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ARTICLES

EXP-1 is an excitatory GABA-gated cation channel

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γ-aminobutyric acid (GABA) mediates fast inhibitory neurotransmission by activating anion-selective ligand-gated ion channels. Although electrophysiological studies indicate that GABA may activate cation-selective ligand-gated ion channels in some cell types, such a channel has never been characterized at the molecular level. Here we show that GABA mediates enteric muscle contraction in the nematode *Caenorhabditis elegans* via the EXP-1 receptor, a cation-selective ligand-gated ion channel. The EXP-1 protein resembles ionotropic GABA receptor subunits in almost all domains. In the pore-forming domain of EXP-1, however, the residues that confer anion selectivity are exchanged for those that specify cation selectivity. When expressed in *Xenopus laevis* oocytes, EXP-1 forms a GABA receptor that is permeable to cations and not anions. We conclude that some of the excitatory functions assigned to GABA are mediated by cation channels rather than by anion channels.

$GABA_A$ receptors, members of the ligand-gated ion channel superfamily, mediate fast inhibitory neurotransmission in both vertebrates and invertebrates. The binding of GABA to these receptors opens an anion-selective channel that predominantly conducts chloride ions[1], thereby inhibiting cell activity. However, there are numerous precedents for GABA acting as an excitatory neurotransmitter. Three mechanisms have been put forward for the excitatory action of GABA. (i) In fetal neurons, intracellular chloride levels are high enough that chloride flows out of the cell through $GABA_A$ receptors and causes depolarization[2,3]. (ii) In adult hippocampal neurons, GABA binding causes an efflux of bicarbonate ions, due to a collapse of the chloride gradient, and is thereby depolarizing[4–6]. (3) Other studies—primarily in invertebrates—have suggested that GABA might mediate excitation via cation currents; however, no cation-selective GABA receptor has been identified at a molecular level[7–10].

The nervous system of the nematode *C. elegans* has 26 GABA neurons[11]. The functions of these neurons have been determined using laser ablation experiments[11]. The DD and VD ventral cord GABA motor neurons innervate the body wall muscles and are required for locomotion[11]. At these synapses, GABA mediates inhibition of the body wall muscle by activating the chloride-selective UNC-49 GABA receptor[12]. By contrast, two GABA motor neurons, one whose cell body is in the dorsorectal ganglion (DVB) and the other a ventral cord motor neuron (AVL), innervate the enteric muscles and are required for enteric muscle contraction[11]. Normally GABA is an inhibitory neurotransmitter, but at these synapses GABA directly causes excitation of the enteric muscles[11].

GABA has been implicated as the excitatory neurotransmitter that mediates enteric muscle contraction for several reasons. First, the GABA motor neurons AVL and DVB directly innervate the enteric muscles and are required for their contraction[11,13]. When AVL and DVB are jointly laser-ablated, enteric muscle contractions are eliminated[11]. Second, killing all other neurons with processes near the enteric muscles does not cause defects in enteric muscle contraction[11]. Third, mutants that do not synthesize GABA lack enteric muscle contractions. The biosynthetic enzyme for GABA, glutamic acid decarboxylase, is encoded by the *unc-25* gene[14]. In *unc-25* mutants, which lack GABA immunoreactivity, enteric muscle contractions are reduced to 12% of the normal frequency[11,15]. Fourth, when *unc-25* mutant adults are bathed in exogenous GABA, AVL and DVB accumulate GABA via the plasma membrane transporter, and enteric muscles contractions are restored[16]. Thus, the lack of enteric muscle contractions in *unc-25* mutants is not due to developmental defects. Fifth, lack of the chloride-selective UNC-49 GABA receptor does not affect enteric muscle contraction, demonstrating that the inhibitory and excitatory actions of GABA are mediated by distinct GABA receptors[12].

In the present study, we show that the *exp-1* gene encodes an excitatory cation-selective GABA receptor that mediates enteric muscle contraction in *C. elegans*.

RESULTS

exp-1 mutants lack enteric muscle contractions

The defecation motor program in *C. elegans* hermaphrodites consists of a series of three independent muscle contractions that are initiated every 50 s when the animal is feeding[15]. The program begins with a posterior body muscle contraction (pBoc) which forces intestinal contents forward, is followed by an anterior body muscle contraction (aBoc) which forces intestinal contents backward, and is concluded by an enteric muscle contraction (Emc) which expels the contents of the intestine[15]. Because the enteric muscle contractions seemed to be mediated by an excitatory activity of GABA, we were interested in genes that disrupt this specific step of the defecation motor program. *exp-1* mutants have normal posterior and anterior body muscle contractions, but lack enteric muscle contractions[15] (**Fig. 1a**). Moreover, these animals exhibit normal locomotion, which requires the inhibitory action

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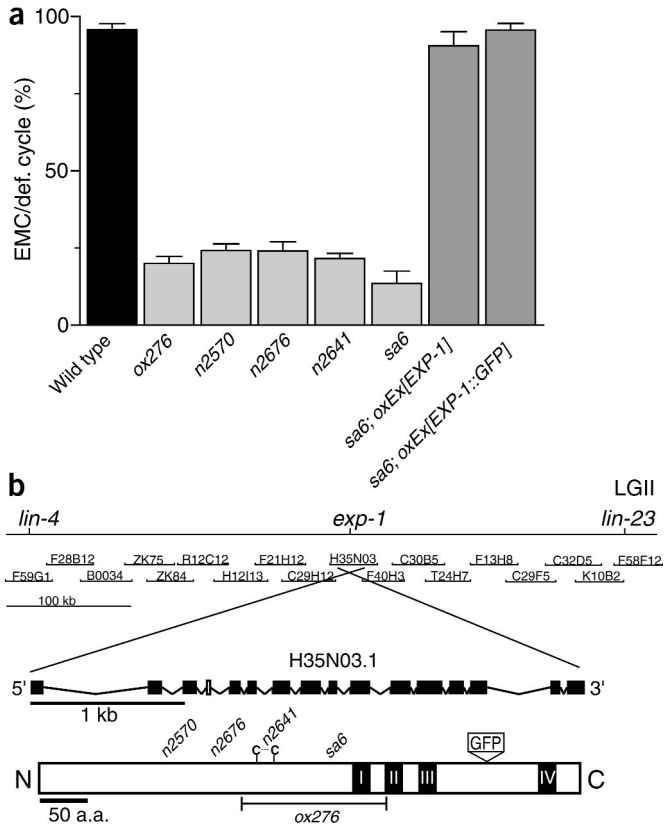

Figure 1 Phenotypic characterization and molecular cloning of *exp-1*. (**a**) *exp-1* mutants (*ox276, n2570, n2676, n2641, sa6*) are defective in enteric muscle contractions. Enteric muscle contractions (EMC) were scored for each defecation cycle (EMC/def. cycle). To determine the percentage of successful enteric muscle contractions per defecation cycle, we scored for the presence of an enteric muscle contraction following a posterior body contraction (Emc/pBoc). Eleven defecation cycles from ten individual young adult animals were scored for each genotype. The strain *sa6; oxEx[EXP-1]* contains an extrachromosomal array containing the *exp-1* genomic rescuing plasmid (pAB03). The strain *sa6;oxEx[EXP-1:GFP]* contains the array expressing the EXP-1:GFP fusion protein (pAB05). Percentage of EMC/def. cycle: wild type, 95.7 ± 2.0%; *ox276*, 20.0 ± 2.3%; *n2570*, 24.2 ± 2.1%; *n2676*, 24.0 ± 3.0%; *n2641*, 21.6 ± 1.7; *sa6*, 13.6 ± 4.0%; *sa6; oxEx[EXP-1]*, 90.1 ± 4.6%; *sa6; oxEx[EXP-1:GFP]*, 95.6 ± 2.2%. Error bars represent s.e.m. (**b**) The *exp-1* locus. Above, the physical map of the *exp-1* region on chromosome II; middle, the exon-intron structure; below, predicted protein structure. The alternatively spliced exon 4 (white exon) is comprised of 18 nucleotides and is only found in the EXP-1B protein. Positions of the mutations in the *exp-1* alleles are denoted on the predicted protein structure.

of GABA. Thus, *exp-1* is the only gene known to be specifically required for the excitatory function of GABA. Mutations in the *exp-1* gene were first isolated in genetic screens for mutants with abnormal defecation cycles[15], and subsequently in screens for genes required for GABA function (E.M.J. & H.R. Horvitz, personal communication), or in targeted gene disruption screens (A.A.B. & E.M.J., unpublished data).

exp-1 cloning

We cloned *exp-1* using standard positional cloning techniques, and it mapped to the interval between *lin-4* and *lin-23* on chromosome II (**Fig. 1b**). Cosmids covering this interval were injected into *exp-1(sa6)*, and the fosmid H35N03 rescued the mutant phenotype. A genomic subclone containing the H35N03.1 open reading frame plus 3.5 kb of upstream promoter sequence was sufficient to rescue the *exp-1* phenotype (**Fig. 1a**). All *exp-1* alleles contained mutations in this open reading frame, thereby proving that we had identified the *exp-1* gene (**Fig. 2** and **Table 1**). The mRNA structure of EXP-1 was determined by reverse transcription and polymerase chain reaction (PCR). We identified two distinct EXP-1 transcripts that differed by the small alternatively-spliced exon 4 (**Fig. 1b**, white exon). The *exp-1* gene encodes a protein that is homologous to ligand-gated ion channel subunits. The predicted protein is comprised of a large extracellular ligand-binding domain with a conserved disulfide bond, four transmembrane domains (M1–M4) and a large intracellular loop between M3 and M4 (ref. 17; **Figs. 1b** and **2**).

EXP-1 is localized to neuromuscular junctions

If *exp-1* encodes the GABA receptor that mediates enteric muscle contractions, then it should be expressed in these tissues. The enteric muscles are comprised of three muscles classes: the intestinal, sphincter and anal depressor muscles (**Fig. 3a**). The open reading frame for green fluorescent protein (GFP) was placed under the control of the 3.5-kb *exp-1* promoter to determine which tissues express the *exp-1* gene. Fluorescence was observed in the intestinal and anal depressor muscles, but not in the sphincter muscle (**Fig. 3b**). Expulsion in hermaphrodites is largely regulated by contraction of the anal depressor muscle, but due to differences in anatomy between hermaphrodites and males, expulsion is regulated by relaxation of the sphincter muscle in adult males[18]. Interestingly, the inhibitory UNC-49B GABA receptor isoform is expressed in the sphincter muscle and not in the anal depressor or intestinal muscles[12]. Thus, complementary expression patterns of excitatory and inhibitory GABA receptors can explain how AVL and DVB can cause muscle contraction in hermaphrodites and muscle relaxation in males.

To visualize the location of the EXP-1 protein, we inserted GFP into the cytoplasmic loop between M3 and M4 of the *exp-1* rescuing subclone (**Fig. 2**). The GFP-tagged protein was able to fully rescue *exp-1(sa6)* mutants (**Fig. 1a**), suggesting that the fusion protein was correctly localized. GFP-tagged receptors were juxtaposed to the preanal ganglion where AVL and DVB innervate the enteric muscles, suggesting that EXP-1 receptors are clustered at sites that may correspond to neuromuscular junctions (**Fig. 3c**).

EXP-1 is an ionotropic GABA receptor

$GABA_A$ receptors are members of the ligand-gated ion channel superfamily that include the nicotinic acetylcholine (nAChR), serotonin (5-HT3R) and glycine receptors (GlyR). Each of these receptor subtypes are oligomeric proteins comprised of five subunits that are pseudo-symmetrically arranged around a central ion channel, such that the M2 domain lines the ion-channel wall[19]. These ligand-gated ion channel subunits all share a common structural and functional homology[20]. A phylogenetic tree indicated that EXP-1 is a member of the ionotropic GABA receptor subunit family (**Fig. 4**; see Discussion). EXP-1 shares 22% and 21% identity with the *C. elegans* UNC-49B and human β2 GABA receptor subunits, respectively, but only 11% identity with the human α7 nAChR subunit. Alignments with GABA receptors indicate that the residues implicated in lining the GABA binding pocket are conserved in the EXP-1 protein[21,22] (**Fig. 2**). EXP-1 possesses the GABA interacting residues found in both the alpha and beta subunits, suggesting that EXP-1 could act as a homomeric receptor because a complete GABA-binding pocket would be created at interfaces between EXP-1 subunits[21,22] (**Fig. 2**). Interestingly, EXP-1 differs significantly from all known GABA

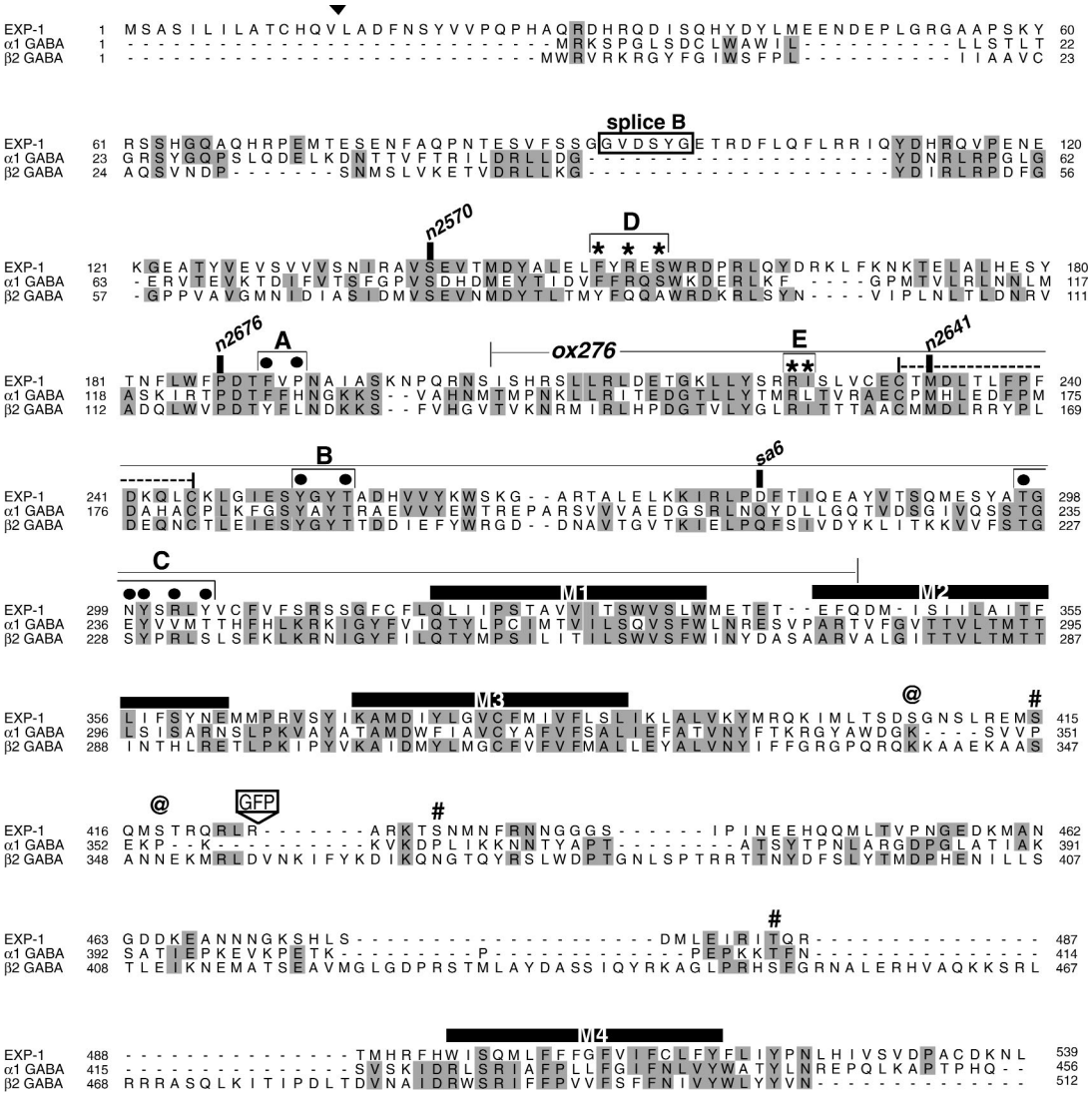

Figure 2 Sequence alignment of EXP-1 and GABA receptor subunits. Deduced polypeptide sequence of *C. elegans* EXP-1 and alignment with the human α1 (CAA32874) and β2 (AAB29370) $GABA_A$ receptor subunits. The alternatively spliced exon 4, which encodes six residues, is boxed (splice B). Sequences were aligned using ClustalX, SeqVu and Bonsai (J. Thomas, University of Washington). Identities are shaded. Arrowhead indicates the predicted signal peptide cleavage site; dashed lines indicate the conserved disulfide bond ($CX_{13}C$) present in all ligand-gated ion channel subunits. The stars and black circles represent residues implicated in lining the GABA binding pocket for the α1 (loops D and E) and β2 (loops A, B, and C) subunits, respectively[21,22,46–50]. The four transmembrane domains are represented by black bars. All *exp-1* mutations are shown; the thin black bar delineates the residues deleted in the *ox276* allele. Predicted PKA and PKC phosphorylation sites are shown as # and @, respectively. The position of the GFP insertion is indicated with a triangle.

receptor subunits within the M2 domain, which lines the ion channel pore and determines ion selectivity[19,23–26] (**Fig. 6**; see Discussion).

To determine whether EXP-1 can form a functional GABA receptor, we examined the functional properties of EXP-1 expressed in *X. laevis* oocytes using two-electrode voltage clamp recording. Oocytes injected with *exp-1* cRNA produced a GABA-evoked current, suggesting that EXP-1 forms a GABA-gated homo-oligomeric receptor (**Fig. 5a**). At –60 mV, application of GABA concentrations from 1 to 1,000 μM produced robust inward currents up to several microamperes (**Fig. 5a**, inset). The EC_{50} (effective concentration for half-maximum response) for GABA activation of EXP-1 receptors was 26.4 ± 1.08 μM (n = 19), which is lower than that determined for the UNC-49B homomeric GABA receptor (43.7 ± 2.9 μM) when expressed in *X. laevis* oocytes[12].

EXP-1 is a cation-selective GABA receptor

To date, all characterized ionotropic GABA receptors form chloride-selective channels. We used current-voltage (*I-V*) analysis to characterize the ionic conductance underlying EXP-1 responses. The GABA-dependent current from oocytes expressing EXP-1 reverses at –5.54 ± 2.20 mV (n = 24) in Ringer's solution (**Fig. 5b**, solid line). This is significantly different from the reversal potential of the worm's chloride-selective UNC-49B GABA receptor (–30 mV)[12] and the chloride equilibrium potential (E_{Cl}) in *X. laevis* oocytes (approximately –20 to –30 mV)[27]. To determine which ionic species permeate the EXP-1 channel, we calculated *I-V* relationships in a number of different extracellular solutions.

To test whether EXP-1 is permeable to chloride, we substituted extracellular chloride with the large anion gluconate. In chloride-free

Table 1 Summary of *exp-1* mutations

Allele	Nucleotide change	Protein change
n2570	TCT → TTT	S140F
n2676	CCC → TCC	P187S
n2641	ATG → ATA	M233I
sa6	GAC → AAC	D280N
ox276	TCGAT(Δ724 bp) ttgt…tga	SI…9aa…stop

The nucleotide mutation and resulting protein change are shown in the table for each allele. All mutations were induced by ethyl methane sulfonate.

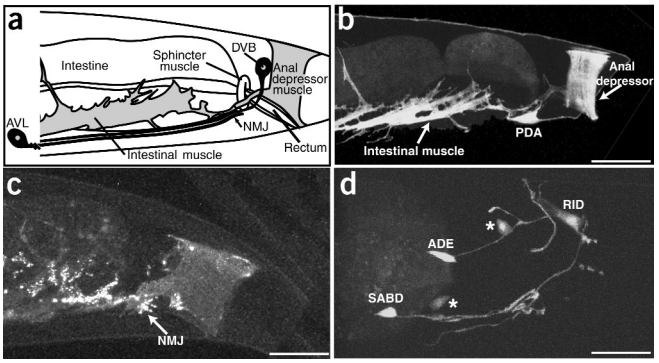

Figure 3 Expression pattern of the *exp-1* gene. (**a**) Schematic diagram of the enteric muscles in *C. elegans*. The enteric muscles are comprised of three muscles classes: intestinal, sphincter and anal depressor muscles. Flattened processes of the GABA motor neurons AVL and DVB abut the enteric muscles and DVB forms a neuromuscular junction at the dorsal edge of the preanal ganglion as depicted[13]. Confocal micrographs of transgenic animals expressing GFP under the control of the *exp-1* gene. (**b**) Transcriptional GFP fusion. Fluorescence was observed in the intestine and anal depressor muscles as well as the neuron PDA. PDA is a motor neuron that receives input from the GABA motor neuron DVB and forms synapses on the dorsal muscles. Activation of this motor neuron may cause dorsal flexure, which could aid in opening the anus. (**c**) Translational GFP fusion. Dim GFP fluorescence is observed in the cell bodies of the intestine and anal depressor muscles. Bright puncta are located at the dorsal surface of the pre-anal ganglion where AVL and DVB contact the enteric muscles (NMJ), indicating a synaptic localization of the EXP-1 protein. Tail of an adult hermaphrodite is shown; anterior is to the left, dorsal is up. (**d**) Head neurons. Confocal micrograph of the right side of the head of an adult hermaphrodite expressing the EXP-1: GFP translational fusion. Fluorescence is observed in the neurons RID, ADE and SABD. In addition, two unidentified neurons (*) were variably expressed in the head ganglion. Anterior is to the right and dorsal is up. Scale bar = 5 μM in all panels.

Ringer's solution, the reversal potential for EXP-1 currents was –5.05 ± 1.08 mV (n = 9) (**Fig. 5b**, dashed line). This value was not significantly different (P = 0.88, paired t-test) from that determined in normal Ringer's and indicates that chloride ions make no significant contribution to the current passing through the EXP-1 channel. If chloride does not pass through the EXP-1 ion channel, then which ions pass through the channel?

The near 0 mV reversal potential suggests that EXP-1 may be a nonselective cation channel. To determine whether EXP-1 is permeable to the monovalent cation sodium (Na^+), we substituted Na^+ with the large cation *N*-methyl-D-glucamine (NMDG). When external Na^+ was completely replaced by NMDG, the EXP-1 reversal potential shifted to –84.34 ± 1.0 mV (n = 8), near the predicted potassium equilibrium potential (E_K) in *X. laevis* oocytes[27] (**Fig. 5c**, dashed line). Replacement of sodium in the external bath with NMDG abolished the GABA-induced inward current, suggesting that EXP-1 is not permeable to large cations (NMDG) and anions (Cl^-), but is permeable to the small cation Na^+. To further investigate ion permeation through the EXP-1 channel, we varied external potassium. When external NaCl was replaced by 115 mM KCl, the reversal potential of EXP-1 expressing cells was –4.12 ± 1.28 mV (n = 13), demonstrating that K^+ ions are also permeant through EXP-1 channels. Using the averaged ΔE_{rev} values measured in KCl Ringer's compared to NaCl Ringer's, a permeability (P) ratio of P_K/P_{Na} = 1.1 ± 0.08 (n = 13) was determined using a modified Goldman-Hodgkin-Katz equation.

Extracellular NaCl dilutions were performed to verify cation selectivity. Extracellular NaCl was reduced from 100% (115 mM) to 50% (57.5 mM) and 25% (28.75 mM). Reversal potentials obtained from the dilution experiments (50%: –19.4 ± 3.19 mV, n = 13; 25%: –42.98 ± 6.52 mV, n = 10) were plotted against the extracellular concentration of NaCl (**Fig. 5d**, solid line). As extracellular NaCl decreased, negative reversal potential shifts were observed, as predicted by the Goldman-Hodgkin-Katz equation for a nonselective cation channel (50%: E_{rev} = –20.24 mV; 25%: E_{rev} = –37.51 mV; **Fig. 5d**, dashed line).

To determine if EXP-1 is permeable to divalent cations, we increased extracellular barium ten-fold (18 mM Ba^{2+}) in the absence of Na^+. If EXP-1 were permeable to Ba^{2+}, then a positive reversal potential shift would be expected because Ba^{2+} would represent the only available cation capable of conducting through the EXP-1 channel in this solution. Instead, GABA-induced responses did not show a significant shift in reversal potential from control conditions (ΔE_{rev} = –0.11 mV, n = 10, $P \geq 0.05$, two-way ANOVA), suggesting EXP-1 has negligible divalent cation permeability (**Fig. 5e**).

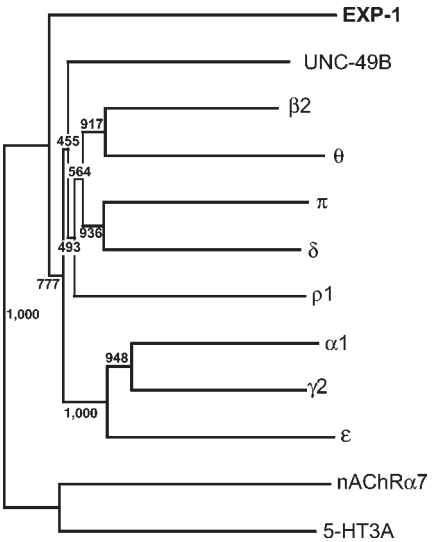

Figure 4 EXP-1 is a unique member of the ionotropic GABA receptor family. Phylogenetic tree of the EXP-1 protein and various ligand-gated ion channel subunits. The intracellular loop was not used in this analysis due to the lack of sequence homology within this domain. The bootstrap method with 'neighbor-joining' search was used to generate the tree. Bootstrap values of 1,000 replicates are indicated on the tree. Alignments were performed using ClustalX, NJPlot (Genetics Computer Group) and Bonsai (J. Thomas). We used human GABA (α1, β2, γ2, δ, ε, ρ1, θ, π), human α7 nAChR, human $5HT3_A$ and *C. elegans* UNC-49B receptor subunits for this analysis.

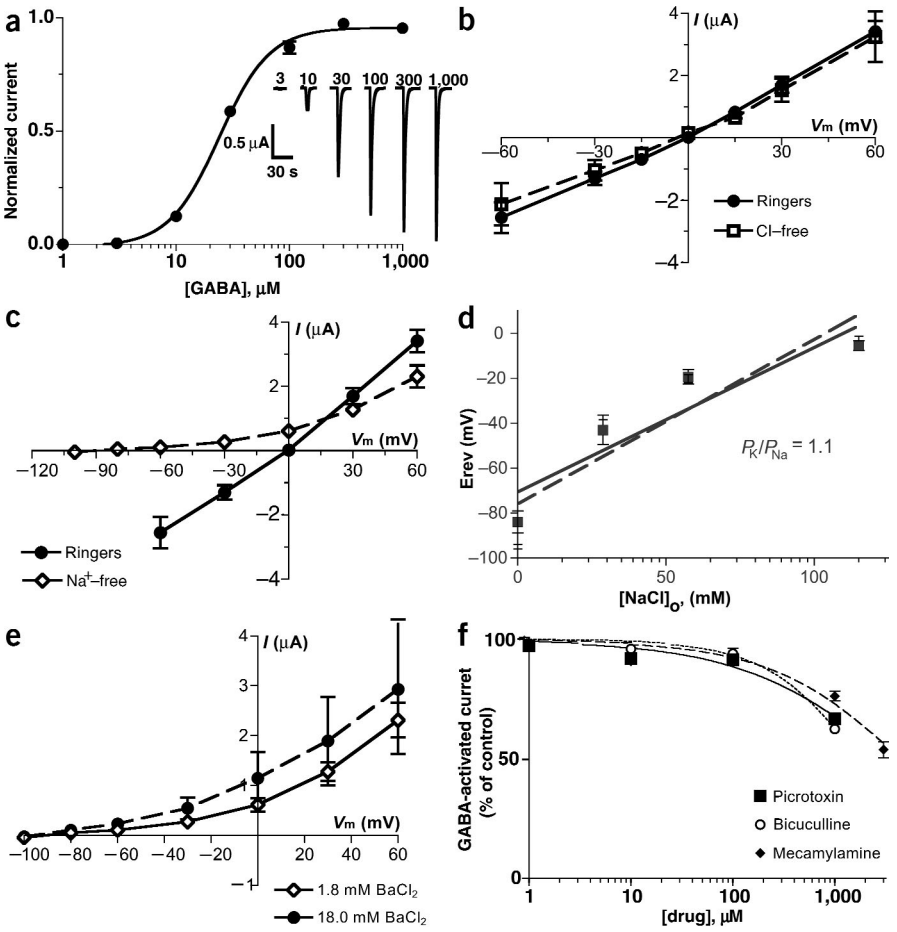

Figure 5 EXP-1 is a GABA-gated, cation-selective channel. (**a**) EXP-1 GABA dose-response curve. EXP-1-expressing oocytes were voltage-clamped at –60 mV and a series of GABA applications (1–1,000 μM) were bath-applied for 5 s. Each point represents the mean current value normalized to the maximum and minimum values. For EXP-1 receptors, EC_{50} = 26.4 ± 1.08 μM and H = 1.8 ± 0.24 (n = 19). Error bars represent s.e.m. Inset, representative traces of EXP-1 dose-response experiments. (**b**–**e**) Ion selectivity of EXP-1 receptors in *X. laevis* oocytes. GABA-evoked (30 μM, 5 s) current responses were recorded at the holding potentials shown. (**b**) Solid line, *I-V* curve determined under standard Ringer's (E_{rev} = –5.54 ± 2.20 mV, n = 24). Dotted line, Cl^- permeability (in Cl^--free Ringer's, chloride ions were replaced with the anion gluconate). The negligible shift in reversal potential demonstrates that chloride ions do not underlie the ionic conductance through EXP-1 channels (E_{rev} = –5.05 ± 1.08 mV, n = 9). (**c**) Na^+ permeability (in Na^+-free Ringer's, Na^+ ions were replaced with the cation NMDG). There was a negative shift in reversal potential (E_{rev} = –84 mV ± 1.0 mV, n = 8) and the inward current was nearly abolished, which suggests that Na^+ is the primary charge carrier through the EXP-1 channel. (**d**) NaCl dilutions. Reversal potentials were measured in solutions with varying NaCl concentrations (50%, 25% and 0%) relative to 100% NaCl Ringer's (115 mM). As expected, the reversal potential became more negative as NaCl concentrations were reduced. EXP-1 channels showed a shift in reversal potential that is indicative of cation-selectivity (solid line) and correlates well with the shift predicted by the Goldman-Hodgkin-Katz equation for nonselective cation channels (dashed line) based on intracellular concentrations of 10.4 mM Na^+ and 108 mM K^+ (ref. 27). (**e**) Ba^{2+} permeability (in 18 mM Ba^{2+} Ringer's, Ba^{2+} was increased tenfold in the absence of sodium to determine divalent permeability). Note that EXP-1 receptors did not show a significant shift in reversal potential ($P \geq 0.05$, two-way ANOVA) or an increased inward conductance under these conditions, which suggests EXP-1 receptors have negligible divalent cation permeability (n = 10). (**f**) EXP-1 pharmacology. Pharmacological dose-response curves were generated by applying EC_{50} GABA alone or with drug at varying concentrations (n = 5–7). Responses were normalized to maximal EC_{50} GABA responses.

EXP-1 pharmacology

EXP-1 is a GABA receptor composed of a conventional GABA binding domain associated with a cation-permeable pore, suggesting that it should be affected by GABA receptor competitive agonists and antagonists but not by pore blockers. As expected, the GABA agonist muscimol activated EXP-1 expressing cells (EC_{50} = 420.5 ± 1.09 μM, n = 5), demonstrating conservation of the GABA binding pocket. Bicuculline is a competitive antagonist of $GABA_A$ receptors[28]; surprisingly, EXP-1 is not blocked by bicuculline (**Fig. 5f**). Bicuculline resistance of EXP-1 receptors is intriguing because the residues implicated in GABA binding are fairly well conserved (**Fig. 2**).

The M2 pore domain of EXP-1 more closely resembles cholinergic receptors (**Fig. 6**). Picrotoxin is regarded as a pore blocker of ligand-gated anion channels[29–31], and as expected, the EXP-1 receptor is not blocked by picrotoxin (**Fig. 5f**). Mecamylamine is regarded as a cation channel blocker[32]. Although the EXP-1 M2 pore domain is analogous to cholinergic receptors at the molecular and functional levels, the EXP-1 receptor is highly resistant to the channel blocking activity of mecamylamine (**Fig. 5f**).

DISCUSSION

Here we have shown that GABA mediates enteric muscle contraction in *C. elegans* by activating the EXP-1 receptor, an excitatory GABA-gated cation channel. EXP-1 is expressed in the enteric muscles and is localized to neuromuscular junctions that receive inputs from the GABA motor neurons AVL and DVB. The cloning and characterization of *exp-1* provide the first molecular and functional evidence that ionotropic GABA receptors can serve as excitatory ligand-gated ion channels by passing cations.

Three mechanisms for excitatory activities of GABA have been proposed: chloride efflux, bicarbonate efflux or cation influx. There are convincing physiological data indicating that chloride and bicarbonate efflux can mediate GABA excitation in particular cell types[2–6]. The receptors that mediate GABA excitation in these cells are likely to be members of the $GABA_A$ family of receptors, that is, they are anion-selective channels that mediate unusual physiological responses because of changes in cellular ion concentrations. However, physiological studies in other systems suggest that GABA mediates excitation by activating cation conductances. For example, Na^+-dependent GABA depolarizations have been documented in the stomatogastric ganglion of crab[8], and in neurons of the snail[9,33] and *Aplysia californica*[10]. Such cellular responses would require the existence of a GABA-gated cation channel. The molecular identification and functional characterization of the EXP-1 receptor directly demonstrates the existence of an ionotropic GABA receptor that is permeable to cations and not anions. It is likely that the excitatory actions of GABA

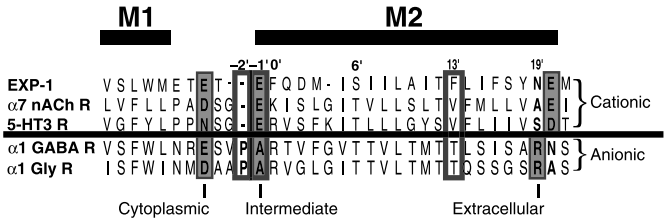

Figure 6 EXP-1 contains the molecular determinants for cation selectivity. EXP-1 resembles ligand-gated cation channels in the M2 domain. The top sequences represent selected M2 domains of cation-selective channels: human α7 nAChR (CAA49778) and the human serotonin 5-HT_{3A} receptor (NP_000860). The bottom two rows represent sequences from anion-selective channels: human α1 GABAR (CAA32874) and human α1 GlyR (NP_000162) for comparison. The residues that are implicated in determining ion selectivity for ligand-gated ion channels are represented in bold boxes. The cytoplasmic, intermediate and extracellular rings of charge are shown in the corresponding sequences[34].

in these other organisms are mediated by receptors related to the EXP-1 GABA receptor. Moreover, in the rat accessory olfactory bulb, a subset of cells respond to GABA with unusually high reversal potentials, indicating that GABA is excitatory to these neurons[7]. The reversal potential of these excitatory GABA responses is dependent on extracellular Na^+ and not Cl^-. Database searches do not, however, return an EXP-1 homolog in the mouse genome. Searches for homologs have revealed that there is another closely related gene to *exp-1* in *C. elegans*: Y46G5A.26.

What are the molecular determinants of cation selectivity in EXP-1? Ion selectivity of ligand-gated ion channels is determined by the M2 pore-forming domain. The M2 domain possesses three rings of charge called the cytoplasmic, intermediate and extracellular charged domains[34]. The cytoplasmic ring is negative in all ligand-gated ion channels, whereas the intermediate and extracellular rings are negative in cation-selective and positive in anion-selective channels[19]. The intermediate ring of charge is located near the intracellular mouth of the channel, where the pore is most constricted, and is thought to provide the electrostatic interaction for cation or anion selectivity[34–38]. The first receptor in which ion charge selectivity was converted was the α7 nAChR[26]. When three residues within the M2 domain of the α7 nAChR were replaced with equivalent residues of the α1 GlyR, cation selectivity was converted to anion selectivity. The residue substitutions were as follows: insertion of a proline residue (–2′P), neutralization of the intermediate ring of charged glutamic acids (E–1′A) and substitution of a valine for threonine (V13′T)[26]. Additionally, the analogous substitutions were shown to convert the cation-selective serotonin-gated 5-HT_{3A} receptor to an anion-selective channel[39].

Recently, the converse changes have been shown to convert the anion-selective α1 glycine receptor into a cation-selective ion channel. Specifically, three changes converted the normally anion-selective α1 glycine receptor to a cation-selective channel: the deletion of a proline residue (P–2′Δ) immediately preceding the intermediate ring of charge, the replacement of the neutral alanine with a negatively-charged glutamic acid at the intermediate ring (A–1′E), and the substitution of threonine for valine at the 13′ position (T13′V)[40]. Of these changes, it appears that the intermediate ring of charge at the –1′ position seems to be the most important, as a single substitution (A–1′E) was sufficient to convert the α1 glycine receptor to select cations over anions[38]. Additionally, the deletion of the proline (P–2′Δ) when coupled with the A–1′E substitution enhances cation permeability almost threefold, suggesting that pore geometry as well as electrostatic charge are important determinants of ion charge selectivity[38].

At the molecular level, the EXP-1 protein is homologous to other GABA receptor subunits in almost all domains. Of particular importance is the conservation between the EXP-1 receptor and $GABA_A$ receptors in the GABA binding motifs (**Fig. 2**). However, alignment of the M2 domain with ligand-gated ion channels reveals that EXP-1 contains the exact molecular determinants sufficient for cation-selectivity. First, EXP-1 has no proline residue at position –2′; second, it contains a negatively-charged glutamic acid at the intermediate ring of charge; and finally position 13′ contains a phenylalanine, which is similar to the hydrophobic valine residue present in cation-selective channels (**Fig. 6**). Interestingly, the extracellular ring in EXP-1 is negatively charged as in other cation channels, rather than positive as in the other anion channels, although this charged residue is not essential for cation selectivity[38,41,42]. The molecular characterization of EXP-1 reveals that GABA receptor ion selectivity is determined by the same set of residues identified in *in vitro* mutagenesis of other members of the ligand-gated ion channel superfamily.

Ligand-gated ion channels are thought to have arisen from a common ancestor over 2.5 billion years ago[43]. It is intriguing to speculate how the EXP-1 receptor subunit might have evolved. First, it is possible that a cation channel accumulated mutations in the extracellular domain to acquire sensitivity to GABA. According to our phylogenetic tree, however, EXP-1 has a closer relationship to GABA receptors than to any cation-permeable ligand-gated ion channel (**Fig. 4**). Second, evolution may have 'recapitulated' the mutagenesis experiments; in other words, there is only one solution for creating a cation channel from the structure of a ligand-gated ion channel—the same as that identified by *in vitro* mutagenesis experiments[26,38]. Mutations at these sites converted an originally anion-selective GABA receptor into a cation-selective GABA receptor. Third, the EXP-1 GABA receptor might have arisen by domain shuffling between a GABA receptor and a cation channel to generate a chimeric receptor with novel properties.

METHODS

Behavioral assays. A defecation cycle (def. cycle) consists of a posterior body contraction (pBoc) followed by an anterior body contraction (aBoc) and is concluded by an enteric muscle contraction (Emc)[15]. To determine the percentage of successful enteric muscle contractions per defecation cycle, we scored for the presence of an enteric muscle contraction following a posterior body contraction (Emc/pBoc). We scored eleven defecation cycles from ten young adult animals at room temperature (22 °C) for each genotype. All strains were grown at room temperature. Error bars represent the standard error of the mean (s.e.m.).

Mapping. Mapping data have been submitted to WormBase (www.wormbase.org).

Transformation rescue and cloning of *exp-1*. Transgenic strains were obtained by microinjection of plasmid and cosmid DNA into the germline[44]. Initial rescue of *exp–1(sa6)* mutants was obtained by co-injecting the fosmid H35N03 at 20 ng/μl with 60 ng/μl pEK1, a plasmid that contains the wild-type *lin-15* gene as a co-transformation marker[45], into *exp-1(sa6); lin-15(n765ts)* animals to generate the array *oxEx288*. The 7.6-kb *exp-1* genomic rescuing subclone (pAB03) was created by isolating an *Nsi*I/*Sca*I restriction fragment of fosmid H35N03. This fragment contained the 3.5-kb *exp-1* promoter, the 3.8-kb H35N03.1 open reading frame, plus 0.31 kb of 3′ sequence. This fragment was ligated into the *Nsi*I/*Hin*cII site of the pGEM-T vector (Promega). pAB03

(20 ng/μl) was co-injected with pEK1 (80 ng/μl) into *exp-1(sa6);lin-15(n765ts)* animals. Stable lines (*oxEx289*) were obtained, which were completely rescued for enteric muscle contractions.

Several complementary DNAs (cDNAs) of the *exp-1* transcript were obtained by reverse transcription of wild-type poly-A^+ selected RNA followed by PCR amplification. 5′ and 3′ primers were chosen based on splice predictions (Intronerator). Fragments were gel purified (Qiagen) and TA-cloned into the pCR2.1 vector (Invitrogen), and sequenced by an automated sequencing machine (Applied Biosystems). Full-length error-free transcripts were obtained. Alternative splicing of the *exp-1* locus was determined by sequencing multiple cDNAs. Exon 4 is absent in the EXP-1A splice form and present in the EXP-1B splice form (**Fig. 1b**). Fifteen cDNA clones were sequenced; 11 of 15 contained exon 4, and 4 of 15 did not.

To identify the molecular lesions of *ox276, sa6, n2676, n2570* and *n2641* we PCR amplified the H35N03.1 open reading frame from single worms of each genotype. We sequenced regions corresponding to exons and deduced the nucleotide mutation for each genotype.

GFP expression constructs. Transcriptional GFP fusion. A 3.4-kb restriction fragment (*Eco*RV/*Eco*RV) of pAB03, which encompasses the *exp-1* promoter, was ligated into a *Hin*cII site of pPD95.75 (1995 Fire Vector Kit) to generate pAB04. This P_{exp-1}:GFP transcriptional reporter was co-injected with pEK1 (30 ng/μl) into *lin-15(n765ts)* animals at 60 ng/μl to generate the extrachromosomal array *oxEx348*.

Translational GFP fusion. To determine the location of the protein, we inserted GFP in-frame within the EXP-1 open reading frame. The GFP coding region from pPD95.75 was amplified with primers tagged with ApaI sites on both the 5′ and 3′ ends. This PCR product was gel-purified and inserted in frame into an ApaI site of pAB03 to generate pAB05. This fusion protein contains GFP in the intracellular loop between M3 and M4 (**Fig. 1b**). This translational *exp-1*:GFP reporter was coinjected at 1 ng/μl with pEK1 at 30 ng/μl and 69 ng/μl of 1-kb DNA Ladder (Gibco) into *exp-1(sa6); lin-15(n765ts)* animals. We obtained stable transgenic lines (*oxEx469*) that were rescued for enteric muscle contractions and showed GFP fluorescence in the enteric muscles.

Electrophysiology of EXP-1. A *Not*I/*Hind*III fragment containing the full-length EXP-1A and EXP-1B cDNAs including the 5′ and 3′ UTRs was cloned into the *Not*I/*Hind*III sites of pSGEM (courtesy of M. Hollmann) for oocyte expression. Capped RNA was prepared using the mMessage mMachine kit (Ambion). *X. laevis* oocytes were collected and injected with 50 ng of cRNA and two-electrode voltage clamp recordings were performed 3–5 d post-injection. The standard bath solution for dose-response and control *I-V* experiments was Ringer's: 115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, 10 mM HEPES (pH 7.2 NaOH). For dose-response experiments, each oocyte was subjected to a 5-s application of GABA (1–1,000 μM) with 2 min of wash between applications.

Ion selectivity experiments. All points are responses to 30 μM GABA application for 5 s. The reversal potential of EXP-1-expressing oocytes were first determined in standard Ringer's (as above). For the chloride permeability test, we used a simplified chloride-free solution, substituting 115 mM sodium gluconate for NaCl and KCl. Sodium dilution experiments were performed in the following solutions: 100% NaCl (115 mM NaCl, 1.0 mM $BaCl_2$, 10 mM HEPES), 50% NaCl (57.5 mM NaCl, 1.0 mM $BaCl_2$, 10 mM HEPES), 25% NaCl (28.75 mM NaCl, 1.0 mM $BaCl_2$, 10 mM HEPES), 0% NaCl (115 mM NMDG, 2.5 mM KCl, 1.8 mM $BaCl_2$, 10 mM HEPES). All NaCl solutions were brought to pH = 7.2 with NaOH or HCl. To determine K^+ permeability, *I-V* experiments were performed in K^+ Ringers: 115 mM KCl, 1.0 mM $BaCl_2$, 10 mM HEPES (pH = 7.2, KOH). For barium permeability, the Na^+ free solution was used except $BaCl_2$ was increased tenfold: 115 mM NMDG, 18 mM $BaCl_2$, 2.5 mM KCl, 10 mM HEPES. Osmolarity was measured for each solution and was maintained at 240 mosm by the addition of sucrose. All recording were done at room temperature. We used 3-M KCl filled electrodes with a resistance between 1-3 MΩ. We used a 3-M KCl agar bridge to minimize liquid junction potentials, and all liquid junctions potentials arising at the tip of the recording electrode were corrected online.

Data analysis. Data acquisition and analysis were performed using Axograph (Axon Instruments) software, and curve fitting and statistical analysis were performed with Prism (Graphpad). Recordings from both EXP-1A and EXP-1B splice forms were performed in parallel. There were no significant differences between the splice forms in GABA binding or *I-V* relationships. All data shown were collected from eggs injected with the full-length EXP-1B isoform. Dose-response curves from individual oocytes were normalized to the maximum and minimum values and averaged for at least 11 oocytes. Normalized data were fit to the four-parameter equation derived from the Hill equation: $Y = Min + (Max - Min)/(1 + 10^{H(\mathrm{LogEC50} - X)})$, where *Max* is the maximal response, *Min* is the response at the lowest drug concentration, *X* is the logarithm of agonist concentration, EC_{50} is the half-maximal response, and *H* is the Hill coefficient. The permeability of potassium relative to sodium was calculated using a modified form of the Goldman-Hodgkin-Katz equation: $\Delta V_{rev} = V_{revK} - V_{revNa} = (RT/F)\ln P_K[K]_o/P_{Na}[Na]_o$, where R is the Gas constant, *T* is the temperature, F is Faraday's constant, and V_{rev} is the potential at which the current is zero. The Goldman-Hodgkin-Katz equation was used to fit the predicted reversal potentials using $[Na^+] = 10.4$ mM and $[K^+] = 108$ mM for intracellular concentrations: $E_{rev} = (RT/F)\ln P_{Na}[Na]_o + P_K[K]_o/P_{Na}[Na]_i + P_K[K]_i$.

EXP-1 GenBank accession number: AY383563.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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