypes that are motile at each time point following a

shift from the acclimation plate to the assay plate. Alleles used were *glr-9(oy180), tax-4(p678),*

osm-9(ky10), and *ocr-2(ak47)*. Each dot is the value from a single assay; n=30 animals per assay.

** and *** indicate different from wild-type at the corresponding time at $P<0.01$ and 0.001,

respectively (one-way ANOVA and Dunnett's test).

Horizontal lines in all scatter plots indicate the mean; errors are SEM. n.s. – not significant.

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Figure S4

Fig. S4. Peptidergic but not cholinergic signaling from I3 is required for salt acclimation.

a) (Left) Mean GCaMP6s fluorescence change in I3 in response to 500 mM NaCl prior to and following acclimation to 250 mM NaCl for 3 hrs. (Right) Peak fluorescence intensity changes calculated from traces on the left. Each dot is the value from a single I3 neuron.

b,d,e,g) Percentage of animals of the indicated genotypes that are motile at each time point following a shift from the acclimation plate (250 mM NaCl for 3 hrs) to 500 mM NaCl. Alleles used were *flp-5(tm10075), flp-6(ok3056), flp-13(tm2427), flp-18(db99), flp-18(tm2179), flp-34(ok3071), nlp-3(tm3023), nlp-7(tm2984), nlp-10(tm6232), nlp-11(syb530), nlp-21(tm2569), dmsr-1(sy1522), dmsr-7(sy1539), frpr-8(sy1362)* and *egl-6(n4537)*. Cre was expressed in I3 under the *glr-9* promoter. Each dot is the value from a single assay; n=30 animals per assay. *, ** and *** indicate different from wild-type at the corresponding time at $P<0.05$, 0.01, and 0.001, respectively (one-way ANOVA and Dunnett's test). For e and g, wild-type and *glr-9* data were interleaved, and are repeated.

c,f) Quantification of time to paralysis of animals of the indicated genotypes in the shown assay conditions (cartoons at top (c) or left (f)). Each dot is the time at which all ten animals in a single assay are immotile. Cre was expressed in I3 under the *glr-9* promoter. Control and experimental animals were examined in parallel in the same assay.

The *glr-9(oy180)* allele was used in all experiments. Horizontal lines in all scatter plots indicate the mean; errors are SEM. Data shown are from 2-3 independent experiments each. n.s. - not significant.

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Figure S5

Fig. S5. Salt acclimation-induced changes in gene expression are partly regulated by GLR-9-mediated signaling.

a) Principal component analysis plot of RNA-Seq data clustered by genotype and condition. Each data point is a single replicate.

b) Venn diagram showing overlap of differentially expressed genes between unacclimated and salt acclimated wild-type animals identified in this and previously published work (Dodd et al., 2018; Rohlfing et al., 2011).

c) A hierarchically clustered heatmap of all differentially expressed genes between acclimated and unacclimated wild-type and *glr-9* mutants.

d) Volcano plots of differential gene expression between unacclimated and salt-acclimated wild-type and *glr-9(oy180)* animals. Horizontal and vertical dashed lines indicate significant at adjusted p-value <0.05 and $\log_2$ fold change >2 or <−2, respectively. A subset of genes involved in osmotolerance and discussed in this work is indicated. Molecules categorized as being involved in stress responses or metabolism, and in extracellular matrix remodeling, are indicated in yellow and purple, respectively.

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Figure S6**

Fig. S6. *glr-9* mutants exhibit reduced recovery following salt stress.

a) Percentage of wild-type or *glr-9(oy180)* mutants that are motile at each time point after being

moved from 50 mM NaCl to 400 mM NaCl. Each dot is the value from a single assay; n=10

animals per assay. * and ** indicate different from wild-type at the corresponding time at

$P<0.05$, 0.01, and 0.001, respectively (one-way ANOVA and Dunnett's test). n.s. – not

significant.

b) Quantification of time to paralysis upon shifting wild-type or *glr-9(oy180)* mutants acclimated

to 250 mM NaCl or 250 mM sucrose to 500 mM sucrose or 500 mM NaCl, respectively. Each

dot is the time at which all ten animals in a single assay are immotile. Wild-type and *glr-9*

mutants were examined in parallel in the same assay. n.s. – not significant.