Betaine acts on a ligand-gated ion channel in the nervous system of the nematode *C. elegans*

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Prior to the advent of synthetic nematocides, natural products such as seaweed were used to control nematode infestations. The nematocidal agent in seaweed is betaine, an amino acid that functions as an osmolyte and methyl donor. However, the molecular mechanisms of betaine toxicity are unknown. We identified the betaine transporter SNF-3 and the betaine receptor ACR-23 in the nematode *C. elegans*. Mutating snf-3 in a sensitized background caused the worms to be hypercontracted and paralyzed, presumably as a result of excess extracellular betaine. These behavioral defects were suppressed by mutations in acr-23, which encodes a ligand-gated cation channel of the cys-loop family. ACR-23 was activated by betaine and functioned in the mechanosensory neurons to maintain basal levels of locomotion. However, overactivation of the receptor by excess betaine or by the allosteric modulator monopentel resulted in hypercontraction and death of the nematode. Thus, monopentel targets a betaine signaling pathway in nematodes.

Humans have long competed with parasitic nematodes for crops, livestock and personal health. Early farmers dealt with nematode infestations by using natural pesticides such as wormwood, tobacco and algae (seaweed), which contain natural phytochemicals that interfere with parasitic nematode development1–3. Seaweed extracts are rich in betaines (δ-aminovaleric betaine, γ-amino-butyric betaine and glycine betaine), and direct application of individual betaines to plants suppresses nematode growth as effectively as algal extract4.

Glycine betaine, or simply ‘betaine’, is a ubiquitous non-canonical amino acid that acts as an osmolyte or as a methyl donor. In addition to its role in metabolism, betaine may have specific roles in the mammalian nervous system. Betaine has anti-epileptic properties5–7 and the transporter BGT-1 is localized to dendritic spines and astrocytes in the mammalian brain8.

In nematodes, betaine arrests larval development2–4, but the molecular target of betaine is not known. The targets of synthetic anthelmintics are often ligand-gated ion channels. The imidazothiazoles directly activate acetylcholine-gated ion channels, which stimulates muscle contraction and paralyzes the worm9,10. The macrocyclic lactones open glutamate-gated chloride channels and inhibit pharyngeal pumping11–13. Amino-acetonitrile derivatives (AADs) are a recently discovered class of compounds that target a nematode-specific ion channel known as ACR-23 in *C. elegans*14. ACR-23 belongs to the nicotinic acetylcholine receptor subfamily, but its homolog in parasitic nematodes, MPTL-1, is not gated by acetylcholine or choline15. The endogenous ligand and biological function of ACR-23 remain to be characterized.

We studied the role of betaine in the nematode *C. elegans* and found that ACR-23 is a betaine receptor. First, we characterized the betaine transporter SNF-3 and observed that mutations in this transporter were subviable in a sensitized background lacking phospholipase Cβ. Second, mutations in acr-23 suppressed this lethality, suggesting that excess betaine acts via ACR-23. Finally, ACR-23 was gated by betaine and potentiated by the AAD monopentel. Thus, ancient and modern anthelmintics act on the same target: a betaine-activated ion channel that is only found in nematodes.

RESULTS

An enhancer screen identifies a betaine transporter

We were able to identify the betaine pathway inadvertently while studying phospholipase Cβ function. In *C. elegans*, mutants lacking the only phospholipase Cβ gene (*egl-8*) exhibit subtle behavioral defects in locomotion and egg-laying, in contrast with the near lethal phenotype of mutants lacking Gαq, the upstream activator of phospholipase Cβ16,17. This modest loss-of-function phenotype in *egl-8* mutants suggests the existence of parallel pathways to inositol signaling that modulate locomotion. To uncover these pathways, we performed an F2 enhancer screen in an egl-8 mutant background. We screened 1,669 haploid genomes and identified a single mutation that caused a synthetic phenotype in the presence of an egl-8 mutation. Mapping, rescue and sequencing experiments revealed that the enhancer was an allele of the snf-3 gene (*sodium neurotransmitter symporter family-3; Supplementary Fig. 1a*). The snf-3 egl-8 double mutants exhibited severe synthetic phenotypes (*Fig. 1a–d*): the strain

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was subviable, uncoordinated and dumpy. The dumpy phenotype was a result of hypercontraction of the muscle, as it could be suppressed by mutations in the contractile apparatus unc-22 (twichin) and unc-54 (myosin) (data not shown). The hypercontracted phenotype of snf-3 egl-8 double mutants was fully synthetic; neither single mutant was hypercontracted (Fig. 1a,b and Supplementary Fig. 1c). The uncoordinated phenotype was also more severe than that of either single mutant (Fig. 1c,d). Multiple allelic combinations exhibited the same synthetic hypercontracted phenotype (data not shown), indicating that the phenotypes are not a result of background mutations.

snf-3 encodes a neurotransmitter transporter of the solute carrier 6 (SLC6) gene family. Members of this transporter family clearly neurotransmitters from the synaptic cleft, including serotonin, dopamine, norepinephrine, GABA and glycine. SNC-3 is orthologous to the vertebrate betaine/GABA transporter (BGT1 or SLC6A12). Although we do not fully understand the genetic interaction between the betaine transporter and PLCβ, the synthetic phenotype provided a selection with which to identify the betaine receptor.

Hypercontraction in snf-3 egl-8 is mediated by acr-23

Mutations in SLC6 transporters often result in constitutive signaling because the neurotransmitter cannot be cleared from the synaptic cleft. For example, mice lacking the dopamine transporter are hyperactive, whereas worms lacking the same gene have defects in their locomotor behaviors. These defects are consistent with the role of dopamine in the control of locomotion.

To determine whether the phenotype of snf-3, egl-8 mutants is a result of constitutive betaine signaling, we screened for suppressors of the snf-3 egl-8 phenotype. Specifically, we mutagenized snf-3 egl-8 double mutants with N-ethyl-N-nitrosourea (ENU) and screened for suppressors of the hypercontracted phenotype (Fig. 1e). We screened 37,000 haploid genomes and obtained 35 suppressors. The phenotypes of the triple mutants fell into three broad classes: those that resembled egl-8 single mutants (snf-3–specific suppressors), those that resembled snf-3 mutants (egl-8–specific suppressors) and those with new phenotypes.

Figure 1 Two genetics screens reveal a betaine receptor. (a) snf-3 was identified as an enhancer of the phospholipase Cβ mutation egl-8(sa47). Shown are images of an adult egl-8(sa47) mutant and an adult snf-3(ox354) egl-8(sa47) double mutant that were isolated in the screen. Mean lengths of 26 (egl-8) and 30 (snf-3, egl-8) adults are shown. (b) Hypercontracted phenotype. We plotted the mean body length of young adult worms. Error bars represent s.e.m. *** P < 0.001 relative to wild type and single mutants. (c) Quantification of locomotion in liquid using a thrashing assay. (d) Average locomotion speed over a 5-min period on an agar plate without food. Speed was determined using an automated worm tracking system. (e) acr-23 was isolated as a suppressor of the snf-3 egl-8 hypercontracted phenotype. Images of adult hermaphrodites of the snf-3(ox354) egl-8(sa47) double mutant (n = 30) and the snf-3(ox354) egl-8(sa47) acr-23(ox429) triple mutant (n = 29). (f,g) Quantification of the hypercontracted (f) and locomotion (g) phenotypes. Data represent the mean ± s.e.m. *** P < 0.001 compared to other mutants. For all histograms, the number of worms tested is shown inside each bar. Statistical significance was determined using one-way ANOVA followed by Tukey post hoc comparisons. # indicates data are the same as shown in b and c.

The strongest snf-3–specific suppressor was the mutation ox429. We identified the suppressor mutation by genome resequencing and by transgenic rescue. ox429 is an allele of the acr-23 gene (acetylcholine receptor-like-23) (Supplementary Fig. 2a). acr-23 encodes a nematode-specific ligand-gated channel subunit and is predicted to function as a cation channel based on its sequence homology to acetylcholine receptors (Supplementary Fig. 2c). The only known function of ACR-23 is that it imparts sensitivity to the new class of anthelmintic drugs known as AADs. Genetic screens for AAD resistance in C. elegans identified 27 independent mutations in a single target, acr-23 (ref. 14), and mutations in related receptor subunits in the same subfamily do not confer resistance to AADs.

acr-23(ox429) fully suppressed the hypercontracted (Fig. 1f) and uncoordinated phenotypes (Fig. 1g) of the snf-3 egl-8 double mutants. For example, the double mutants were completely paralyzed in liquid (0% of wild-type rate; Fig. 1g), whereas the snf-3 egl-8 acr-23 triple mutants thrashed actively (45% of wild-type rate; Fig. 1g). A deletion allele, acr-23(ox2804), also suppressed the double mutants (Fig. 1f–g). The triple mutants still exhibited egl-8 phenotypes, including lethargy (Fig. 1g), constipation (data not shown) and a mild egg-laying defect (data not shown), suggesting that acr-23 mutations specifically suppress snf-3, but not egl-8, pathways.

SNF-3 is a Na+Cl−-dependent betaine transporter

The vertebrate ortholog of SNF-3, betaine/GABA transporter 1 (BGT1 or SLC6A12), has two substrates, betaine and GABA. However, it has a higher affinity for GABA (Km = 93 μM) than for betaine (Km = 398 μM). In contrast, we found that SNF-3 transported betaine, but not GABA. Betaine is a noncanonical amino acid that is present in all organisms (Fig. 2a). It acts as an osmolyte to maintain cell volume during stress and as a methyl donor in the conversion of homocysteine to methionine. Betaine is acquired either by dietary uptake using a plasma membrane transporter or is generated as an oxidation product of choline. Ultimately, betaine is metabolized to glycine.
To rapidly screen for the relevant substrate, we expressed SNF-3 in *Xenopus* oocytes and applied potential transport molecules. SLC6 transporters co-transport ions along with substrate molecules and are therefore electrically. We tested candidate substrates and found that only betaine induced a specific current (Supplementary Fig. 3a). In contrast with BGT1, SNF-3 did not transport GABA in our experiments.

To characterize the transport kinetics of SNF-3, we measured the accumulation of radio-labeled betaine in human retinal pigment epithelial (HRPE) cells, which do not express an endogenous betaine transporter. The expression of SNF-3 increased betaine uptake by more than 100-fold (108 ± 3 pmol per 10⁶ cells per min) over control cells (0.8 ± 0.1 pmol per 10⁶ cells per min). SNF-3-mediated transport was saturable with a Kₘ of 320 ± 50 µM (Fig. 2b); this value is similar to the affinity of BGT-1 for betaine (398 µM)²⁴. As expected for protein-mediated transport, activity was saturable over a range of concentrations (Fig. 2b and Supplementary Fig. 3b) and voltages (Supplementary Fig. 3c). Similar to other SLC6 transporters, SNF-3 requires sodium and chloride to move substrates into cells against their chemical gradient. The absence of sodium (in N-methyl-D-glucamine (NMDG) chloride solution) or the absence of chloride (sodium gluconate solution) abolished betaine uptake (Fig. 2c and Supplementary Fig. 3d), and transport was voltage dependent (Supplementary Fig. 3e). The Michaelis constant of SNF-3 for sodium (K⁺Na) was 37 ± 1 mM with a Hill coefficient of 2.2 ± 0.1 (Supplementary Fig. 3f), and the corresponding value for chloride (K Cl⁻) was 12 ± 1 mM with a Hill coefficient of 1.0 ± 0.1 (Supplementary Fig. 3g).

In contrast with BGT1, SNF-3 did not transport GABA in our biochemical assays, and we observed this lack of GABA transport activity functionally. In the nematode, the GABA transporter is encoded by *snf-11* (ref. 28). Overexpression of the SNF-3 transporter is unable to compensate for the absence of the GABA transporter SNF-11 even when overexpressed under the *snf-11* promoter, indicating that SNF-3 cannot transport GABA in *vivo*²⁸. The presence of a dedicated transporter suggests that betaine may have a specific role in cell signaling.

**SNF-3 is required for betaine clearance**

Members of the SLC6 transporter family clear neurotransmitters from synapses and the extrasynaptic space, thereby limiting their activity. To determine where SNF-3 functions, we examined *snf-3* expression using a functional *snf-3–GFP* transgene (Supplementary Fig. 1b). Transgenic worms displayed strong expression in the excretory canal, tail hypodermal cells, epidermis and vulval epithelia cells (Fig. 3a). SNF-3 was also expressed in some neurons, including the excretory canal–associated neuron (Fig. 3a), and some sensory neurons in the head including ILS, OLS, ADE and AQR (Supplementary Fig. 2d,e).

We were able to rescue the hypercontraction and the locomotory phenotypes of *snf-3 egl-8* double mutants by expressing wild-type SNF-3 using the native *snf-3* promoter or using tissue-specific promoters for the excretory canal or skin (glt-3 and pdl-2 promoters, respectively; Fig. 3b). Notably, expressing SNF-3 in tissues that do not normally express the gene, such as the intestine, chemosensory neurons or acetylcholine motor neurons, also rescued the hypercontracted phenotype (Fig. 3b). Rescue required expression during larval development: expression of SNF-3 in the intestine during larval development (*vha-6* promoter), but not in the adult (*vit-2* promoter; Fig. 3b), rescued the hypercontracted phenotype. This stage-dependent rescue is consistent with the retarded larval development of *snf-3* mutants (Supplementary Fig. 2e). The non-cell autonomous rescue of the *snf-3 egl-8* phenotypes suggests that the hypercontracted and locomotory phenotypes are caused by a lack of betaine clearance; presumably any tissue, not just the epidermis and excretory canal, can remove the excess betaine.

In contrast with *snf-3 egl-8* was required in the nervous system, but not epidermis (Fig. 3c). Further analyses of EGL-8 revealed that the observed synthetic hypercontraction was a result of a defect in acetylcholine neurons (unc-17 promoter). Expression in sensory neurons (data not shown) or in ventral cord motor neurons (data not shown) did not rescue the double-mutant phenotypes. Thus, the functional requirements for EGL-8 and SNF-3 are in separate tissues; the transporter SNF-3 functions primarily in the epidermis to clear betaine from the extracellular space, whereas EGL-8 is required in the nervous system to modulate neuronal activity.

**Exogenous betaine paralyzes snf-3 mutants**

These data suggest that the phenotypes caused by *snf-3* mutants are a result of excess betaine in the extracellular space. If true, then high levels of exogenous betaine should mimic *snf-3* phenotypes. We grew *C. elegans* on different concentrations of betaine and tested their locomotion in liquid. Wild-type worms were not affected by 50 mM betaine (Fig. 3d). *egl-8* mutants were hypersensitive to 50 mM betaine and became sluggish in liquid, but they did not become hypercontracted. *snf-3* mutants were strongly hypersensitive to 50 mM betaine and were paralyzed in liquid. The toxic effect of betaine was mediated by ACR-23, as *snf-3 acr-23* double mutants were resistant to exogenous betaine (Fig. 3d). Wild-type worms grown at a higher
Figure 3  SNF-3 is required for betaine clearance. (a) Expression pattern of snf-3–GFP. Images show the head, tail, lateral midbody and vulva region of a transgenic hermaphrodite expressing GFP fused to genomic snf-3 (Psnf-3::snf-3–GFP::snf-3 UTR). All images are of adult hermaphrodites; anterior is to the left. CAN, excretory canal–associated neuron. (b) snf-3 tissue-specific rescue of the hypercontracted (above) and locomotion (below) phenotype. Top, expression of snf-3 cDNA in excretory canal (vha-6 promoter), skin (pdf-2 promoter), intestine (vha-6 promoter), chemosensory neurons (odr-4 promoter) or acetylcholine (ACh) neurons (Punc-17) rescued the body morphology of snf-3, egl-8 double mutants to normal (egl-8 phenotype). Expression in the adult intestine (vit-2 promoter) did not rescue the phenotypes. Error bars represent s.e.m. ***P < 0.001 compared with the snf-3 egl-8 double mutant. The differences between the egl-8 control and rescue of the snf-3 egl-8 double mutant by expression of snf-3 (+) in the excretory canal (P = 0.0088) or in the acetylcholine motor neurons (P = 0.0031) were statistically significant. Bottom, for the locomotion phenotype, the differences between egl-8 control and rescue in the skin (P = 0.0003) and intestine (P = 0.0048) rescue were statistically significant. Expression of SNF-3 in the acetylcholine motor neurons did not rescue snf-3 egl-8 locomotory defects (P = 0.37), possibly because SNF-3 activity is electrogenic and interferes with motor neuron function, ns indicates not statistically significant. Native tissues are cells that expressed the snf-3 gene, whereas non-native tissues are cells in which snf-3 gene expression was not detected. 11x outcrossed snf-3, egl-8 is EG7513. Error bars represent s.e.m. The number of worms tested is shown inside each bar. (c) Tissue-specific rescue of the egl-8 hypercontracted (top) and locomotion (bottom) phenotypes. egl-8 cDNA was expressed in all cells (dpy-30 promoter), neurons (rab-3 promoter), acetylcholine neurons (unc-17 promoter), intestine (vha-6 promoter) or epidermis (dpy-7 promoter). Expression of EGL-8 in acetylcholine neurons (unc-17 promoter) generated transgenic worms that were long and loopy-coilers. The number of worms tested is shown inside each bar. Error bars represent s.e.m. ***P < 0.001. Statistical significance was determined using one-way ANOVA followed by Tukey post hoc comparisons. (d) Exogenous betaine slowed locomotion. (e) Overexpression of SNF-3 conferred resistance to excess betaine. The number of worms tested is shown inside each bar. Error bars represent s.e.m. Statistical significance was determined using one-way ANOVA followed by Tukey post hoc comparisons. ***P < 0.001, **P < 0.01, ns (not significant) P > 0.05. (f) Average concentrations of total betaine in whole worms. These metabolites were identified using proton NMR spectroscopy. Statistical significance was determined using a one-way ANOVA on each metabolite with Bonferroni correction. n = 8,000 worms per strain. ns (not significant) P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001. Alleles are egl-8(sa47) and snf-3(ox354), unless indicated otherwise.

concentration of betaine (250 mM) exhibited slowed swelling in the presence of betaine (Fig. 3e), whereas worms overexpressing the SNF-3 transporter were resistant to 250 mM betaine (Fig. 3e). These data suggest that excess betaine suppresses locomotion and that the SNF-3 betaine transporter clears betaine from the extracellular space.

snf-3 mutants exhibited locomotory phenotypes even if excess betaine was not applied (Fig. 1c,d), suggesting that betaine may already be present in worms. To determine betaine levels in C. elegans, we performed proton nuclear magnetic resonance (NMR) spectroscopic analysis on the worm metabolome. Betaine was present in extracts of worms at 7.0 ± 1.0 µM per mg of dry pellet (Fig. 3f), whereas the concentrations of GABA and acetylcholine were below detection (data not shown). Total betaine content did not change in snf-3 mutants; however, a redistribution to the extracellular space would not be detectable in assays of whole worms. Notably, concentrations of the metabolites choline and glycine were higher than the concentrations of betaine in whole worms. These metabolites were identified using proton NMR spectroscopy. Statistical significance was determined using a one-way ANOVA on each metabolite with Bonferroni correction. n = 8,000 worms per strain. ns (not significant) P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001. Alleles are egl-8(sa47) and snf-3(ox354), unless indicated otherwise.
ACR-23 forms a homomeric betaine receptor

To test whether betaine acts directly on the ACR-23 receptor, we expressed ACR-23 in *Xenopus* oocytes and examined its response to various ligands. ACR-23 forms an ion channel that is activated by betaine, but not by acetylcholine, choline, glycine, or GABA (Fig. 4a). The sensitivity of ACR-23 for betaine is relatively weak; the half maximal concentration (EC_{50}) for betaine is 1.4 mM with a Hill coefficient indicating betaine concentration. (Fig. 4b). ACR-23 was potassium permeable. Representative I-V curves in modified Ringer’s solution containing Na⁺ (n = 9) or NMDG (n = 8). (e) ACR-23 was potassium permeable. Representative I-V curves in modified Ringer’s solution containing Na⁺ (n = 7) or K⁺ (n = 7). (f) ACR-23 was not permeable to the divalent cation Ba²⁺. The reversal potential of betaine-evoked currents was not altered when the extracellular Ba²⁺ concentration was increased tenfold (n = 9). (g) Monepantel allosterically modulated ACR-23. A representative trace shows current induced by 1 mM betaine in the absence (above) or presence (below) of 300 pM monepantel in oocytes expressing ACR-23 (ref. 11).

**Figure 4** ACR-23 is a betaine-activated ion channel. (a) *Xenopus laevis* oocytes expressing ACR-23 responded to a 20-s pulse of 1 mM betaine, but not 1 mM acetylcholine, choline, glycine or GABA. The mean betaine-evoked response was 1.3 ± 0.13 µA for oocytes injected with 0.1 ng of cRNA (n = 9 oocytes). (b) Representative betaine-evoked currents from oocytes expressing wild-type ACR-23 (ACR-23(WT)) or ACR-23(I301N) stimulated with the indicated betaine concentration. (c) Betaine sensitivity of the wild-type ACR-23 channel (black line, n = 6 oocytes) and ACR-23(I301N) gain-of-function channel (red line, n = 5). EC_{50} was 1.4 mM for the wild type and 166 µM for ACR-23(I301N) (P = 0.0017). Error bars represent s.e.m. Statistical significance was determined using two-tailed unpaired Student’s t test with Welch’s correction. (d–f) ACR-23 is a monovalent cation channel. Insets indicate predicted ion flux under physiological conditions. (d) ACR-23 was permeable to sodium. Representative I-V curves in modified Ringer’s solution containing Na⁺ (n = 9) or NMDG (n = 8). (e) ACR-23 was potassium permeable. Representative I-V curves in modified Ringer’s solution containing Na⁺ (n = 7) or K⁺ (n = 7). (f) ACR-23 was not permeable to the divalent cation Ba²⁺. The reversal potential of betaine-evoked currents was not altered when the extracellular Ba²⁺ concentration was increased tenfold (n = 9). (g) Monepantel allosterically modulated ACR-23. A representative trace shows current induced by 1 mM betaine in the absence (above) or presence (below) of 300 pM monepantel in oocytes expressing ACR-23 (ref. 11).
ACR-23 acts in the nervous system to regulate locomotion

To determine where ACR-23 functions during locomotion, we characterized the expression pattern of the acr-23 gene using transcriptional mCherry reporter constructs (Fig. 5a and Supplementary Fig. 2b). 

acr-23 was expressed strongly in the six mechanosensory neurons, multiple interneurons and body muscles. ACR-23 expression in muscle was high in larvae and weak in adults. The expression of acr-23 in the larval body muscles was consistent with the hypercontraction phenotype observed in snf-3 egl-8 double mutants.

The lack of expression in motor neurons was surprising to us because mutants lacking acr-23 (Supplementary Fig. 1c) exhibited mild swimming defects (Fig. 1g), were lethargic when crawling on an agar plate (Fig. 5b,c) and their movement was interrupted by frequent pauses. snf-3 mutants and snf-3 acr-23 double mutants were equally sluggish (Figs. 1g and 5b,c), suggesting that the effect of betaine on locomotion is mediated by different receptors.

To determine where ACR-23 functions during locomotion, we rescued the mutant using tissue-specific promoters. Expression in the nervous system rescued the locomotory phenotypes of acr-23 mutants (Fig. 5d). Expression in the mechanosensory neurons also partially restored motor activity (Fig. 5d), consistent with the known role of these cells in controlling basal levels of locomotion. Thus, as with egl-8, acr-23 is required in the nervous system. These data suggest that phospholipase Cβ limits ACR-23 function in the nervous system, either directly or indirectly.

Gain-of-function ACR-23 resembles snf-3 egl-8 mutant

Together, these data suggest that an increase in betaine levels in the snf-3 egl-8 double mutants inappropriately activates ACR-23 and leads to hypercontraction. To demonstrate that constitutive activation of ACR-23 could generate the hypercontracted phenotype, we generated an ACR-23 receptor with a higher ligand sensitivity. The second transmembrane domain forms the pore in ligand-gated ion channels and the residues are numbered 0′ to 19′ by convention.35

Mutations at the 13′ position of the pore are known to increase the ligand sensitivity and conductance of cys-loop ion channels, probably by affecting transitions to the open state.31 We engineered a variant at the 13′ position, ACR-23(1301N), based on known gain-of-function mutations in a related receptor (Supplementary Fig. 2c). Similar to other receptors,31 ACR-23(1301N) was more sensitive to betaine and the channel desensitized less than the wild-type receptor (Fig. 4c).

The EC50 for the receptor decreased eightfold, from 1,400 M to 166 µM (Fig. 4c).

This hypersensitive variant of ACR-23 in transgenic worms recapitulated the snf-3 egl-8 double mutant phenotype. Most ACR-23(1301N) transgenic worms died duringlarval development. Those that escaped lethality and reached adulthood were severely hypercontracted (Supplementary Fig. 2d and 2e) and uncoordinated (Fig. 5f), closely resembling the phenotype of snf-3 egl-8 mutants. We also evaluated the tissue-specific effects of the gain-of-function ACR-23(1301N) mutation. Expressing ACR-23(1301N) in body
Using two forward genetic screens, we uncovered the molecular pathway that leads to betaine toxicity in nematodes. ACR-23 is a betaine-gated cation channel expressed in body muscle and neurons. The betaine transporter SNF-3 removed betaine from the extracellular space, and 3- and -mutants exhibited subtle locomotory phenotypes. These phenotypes were exacerbated by mutations in phospholipase Cβ. In the -egl-8 double mutant, constitutive activation of ACR-23 led to hypercontraction, paralysis and sometimes death as a result of a combination of effects on the nervous system and muscle. Why phospholipase Cβ mutations are synthetic with mutations in the transporter is not clear; however, one possibility is that phospholipase Cβ is required to downregulate ACR-23 itself or the activity of the neurons in which it functions. Thus, the elimination of the receptor by mutation restores muscle function and locomotion, reversing the effect of the 3- and egl-8 mutations.

In addition to its natural ligand betaine, ACR-23 is also the target of a class of anthelmintic drugs known as AADs, which contain the active compound monepantel14. Monepantel induces muscle hypercontraction, spasmodic pharyngeal contraction, paralysis and death in . These characteristics are shared with other anthelmintic drugs that activate ligand-gated ion channels in the nematode. Unlike these receptor agonists, monepantel acted as an allosteric modulator of ACR-23, potentiating betaine signaling during development, resulting in nematode death. A drawback of monepantel is that some of the genera lacking the acr-23 gene include parasitic nematodes23, such as and , which represent a major public health concern in developing countries. Unlike acr-23, -3 has an ortholog in most parasitic nematodes species with a sequenced genome. Thus, the betaine–SNF-3 pathway offers a unique set of targets that can be used to improve AAD efficiency or to develop new anthelmintic drugs.

What then is the normal role of betaine in the nematode? Betaine signaling was required in for basal levels of locomotion: acr-23 mutants were sluggish when crawling on agar. The effect on locomotion was mediated in part via ACR-23 receptors expressed in the mechanosensory neurons. The mechanosensory neurons innervate the locomotory command neurons to stimulate touch-induced movement as well as spontaneous levels of locomotion. The mechanosensory neuron dendritic processes run adjacent to the epidermis and, in some cases, are completely embedded in the epidermal cells expressing the betaine clearance transporter SNF-3. We cannot exclude the possibility that betaine is acting as a conventional neurotransmitter, that is, that it is released onto neurons and muscles at synapses. However, it is also possible that betaine is released by the epidermis rather than by neurons. In this model, the skin may both release betaine onto the mechanosensory processes and clear it from the space between the skin and dendrite, acting as both source and sink for this neurotransmitter.

Betaine has been shown to have anticonvulsant properties in vertebrate nervous system. The mammalian nervous system contains many uncharacterized ligand-gated ion channels and protein-coupled receptors. Some of these could potentially mediate betaine anticonvulsive properties.

Methods
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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Author Contributions
A.S.P. designed and performed genetics screens and behavior analyses, and wrote the paper. Y.-J.F., G.J. and V.G. designed and performed the physiological analyses of the betaine transporter. P.M. performed the physiological analyses of the betaine receptor. J.L.G. and C.C. measured betaine concentrations in the worm using NMR spectroscopy. E.A.M. and K.J.M. helped J.L.G. with nematode cultures. E.M.J. provided guidance and edited the paper.

Competing Financial interests
The authors declare no competing financial interests.

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The speed of the worm was determined using an
We mutagenized EG4094
We transferred well-fed young adult worms
We mutagenized EG7081
restoration of the hypercontracted phenotype observed in simple
tions of these two constructs, we obtained over ten transgenic lines with the
with 2 ng
PCR fragments from wild-type genomic DNA of the T13B5.1 gene. To iden-
tify the molecular lesion in
snf-3(ox354), we sequenced snf-3 PCR fragments
generated from snf-3(ox354) egl-8(sa47) double mutant worms.
Suppressor screen. We mutagenized EG7081 snf-3(ox354) egl-8(sa47) with
20 nM ENU. B0348 is a cosmid that
snf-3(ox354) was rescued by injecting snf-3(ox354), egl-8(sa47) worms
with a pool of cosmids F45D11, M01D1 and C07D2 along with 2 ng µl-1
Pmyo-2::GFP co-injection marker. Each cosmid was injected at 20 ng µl-1.
Cosmid C07D2 alone was sufficient to rescue the snf-3(ox354) enhancer
defects. We were able to rescue snf-3(ox354) by injecting four overlapping
PCR fragments from wild-type genomic DNA of the T13B5.1 gene. To iden-
tify the molecular lesion in snf-3(ox354), we sequenced snf-3 PCR fragments
generated from snf-3(ox354) egl-8(sa47) double mutant worms.

Suppressor screen. We mutagenized EG7081 snf-3(ox354) egl-8(sa47) with
20 nM ENU. We screened the F2 progeny for non-hypercontracted and non-
unc-122. We generated EG6888 from these injections. To visualize neurons express-
ing SNF-3
snf-3(ox354) was rescued by injecting
acr-23(ox429), snf-3(ox354) egl-8(sa47) worms.

Molecular biology. To create a full-length genomic snf-3 DNA plasmid, we
exissed a 15-kb genomic fragment of snf-3 from C07D2 using Stul and restric-
tion digest and ligated it into pLTMUS38 (pADA49). To generate a fluores-
cently tagged SNF-3, we cut the GFP with MluI from the pPD114.24 vector
A. Fire, Stanford University), and ligated it into the largest
cently tagged SNF-3, we cut the GFP with MluI from the pPD114.24 vector
snf-3(ox354) exon 8 were inserted into the pDONR P4- P1 vector.

To create a full-length genomic acr-23 construct, we divided the genomic
region into three fragments that were inserted into different MultiSite Gateway
pDONR vectors. A 2.9-kb promoter and the full genomic region except for the
last two exons of the gene were inserted into the pDONR P4- P1 vector.
The 4-kb intron and exon 8 were inserted into the pDONR 221 vector, and
the last exon and the 3′ UTR (252 bases) were inserted into pDONR P2R-P3 vector.
These three fragments were recombined with pDEST R4-B3 vector to
create pADA246. To create ASP268, we inserted mCherry in operon between
the last exon of the gene and the 3′ UTR in the pDONR P2R-P3 vector using
Gibson Assembly Cloning technology (New England Biolab). The intergenic
region of the gdp-2 gdp-3 was used to express mCherry from an operon.
All the constructs used for tissue specific rescues were created using the
MultiSite Gateway Technology. An acr-23 cDNA containing an ATG was
inserted into the pDONR 221 vector. In this case, the corresponding promot-
ers (Pmyo-3, Prab-3 and Pnrec-7) in slot 1 lack ATG.

Confocal microscopy. We acquired images of fluorescently tagged fusion
proteins in living C. elegans with a 63x 1.4 NA objective on a Pascal LSM5
confocal microscope (Carl Zeiss).

Body length assay. We selected L4 worms the day before the assay for each
genotype studied. On the day of the experiment, we anesthetized adult worms
with 15 mM sodium azide in M9 solution on freshly made 2% agarose pads.
We used a plan-Neofahr 10x 0.3 NA objective on a Pascal LSM5 confocal
microscope. The size of the worm was determined using a build-in function
on the LSM5 image browser.

Growth assay. We plated 10 L4 larvae on NGM plates 12 h before we
started the experiment. We transferred the adults every 6.5 h (at 22.5 °C)
or overnight (12 h) at 15 °C. After the animals were transferred, we counted
the number of eggs laid. We monitored hatching rate and development
rate to adulthood. Adulthood was determined by the presence of a vulva.
The percentage of worms was normalized to each plate.

Betaine assay. We top-spreaded NGM plates with betaine to obtain a final
concentration of 50 and 250 mM. These plates were allowed to dry at 22.5 °C
for 24 h and then seeded with the E. coli strain OP50. On the day of the experi-
ment, we added three L4 worms to each plate. The plates were coded so the
experimenter was blind to betaine concentrations and genotype of the strains.
The first and second generations progeny were then tested in a thrashing assay.
For the snf-3 overexpression line (EG8093), the array oxEx1208 was crossed
out from strain EG5051 and crossed twice against N2.

Thrashing assay for locomotion. Single young adult worms were placed in
a microtiter well in a 96-well plate without agar, containing 150 µl of water.
Assays in M9 yielded similar results (data not shown). The worm was allowed
to acclimate for 2 min and we counted thrashes for 60 s. One thrash reflects the
bending of the body from the midbody toward one side of the animal and back
again. The value obtained is doubled to reflect the true number of bends.

Crawling assay for locomotion. We transferred well-fed young adult worms
to a food-free NGM plate (100 mm). Worms were allowed to acclimate for
5 min and we counted the number of body bends generated by each worm
every 20 s. Each worm was tested four times.

Crawling speed assay. The speed of the worm was determined using an
automated worm tracking and analysis system. A single young adult worm
was transferred to a food-free NGM plate (100 mm) placed on a motorized
microscope stage (OptiScan ES111, Prior Scientific). After a 1-min acclimation
period, the worm was aged at 5 frames per s for 5 min using a VGA FireWire
camera (XCD-V60, Sony) mounted on a Leica M55 stereomicroscope. A cus-
tom MATLAB program (MathWorks) was used to control the camera and
the motorized stage and to determine the mean locomotion speed. The speed
was calculated based on the distance traveled by the worm over 5 min.

Statistical analysis. Statistical analyses were performed using Kaleidagraph
3.6 and GraphPad Prism 6. All grouped data are reported as means ± s.e.m.
Statistical significance between genotypes was determined using one-way ANOVA followed by Tukey post hoc comparisons. We used two-tailed unpaired Student’s t test with Welch’s correction to determine the difference between wild-type and the gain-of-function (Fig. 4c). Logarithmic normal distribution was assumed for betaine EC50 values, so we use the PEGL0 values for statistical analysis. P < 0.05 was considered statistically significant. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications in the field (thresh assay42, speed43, body bends21 and body length63). Data distributions were assumed to be normal, but this was not formally tested. Data collection and analysis were not always performed blind, but we specifically state which experiments were performed blind in the Online Methods. All of the data were collected and processed side by side in a randomized manner.

SNF-3 transport assay in HRPE cells. HRPE cells were transfected using a vaccinia virus expressing snf-3 cDNA as described previously.5,47. HRPE cells grown in 24-well plates were infected with a recombinant vaccinia virus (VTF7-3). This virus carries the gene for T7 RNA polymerase. The virus was allowed to adsorb onto the cells for 30 min at 37 °C with gentle shaking of the plate. Cells were then transfected with the plasmid DNA (empty vector or pSPORT1 or snf-3 cDNA construct) using the lipofection procedure (Invitrogen). The cells were incubated at 37 °C for 12 h and then assayed for transport activity. The uptake of [14C]betaine (2.5 μM) was determined at 37 °C. In most experiments, the media consisted of 25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, and 5 mM glucose. In experiments in which the cation and anion dependence of the transport process was investigated, NaCl was replaced isosmotically by sodium gluconate or NMDG chloride. Uptake measurements were routinely made in parallel in control cells transfected with vector alone and in cells transfected with the vector-cDNA construct. The uptake activity in cDNA-transfected cells was adjusted for the endogenous activity measured in control cells to calculate the cDNA-specific activity. Experiments were performed in triplicate, and each experiment was repeated at least three times. Results are presented as means ± s.e.m. The kinetic parameters, Michaelis–Menten constant (Km) and maximal velocity (Vmax) were calculated by fitting the data to a Michaelis–Menten equation describing a single saturable transport system. Analysis was done by nonlinear regression, and the resultant values for the kinetic parameters were confirmed by linear regression.

SNF-3 analysis. The procedures for cRNA in vitro transcription, Xenopus laevis oocyte isolation, microinjection, superfusion, voltage clamping and data analysis have been described previously.42,44. Briefly, the plasmid SNF-3-pSPORT1 was linearized with NotI and transcribed to cRNA using the mMESSAGE mMACHINE RNA transcription kit (Ambion). The expression of SNF-3 was initially detected by comparing the uptake of (2.5 μM) [14C]-betaine (55 mCi mmol−1, American Radiolabeled Chemicals) in water-injected oocytes versus SNF-3–injected oocytes. The electrophysiological characteristics of the heterologously expressed SNF-3 were then studied using a GeneClamp 500 (Axon Instruments). Kinetic parameters for the saturable transport of SNF-3 were calculated using the Michaelis–Menten equation. Data were analyzed by nonlinear regression and confirmed by linear regression.

ACR-23 analysis. ACR-23 analysis was performed as described previously.47. To generate plasmid constructs for Xenopus oocyte expression, we subcloned full-length error-free ACR-23 cDNAs and ACR-23 gain-of-function (ACR-23[1301N]) into the pSGEM expression vector using the SLIC cloning technique36 to produce pADA206 and pADA278. RNAs were prepared using the T7 mMessage mMachine kit (Ambion). Capped ACR-23 (0.1–5 ng total) or ACR-23[1301N] (0.5–2.5 ng total) RNAs were injected into Xenopus oocytes. 1–4 d post-injection, two-electrode voltage-clamp recordings were performed. Voltage was clamped at ~60 mV. The standard bath solution was Ringer’s: 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 10 mM Hepes, pH 7.2 (NaOH). Each oocyte was subjected to 20-s applications of agonist with a 2-min wash between test applications. Expression of ACR-23[1301N] was very toxic to Xenopus oocytes. We only used healthy oocytes with low channel expression for further analysis of this receptor. Eight oocytes were used to characterize the betaine concentration–response relationship of ACR-23[1301N], but individual oocytes were not tested through the full range of active concentrations. Three oocytes were tested at 0.001–0.003 mM, five were tested at 0.01–0.3 mM, and all were tested at 1–10 mM. Curves were fit to data from the five oocytes with data in the linear response range to perform statistical analysis of EC50 values.

Ion selectivity. For voltage-ramp experiments, BaCl2 was replaced by BaC12 in the extracellular solutions to eliminate possible contributions from the endogenous Ca2+–activated chloride channel. To assay sodium permeability, we substituted 115 mM NMDG chloride for NaCl. Thus, Na-free solution contains 115 mM NMDG, 2.5 mM KCl, 1.8 mM BaCl2, and 10 mM Hepes, pH 7.2 (HCl). To compare sodium and potassium permeability, NaCl was replaced with equimolar KCl: 115 mM KCl, 2.5 mM NaCl, 1.8 mM BaCl2, 10 mM Hepes, pH 7.2 (KOH). To assay barium permeability, BaCl2 was increased tenfold: 115 mM NaCl, 18 mM BaCl2, 2.5 mM KCl, 10 mM HEPES, pH 7.2 (NaOH). The osmolality of each solution was adjusted to 300 mOsm with sucrose. Reversal potentials were measured using 2-s voltage ramps (~100 mV to +60 mV) during sustained betaine application. Reversal potential shifts were similar at various time points after initiating betaine perfusion. Ramps at 20 s were analyzed for the potassium substitution experiment because of changing rectification characteristics at later time points, whereas ramps at 60 s were used for all other analyses. An agar bridge was used to minimize changes in junction potentials when switching solutions. Small residual voltage offsets due to differing ion composition in perfusion buffers were measured relative to standard solution before each voltage ramp recording, and were subtracted from the amplifier command voltages during data analysis. Data analysis was performed using Igor software (WaveMetrics). All chemicals were purchased from Sigma Chemical.

Monepantel. Commercially available Zolvix (25 mg ml−1 monepantel, Novartis) was diluted in DMSO to make a 10 mM stock solution of monepantel. The stock solution was diluted in water to make a 10 μM working stock in 0.1% DMSO. Final concentrations were achieved by diluting in Ringer’s to make the experimental perfusion solutions. 1 mM betaine was applied for 60 s either alone or in the presence of 0.3 mM monepantel following a 40-s pre-application of monepantel alone. All perfusion buffers were supplemented with 0.1% DMSO. Peak current magnitudes in three 10-s time windows were compared in the presence and absence of monepantel immediately after applying monepantel alone, immediately after initiating betaine application and 50 s after sustained betaine application.

Culture of nematodes. Strain genotypes were blinded to the experimenter. Early embryos were isolated by alkaline hypochlorite treatment and plated after synchronization on NGM plates seeded with HB101 bacteria as a food source at 20 °C. For each biological replicate, ~8,000 worms were harvested, washed twice with M9, twice with water and stored at ~80 °C until extraction. Eight samples were grown for each strain.

Extraction procedure for metabolites. Metabolites from whole nematodes were extracted using a methanol-chloroform procedure. 600 μl of a methanol-chloroform mix (2:1, vol/vol) was added to the frozen nematodes. Samples were homogenized using a Polytron and sonicated for 15 min. 200 μl each of chloroform and water were added, the samples centrifuged and the aqueous layer separated from the lipid one. The procedure was repeated twice. The aqueous layer was dried overnight in an evacuated centrifuge.

Analyis of aqueous extracts. The dried extracts were rehydrotated in 600 μl of D2O containing 0.05 mM sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeteriopropionate (TSP; Cambridge Isotope Laboratories) as an internal standard. The samples were analyzed using an AVANCE II+ NMR spectrometer operating at 500.13 MHz for the 1H frequency (Bruker) using a 5-mm TXI probe. Spectra were collected using a solvent suppression pulse sequence based on a one-dimensional NOESY pulse sequence to saturate the residual 1H water signal (relaxation delay = 2 s, t1 increment = 3 μs, mixing time = 150 ms, solvent presaturation applied during the relaxation time and the mixing time). 196 transients were collected into 16 K data points over a spectral width of 12 ppm at 27 °C for the adult samples.

Conflict of interest.

Data Availability.

Acknowledgments.

Funding.
Analysis of the metabolic profile of the bacteria. 400 µl of the bacteria (HB101) broth used to feed worms was mixed with 600 µl of water and centrifuged. The supernatant was collected and dried in an evacuated centrifuge. The dried samples were rehydrated in 600 µl of D2O, containing 0.05 mM TSP as an internal standard and analyzed by NMR spectroscopy in the same way as the extracts described above. 128 transients were collected for these samples.

Data processing. NMR spectra were processed using an ACD one-dimensional NMR processor (version 13, ACD). Free induction decays were Fourier transformed following multiplication by a line broadening of 1 Hz, and referenced to TSP at 0.0 ppm. Spectra were phased and baseline corrected manually. Each spectrum was integrated using 0.02 ppm integral regions between 0.5 and 4.5, and 5.5–9.5 ppm. Each spectral region was normalized to a total integral value of 1,000. In addition the resonances of selected metabolites (betaine, choline and glycine) were integrated using the software package Chenomx version 7.1. The integrals were normalized to total pellet dry weight.

Multivariate analysis of metabolic profiles. Each set of metabolic profiles obtained were analyzed by multivariate analysis. Data sets were imported into SIMCA-P 12.0 (Umetrics) for processing using PCA and PLS-DA (a regression extension of PCA used for supervised classification). Proton NMR data were Pareto scaled, in which each variable was centered and multiplied by 1/(Sk)^1/2 where Sk is the s.d. of the variable.

Transgenic worms. The plasmids used in this study were generated using standard molecular biology techniques and Gateway technology (Invitrogen). All transgenic strains were generated by standard microinjection techniques.

Strains. The Bristol N2 strain was used as the wild type; worms were raised at 22 °C on NGM plates seeded with OP50 E. coli. All other strains generated for this work are listed in Supplementary Table 1.
Erratum: Betaine acts on a ligand-gated ion channel in the nervous system of the nematode *C. elegans*

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In the version of this article initially published, Aude S. Peden was included under “These authors contributed equally to this work.” Only the second and third authors contributed equally. The error has been corrected in the HTML and PDF versions of the article.