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The *Caenorhabditis elegans snf-11* Gene Encodes a Sodium-dependent GABA Transporter Required for Clearance of Synaptic GABA[D]

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Sodium-dependent neurotransmitter transporters participate in the clearance and/or recycling of neurotransmitters from synaptic clefts. The *snf-11* gene in *Caenorhabditis elegans* encodes a protein of high similarity to mammalian GABA transporters (GATs). We show here that *snf-11* encodes a functional GABA transporter; SNF-11–mediated GABA transport is Na^+ and Cl^- dependent, has an EC_{50} value of 168 μM, and is blocked by the GAT1 inhibitor SKF89976A. The SNF-11 protein is expressed in seven GABAergic neurons, several additional neurons in the head and retrovesicular ganglion, and three groups of muscle cells. Therefore, all GABAergic synapses are associated with either presynaptic or postsynaptic (or both) expression of SNF-11. Although a *snf-11* null mutation has no obvious effects on GABAergic behaviors, it leads to resistance to inhibitors of acetylcholinesterase. In vivo, a *snf-11* null mutation blocks GABA uptake in at least a subset of GABAergic cells; in a cell culture system, all GABA uptake is abolished by the *snf-11* mutation. We conclude that GABA transport activity is not essential for normal GABAergic function in *C. elegans* and that the localization of SNF-11 is consistent with a GABA clearance function rather than recycling.

INTRODUCTION

Synaptic signaling is modulated by a variety of pre- and postsynaptic mechanisms. Complex signaling pathways regulating synaptic vesicle fusion, for example, have been defined in a number of experimental systems (reviewed in Südhof, 2004). Recently, plasma membrane neurotransmitter transporters have been shown to play a significant role in maintaining appropriate levels of neurotransmitters in the synaptic cleft. Proteins in the sodium-dependent neurotransmitter symporter family (Pfam designation, SNF) transport neurotransmitters (and related molecules) from the synaptic cleft in a Na^+- and Cl^--dependent manner, thereby terminating synaptic transmission and providing a means of recycling neurotransmitters. SNFs can rapidly redistribute between plasma membrane and vesicular membranes; this is regulated by cycles of phosphorylation and dephosphorylation (reviewed in Deken *et al*., 2001). SNFs are also the focus of much interest as drug targets; drugs blocking specific SNFs are currently used to treat a number of pathological conditions, including depression and epilepsy.

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There are at least 18 members of the SNF family in humans, and 14 each in *Drosophila melanogaster* and *Caenorhabditis elegans*. Sequence comparisons suggest that humans, fruit flies, and nematodes share a core set of SNF proteins, which include the dopamine, serotonin, and GABA transporters. In addition, each species has a unique set of SNF proteins. We chose the nematode *C. elegans* as a model for studying SNF function because it is well-suited for genetic analysis, has a simple nervous system, and the complete sequence of its genome has been determined. When this study was initiated, there were already two characterized SNF members in *C. elegans*: *dat-1* (T23G5.5) encodes the dopamine transporter (Nass *et al*., 2001) and *mod-5* (Y54E10BR.7) encodes the serotonin transporter (Ranganathan *et al*., 2001). By our analysis, there are 12 additional family members in the *C. elegans* genome. We have assigned them the *C. elegans* gene designation "*snf*," consistent with the Pfam designation.

The GABAergic system in *C. elegans* mediates several distinct behaviors, and mutations affecting GABAergic function result in several easily scorable mutant phenotypes (reviewed in Schuske *et al*., 2004). The 26 *C. elegans* GABAergic cells modulate locomotion (DD and VD motor neurons), foraging (RMEs), and defecation (AVL and DVB) behaviors (McIntire *et al*., 1993b; Schuske *et al*., 2004). Mutations in the GABA biosynthetic enzyme glutamic acid decarboxylase (GAD) (*unc-25*; Jin *et al*., 1999), vesicular transporter (*unc-47*; McIntire *et al*., 1997), and excitatory and inhibitory receptors (*exp-1*; Beg and Jorgensen, 2003; *unc-49*; Bamber *et al*., 1999), have been described in detail. In addition, earlier studies had demonstrated uptake of exogenous GABA into a subset of *C. elegans* neurons (McIntire *et al*., 1993b).

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Two *C. elegans* genes, *snf-3* and *snf-11*, encode proteins with significant similarity to mammalian GABA transporters. We show here that *snf-11* encodes a functional GABA transporter with properties similar to the high-affinity mammalian transporter GAT1, we describe the expression pattern for the gene, and we characterize the phenotypes of *snf-11* null mutants.

MATERIALS AND METHODS

Growth and Culture of C. elegans Strains

C. elegans was grown on modified nematode growth medium using standard +methods (Brenner, 1974). The deletion mutations *snf-11(ok156)* and *snf-3(ok293)* were provided by the *C. elegans* Gene Knockout Consortium, and *snf-11(tm625)* was provided by Shohei Mitani (Tokyo Women's Medical College, Tokyo, Japan). A strain containing a *Pmyo-3*::GFP transgene (*ceEx64*) was provided by Ken Miller (Oklahoma Medical Research Foundation). Additional strains were provided by the *Caenorhabditis* Genetics Center (St. Paul, MN).

Isolation of snf-11 cDNAs

We used a PCR-based screening approach to identify and enrich for *snf-11* cDNAs in the RB1 cDNA library. Nested primers were used to amplify a fragment of the *snf-11* cDNA from the library. The "titer" of the cDNA in the library was then determined by dilution and PCR amplification. The library was then diluted accordingly and used to infect host bacteria in a 96-well microtiter tray. Phage were then harvested, pooled, and analyzed by a second round of PCR. Repeated rounds of PCR amplification, dilution of phage, and phage growth achieved significant enrichment of the target cDNA. Sequencing of the ends of the cDNA indicated that the insert was nearly full length, missing only six base pairs of sequence at the 5′ end (determined by 5′ rapid amplification of cDNA ends). A primer was synthesized with the missing 5′ sequences and used with a reverse primer in the 3′-untranslated region to amplify the full-length cDNA (using the Expand High Fidelity PCR kit; Roche Diagnostics, Indianapolis, IN).

snf-3 cDNA

We obtained two putative *snf-3* cDNA clones from Yuji Kohara (National Institute of Genetics, Mishima, Japan): yk802e9 contains a presumed missense mutation (A1979G) that converts Q660R, and yk39f1 is a partial cDNA that starts 13-base pairs into exon 2. A 1-kb BamHI fragment from yk802e9 was inserted into the BamHI site of yk39f1 to yield a full-length *snf-3* cDNA.

Expression and Reporter Constructs

Modular *C. elegans* expression vectors (Fire *et al*., 1990) and GFP-containing reporter vectors (Miller *et al*., 1999) were obtained from Andy Fire (Stanford University School of Medicine, Stanford, CA). Transformation experiments involved injecting either plasmids (with "RM#" designations) or PCR fusion products (with "FRM" designations). PCR fusion products (Horton *et al*., 1989; Hobert, 2002) were generated using PCR products amplified from either green fluorescent protein (GFP)- or cDNA-containing plasmids, fused to PCR products derived from the *snf-11* promoter region of cosmid T03F7.

The promoter for *Punc-25*::GFP and *Punc-25*::YFP plasmids was derived from pSC325 (Jin *et al*., 1999). Transgenic animals carrying either *Punc-25*::GFP or *Punc-25*::YFP robustly express GFP/yellow fluorescent protein (YFP) in the RMEs and ventral nerve cord GABAergic cells, but not detectably in the AVL, DVB, and RIS cells. In this respect, our constructs resemble pSC380 and similar constructs (Jin *et al*., 1999). Jin *et al*. (1999) note that extensive RNA processing may be necessary for expression of *unc-25* reporters in AVL, DVB, and RIS.

Transgenic Methods

Transgenic nematodes were obtained by microinjection of DNA, essentially as described by Mello *et al*. (1991). Transformation markers included the pBX plasmid (Heinke and Ralf Schnabel, Max-Plank-Institute fur Biochemie, Martinsried, Germany), which rescues the temperature-sensitive lethality of *pha-1(e2123)* mutants, and the RM#611p plasmid, which contains the *unc-25* promoter driving expression of GFP. In some experiments, GFP fusions were used as transformation markers.

Xenopus Oocyte Preparation and Injection

Xenopus oocytes were prepared as described previously (Quick *et al*., 1992). Briefly, oocytes were defolliculated in collagenase A (Roche Diagnostics) and maintained at 18°C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, and 1.8 mM $CaCl_2$) supplemented with 2.5 mM Na pyruvate, 50 μg/ml gentamicin, and 5% horse serum. The medium was changed daily. Increased expression of SNF-11 protein was achieved by inserting the alfalfa mosaic virus 5′-untranslated region upstream of the start codon, and a poly(50)-A tail downstream of the stop codon. cRNA synthesis was performed using mMessage mMachine (Ambion, Austin, TX). Individual oocytes were injected with 50 ng of cRNA twice: 1 and 4 d after the oocyte preparation. Expression was found to be maximal 4–9 d after the second injection.

[^{3}H]GABA Uptake Assays

Individual oocytes were placed in multiwell plates in ND96. At timed intervals, GABA was added to a final concentration of 1 mM unless otherwise indicated; the concentration of [^{3}H]GABA was 60 nM. The reaction was terminated by rapid removal of the oocyte followed by six washes in ice-cold ND96. Subsequently, the oocyte was solubilized in 10% SDS at 60°C for 2 h, and [^{3}H]GABA uptake was determined by liquid scintillation spectrometry. For all uptake experiments, specific uptake at each concentration was determined by subtracting total uptake from nonspecific uptake measured in sister oocytes from the same oocyte batch.

Behavioral Assays

Swimming (thrashing), pharyngeal pumping, and defecation were all measured on young adult hermaphrodites raised at 20°C as described previously (Miller *et al*., 1996). Acute aldicarb assays were performed as described previously (Lackner *et al*., 1999; Nurrish *et al*., 1999) using 2 mM aldicarb. Paralysis was defined as the complete absence of spontaneous and provoked (prodded with a worm pick) locomotion. GABA transport-dependent behavioral assays were performed as described by McIntire *et al*. (1993b), except that the final concentration of GABA was 70 mM.

Generation and Purification of Polyclonal Antibodies

α-SNF-11 antibodies were raised against a synthetic peptide (NH_2-CRPDIE-IKGAMENAEN-COOH) corresponding to a predicted antigenic region in the carboxyl tail of SNF-11. The peptide was coupled to goat serum albumin with glutaraldehyde and injected into two goats (G379 and G380). Polyclonal antibodies were affinity purified against SNF-11 peptide (coupled to rabbit serum albumin) immobilized on nitrocellulose membranes as described previously (Duerr *et al*., 1999).

Immunofluorescence Staining and Microscopy of Nematodes

Nematodes were stained using a freeze-fracture procedure adapted from Duerr *et al*. (1999), with major modifications by Kiely Grundahl (Oklahoma Medical Research Foundation). Nematodes were freeze fractured (with no subbing agent) and then washed from slides in ice-cold methanol. Nematodes were pelleted in 50-ml conical tubes, transferred to microfuge tubes, and then pelleted again. Nematodes were then fixed in acetone for 2 min, washed in phosphate-buffered saline (PBS), and then blocked with 10% donkey serum in antibody buffer (0.5% Triton X-100, 1 mM EDTA, and 0.1% bovine serum albumin in PBS with 0.05% sodium azide). Primary antibody incubations (1:50–1:100) were done overnight at 4°C. After thorough washing with antibody buffer, nematodes were incubated in secondary antibodies overnight at 4°C. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). After washing, nematodes were mounted in anti-fade medium.

Primary Cell Culture

Embryonic cells were isolated and cultured in a procedure modified from Christensen *et al*. (2002). Briefly, worms were grown in 2xYT liquid media with *Escherichia coli* (OP50 strain) and harvested by gentle centrifugation. Eggs were released by agitating worms in a bleach/NaOH solution, chitinased, and passed through a 5-μm filter in an osmotically balanced egg buffer. Dissociated cells were plated onto peanut lectin-coated coverslips at a density of $\sim 1 \times 10^6$ cells/ml and allowed to differentiate and grow at 20°C in L-15–10 media for 7–10 d. In GABA uptake experiments, the growth media were replaced with fresh media containing 840 μM GABA, and cells were incubated for 1 h at 23°C. For immunofluorescence, coverslips were washed in egg buffer, and fixed immediately with 1% formaldehyde, 0.20% glutaraldehyde in 0.1 M PIPES for 10 min at 23°C. After washing in PBS, cells were incubated 60–90 min at 23°C in a PBS-based blocking buffer containing 0.5% porcine gelatin, 0.01 M glycine, 0.5% Tween 20, 0.05% sodium azide, 1 mM EDTA, and 10% donkey serum. Primary antibody staining was at 23°C for 45–60 min with rabbit α-GABA (Sigma-Aldrich, St. Louis, MO), and mouse and chicken α-GFP antibodies (Chemicon International, Temecula, CA) diluted 1:1000 in the blocking buffer without donkey serum. After three PBS washes, cells were incubated at 23°C for 45–60 min in a secondary antibody solution consisting of Cy3-conjugated donkey α-rabbit $F(ab')_2$ and Cy2-conjugated donkey α-mouse and α-chicken $F(ab')_2$ (The Jackson Laboratory, Bar Harbor, ME), each diluted 1:1000 in the above-mentioned buffer. Coverslips were then mounted on slides with ProLong anti-fade medium (Invitrogen, Carlsbad, CA).

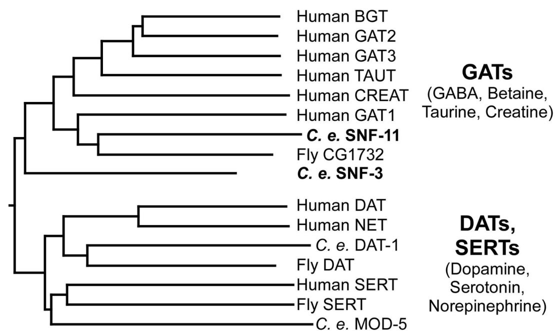

Figure 1. Similarity tree of Human, *Drosophila*, and *C. elegans* SNF proteins, showing the core set of transporters (GABA, dopamine, and serotonin). Tree was constructed in AlignX (Vector NTI) using the neighbor joining method of Saitou and Nei (1987). Additional protein clusters (amino acid transporters and "orphan" transporters) were omitted for clarity. SNF-11, Fly CG1732, and GAT1 are closely related, whereas SNF-3 is more distantly related to GAT1 and other mammalian GABA transporters. GenBank accession numbers: *C. elegans* DAT-1, NP_499043; *C. elegans* MOD-5, NP_491095; *C. elegans* SNF-3, ABF20556 (this study); *C. elegans* SNF-11, ABF20555 (this study); Fly CG1732, NP_651930; Fly DAT, NP_523763; Fly SERT, NP_523846; Human BGT, NP_003035; Human CREAT, NP_005620; Human DAT, NP_001035; Human GAT1, NP_003033; Human GAT2, NP_057699; Human GAT3, NP_055044; Human NET, NP_001034; Human SERT, NP_001036; Human TAUT, NP_003034.

Microscopy and Imaging

Images were collected on a Leica TCS NT confocal microscope. Nematode images were collected at 10 or 40× magnification, and 512 × 512 or 1024 × 1024 pixels with 0.5-μm Z-steps. Cell culture images were collected at 100× magnification with 2× zoom and 512 × 512 pixels with 0.2-μm Z-steps. Images were cropped to size, assembled, and annotated using Adobe Photoshop 5.5 (Adobe System, Mountain View, CA). All images within a given experiment were collected in the same session using identical settings and were processed identically for publication.

RESULTS

The snf-11 Gene

Genomic analysis reveals that the *C. elegans* genes *snf-11*, and, to a lesser extent, *snf-3* encode proteins with high degrees of similarity to mammalian GABA transporters (Figure 1). The *snf-11* gene contains six exons and encodes a 12-transmembrane domain protein with the typical structure of the SNF proteins (Figure 2). A gene encoding a seven-transmembrane domain receptor, *srh-45*, is just downstream of *snf-11* (Figure 2), raising the possibility that the two genes were jointly transcribed in an operon (Spieth *et al.*, 1993). However, reporter expression studies indicated that transcription of *srh-45* requires only the small (383 base pairs) region just upstream of *srh-45* and is not affected by any sequences within or upstream of the *snf-11* coding sequence; thus, *snf-11* does not seem to be part of an operon.

SNF-11 Is a GABA Transporter

To determine whether *snf-11* encodes a bona fide GABA transporter, we constructed a full-length *snf-11* cDNA (see *Materials and Methods*), injected *Xenopus* oocytes with cRNA transcribed from this cDNA, and assayed for GABA uptake. We found that injection of *snf-11* cRNA resulted in significant GABA transport, indicating that *snf-11* encodes a func-

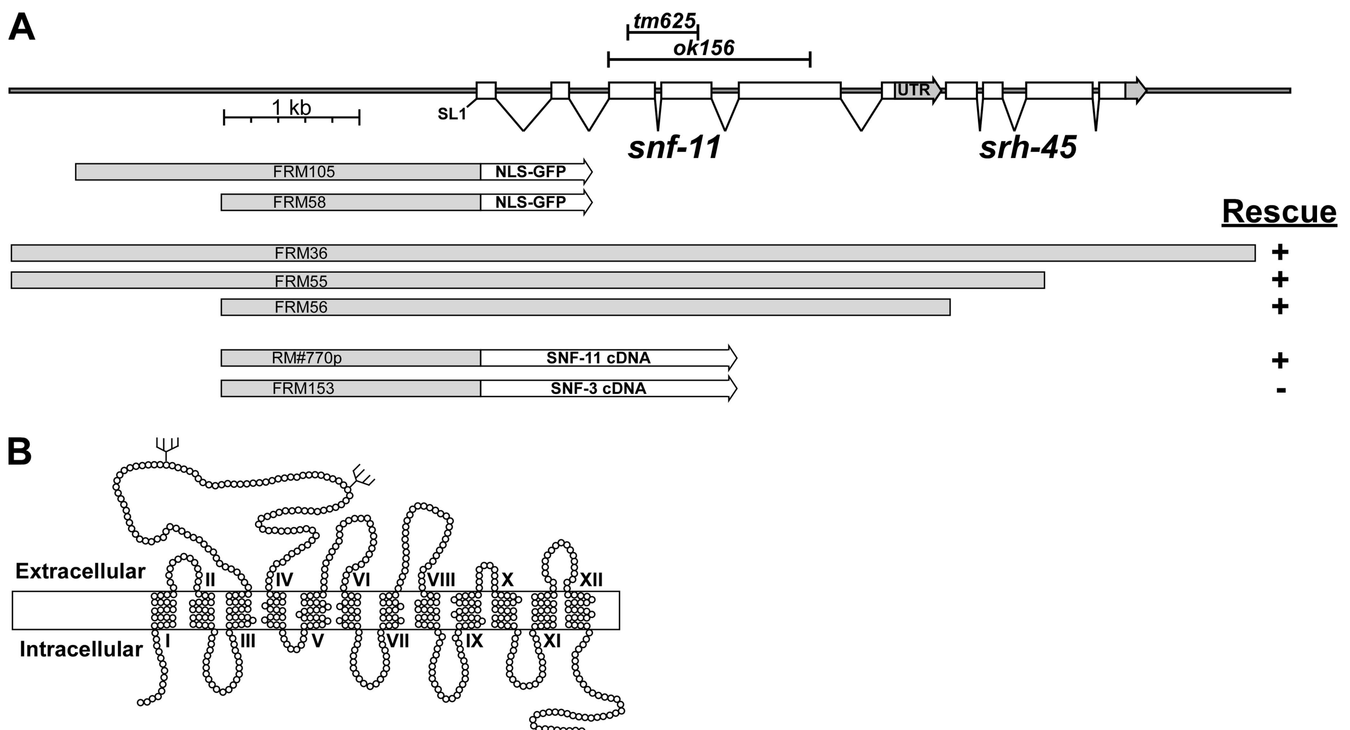

Figure 2. (A) Diagram of the *snf-11* gene, showing intron-exon structure, and location of the *ok156* and *tm625* deletion breakpoints. Note the position of the adjacent gene, T03F7.2, which encodes a seven-transmembrane G-protein–coupled receptor. Also shown are the rescuing fragments, reporters (plasmids and PCR products), and cDNA constructs described in this study. The ability of PCR products or clones to rescue the GABA transport-dependent phenotypes and proper cellular expression is also indicated. (B) Predicted topology of the SNF-11 protein. Like other members of the SNF family, SNF-11 is thought to have 12 transmembrane domains and cytoplasmic amino and carboxy termini. The two potential sites of glycosylation, in the large extracellular loop between the third and fourth transmembrane domains, are also indicated.

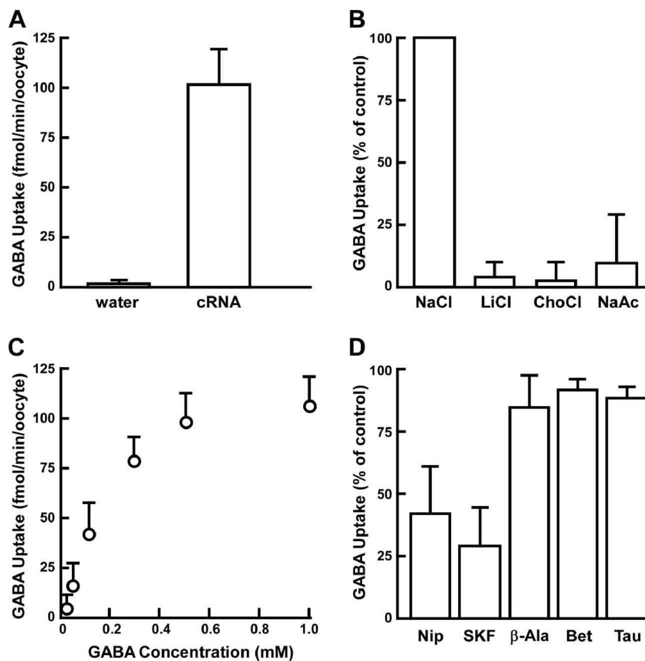

Figure 3. *snf-11* encodes a sodium- and chloride-dependent GABA transporter. (A) GABA uptake in *snf-11*–injected oocytes. Oocytes were injected with water or *snf-11* cRNA and subjected to GABA uptake assays. The assay time was 30 min; data are from eight oocytes/condition. (B) Uptake is Na^+ and Cl^- dependent. GABA uptake assays were performed in saline containing 96 mM of the following salts: NaCl, LiCl, choline chloride, or sodium acetate. Data are presented relative to uptake in NaCl, and represent 13–17 oocytes per condition. (C) Saturation analysis of GABA uptake. Data are from six to nine oocytes per condition per data point. Uptake parameters based upon Eadie–Hofstee analysis: $EC_{50} = 168 \pm 21$ μM, $V_{max} = 122 \pm 14$ fmol/oocyte/min. (D) GABA uptake competition using the following drugs: 1 mM nipecotic acid, 1 mM SKF89976A, 10 mM β-alanine, 10 mM betaine, and 10 mM taurine. Data are reexpressed relative to uptake in untreated oocytes and represent six to 13 oocytes per condition.

tional GABA transporter. The SNF-11–mediated GABA transport is Na^+ and Cl^- dependent, has an EC_{50} value of 168 μM, and is blocked by the GAT inhibitors nipecotic acid and SKF89976A (Figure 3). GABA transport was not inhibited by β-alanine, betaine, or taurine, indicating that these compounds are not substrates.

Expression and Localization of SNF-11 in C. elegans

We raised SNF-11–specific polyclonal antibodies to determine the expression pattern of SNF-11 in *C. elegans*. In healthy young adults, the α-SNF-11 antibody strongly stained the four RME neurons (RMED, RMEV, RMEL, and RMER; Figure 4). Faint staining of three additional GABAergic neurons (AVL, DVB, and RIS; Table 1) was sometimes observed. All seven of these neurons have been shown previously to possess GABA uptake activity (McIntire *et al.*, 1993b). Several non-GABAergic neurons, including RID, also seemed to stain. We observed staining of both the processes and the soma of each neuron. In RMED and RMEV, a punctate staining pattern was observed in the posteriorly directed processes, possibly corresponding to synapses (Figure 4). The ventral nerve cord DD and VD inhibitory motor neurons did not stain. Faint staining of the body wall, anal, and uterine muscles with the α-SNF-11 antibody was observed in some animals. In *snf-11(ok156)* mutants, α-SNF-11 staining was completely absent, confirming the specificity of the staining (Figure 4).

We also generated two *snf-11* transcriptional reporters, with 3.0 and 1.9 kb of upstream sequence, respectively, driving GFP expression (Figure 2). These *Psnf-11*::GFP fusions are expressed in the same neurons (RMEs, AVL, DVB, RIS, and RID; Figure 5 and Table 1) that stained with the α-SNF-11 polyclonal antibodies. We also noted expression in two additional neurons near the pharynx as well as two neurons in the retrovesicular ganglion. There were no apparent differences in expression between the two reporters, suggesting that the 1.9-kb region is sufficient to drive expression in all *snf-11*–positive cells. In contrast to observations reported previously (Jiang *et al.*, 2005), we did not observe *snf-11* expression in the ventral cord inhibitory (DD and VD) motor neurons (Figure 5). However, we did observe robust expression in the body wall, anal, and uterine muscles that was not noted previously (McIntire *et al.*, 1993b; Jiang *et al.*, 2005). In young animals, expression of the *Psnf-11*::GFP reporter in muscle cells is the most prominent aspect of the expression pattern (Figure 5A).

Phenotypes of snf-11 Mutants

The *snf-11(ok156)* mutation is associated with a 1491-base pair deletion that removes more than two-thirds of the *snf-11* coding sequence (Figure 2); it is almost certainly a null allele. A second allele, *snf-11 (tm625)*, is associated with a 529-base pair deletion and a seven-nucleotide insertion; this allele is also likely to be null. We expected a priori that eliminating GABA transport function would result in a "hyper-GABAergic" phenotype. The 26 GABAergic cells in *C. elegans* modulate three known behaviors; locomotion (DD and VD motor neurons), foraging (RMEs), and enteric muscle contraction/defecation (AVL/DVB) (reviewed in Schuske *et al.*, 2004). To our surprise, we found that a *snf-11* null mutation had no dramatic effects on any of these GABAergic behaviors. Thrashing rates in liquid were not significantly different from those of wild-type (N2) animals [*snf-11(ok156)* = 163 ± 2.4, N2 = 160 ± 3.6 (thrashes per minute)]. Likewise, the enteric muscle contraction step of the defecation motor program was normal [*snf-11(ok156)* = 99 ± 1%, N2 = 92 ± 3%], although there was a small but statistically significant difference between mutant and wild-type animals. The foraging behavior seemed to be completely normal in *snf-11* mutants.

Because the *snf-11* null mutation did not result in a dramatic GABAergic phenotype, we used a pharmacological approach to look at more subtle defects. *C. elegans* mutants deficient in GABA are hypersensitive to the effects of the acetylcholinesterase inhibitor aldicarb, presumably because GABA normally antagonizes the action of acetylcholine (ACh) on the body wall muscles. A priori, we expected that *snf-11* mutants would be at least slightly resistant to aldicarb because the elevated levels of extracellular GABA would offset the effects of aldicarb to some degree. Therefore, we used an aldicarb acute response assay (Lackner *et al.*, 1999; Nurrish *et al.*, 1999) to monitor the response of *snf-11* mutants to 2 mM aldicarb. We found that *snf-11* mutants are resistant to aldicarb (Figure 6). This resistance was dependent on endogenous GABA synthesis, however, because *unc-25; snf-11* double mutants were as hypersensitive as *unc-25* single mutants.

We also used an approach developed by McIntire *et al.* (1993b) to create a contrived "GABA transport dependent" phenotype. As noted above, *unc-25(null)* mutants are defective in GABA-mediated behaviors, including defecation and

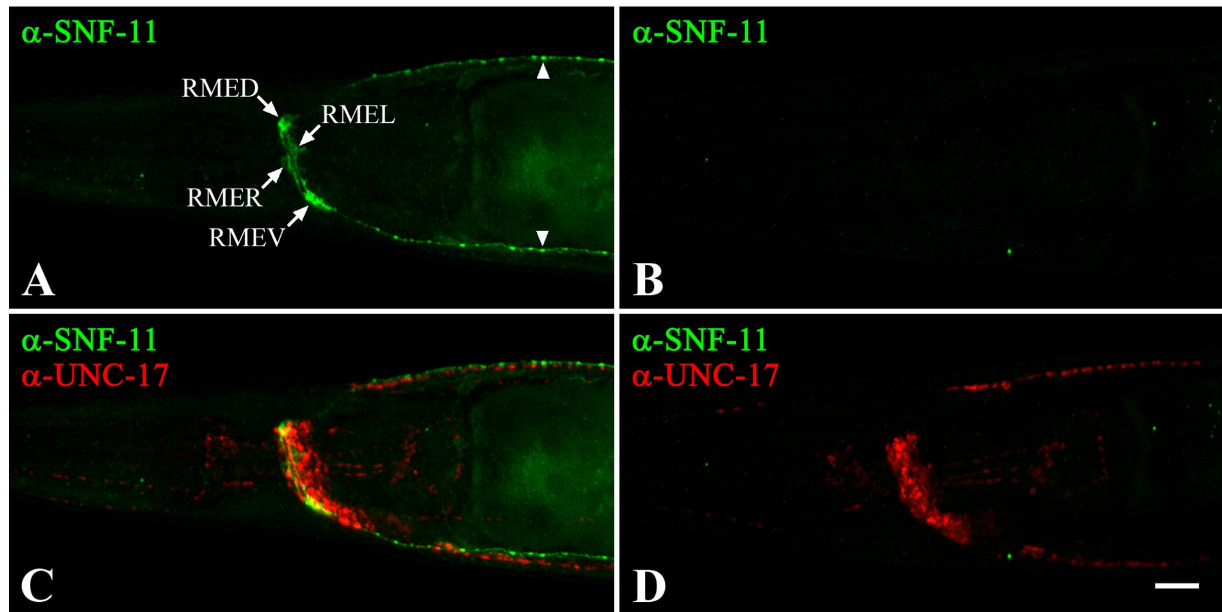

Figure 4. SNF-11 localization: N2 (A and C) and RM2710 [*snf-11(ok156)*] (B and D) stained with α-SNF-11 polyclonal antibodies (goat 387; shown in green) and an α-UNC-17 (vesicular ACh transporter [VAChT]) monoclonal antibody (shown in red). A and B are single-channel images of α-SNF-11 staining, whereas C and D are merged images. Head region, anterior is to the left and ventral is down. The four RME neurons are indicated with arrows, and the processes (RMED, top and RMEV, bottom) are indicated with arrowheads. Note the punctate appearance of the processes. A second dorsal process, probably from RID, can be seen just below the RMED process. In *snf-11(ok156)* mutants, α-SNF-11 staining is completely absent (B and D). Mutant and wild-type images were collected and processed using identical settings. Bar, 10 μm.

foraging. However, application of exogenous GABA rescues the defecation and foraging defects in *unc-25* mutants. Behavioral rescue is not only dependent on GABA but also on GABA transport—treatment with the GAT inhibitor nipecotic acid blocks behavioral rescue (McIntire *et al.*, 1993b). Similarly, we found that the *snf-11(ok156)* mutation blocks GABA-mediated rescue of the *unc-25* defecation defect (Figure 7). The *snf-11* mutation also blocked rescue of the RME-mediated foraging defect. A null mutation in the second potential GABA transporter gene, *snf-3*, had no effect on GABA transport in this assay (Figure 7), suggesting that *snf-11* encodes the sole GABA transporter in the defecation and foraging neurons.

To confirm that these mutant phenotypes were specifically associated with the loss of *snf-11* function, we generated transgenic lines carrying the cosmid T03F7 or PCR fragments spanning the *snf-11* gene (Figure 2). We found that a genomic fragment containing just the *snf-11* gene (including 1.9 kb of upstream sequence) was sufficient to rescue the GABA transport dependent phenotypes. The downstream gene, *srh-45*, was not required for rescue. In addition, the GABA transport dependent phenotypes were rescued by driving expression of the *snf-11* cDNA with the 1.9-kb *snf-11* promoter (Figures 2 and 7). In contrast, these phenotypes were not rescued by driving expression of the *snf-3* cDNA with the *snf-11* promoter (Figures 2 and 7).

GABA Uptake in C. elegans *Embryonic Cell Culture Is Blocked by a snf-11 Null Mutation*

Earlier studies on GABA uptake in *C. elegans* relied on whole-worm treatment and staining techniques; worms were exposed to high levels of exogenous GABA, followed by fixation and staining with α-GABA antibodies (McIntire *et al.*, 1993b). This technique has provided valuable insights

Table 1. GABA-synthesizing and GABA-transporting cells

Class	Cell	Type	GABA synthesis (UNC-25)[a]	GABA transport (SNF-11)
1	DD/VD	Inhibitory motor neurons (19)	+	−
2	RME	Inhibitory motor neurons (4)	+	+
	AVL	Excitatory motor neuron	+	+
	DVB	Excitatory motor neuron	+	+
	RIS	Interneuron	+	+
3[b]	RID	Motor neuron	−	+
	Muscle	Body wall, uterine, anal muscles	−	+

[a] Data from Jin *et al.* (1999). This same set of 26 cells also express the UNC-47 vesicular GABA transporter (McIntire *et al.*, 1997) and contain GABA (McIntire *et al.*, 1993a).

[b] Not listed in class 3 are four unidentified cells, two in the head and two in the retrovesicular ganglion (see Figure 5).

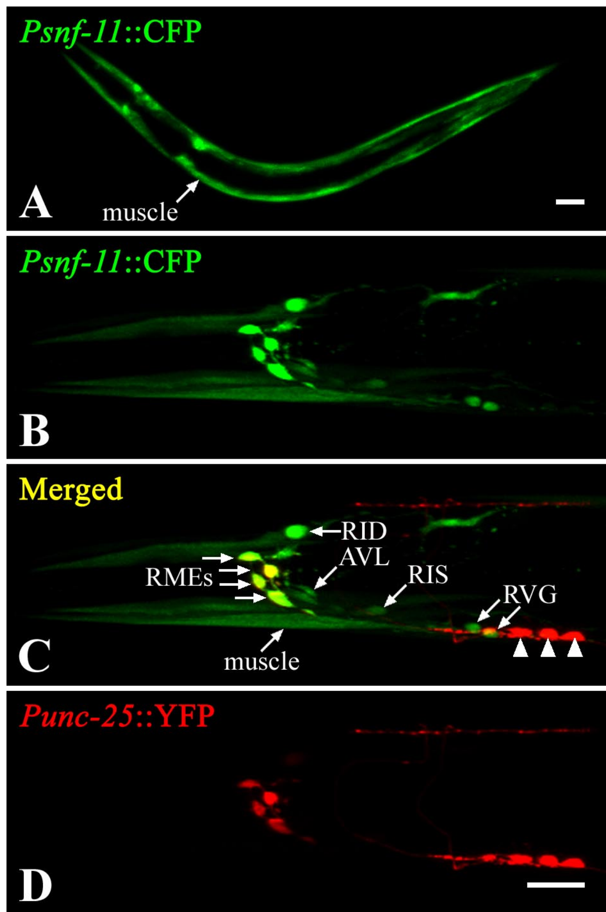

Figure 5. Expression of a *Psnf-11*::CFP reporter in *C. elegans*: (A) Low-magnification image of a young larva expressing *Psnf-11*::NLS-CFP. Arrow indicates body wall muscles. Muscle expression of the reporter is the most obvious aspect of the expression pattern. (B, C, and D) Head region of a young adult hermaphrodite expressing *Psnf-11*::NLS-CFP (green) and *Punc-25*::YFP (red) reporters. Several of the cells expressing *snf-11* are indicated with arrows (RMEs, AVL, RIS, RID, and body wall muscles). Arrowheads indicate several ventral cord GABAergic cells (VD and DDs); these cells do not express *snf-11*. There are several additional unidentified *snf-11*–expressing cells in the head and retrovesicular ganglion. Anterior is to the left and ventral is down. Bar, 10 μm.

into GABA uptake in *C. elegans*, but it has several limitations that reduce its usefulness. The *C. elegans* cuticle is relatively impermeable; consequently, an extremely high level of GABA is required to detect uptake. The cuticle also makes staining with antibodies technically difficult. To overcome these limitations, we took advantage of the newly developed *C. elegans* embryonic cell culture techniques to study GABA uptake. We used this approach to demonstrate GABA uptake in a variety of cell types in cell culture using physiological concentrations of GABA. In combination with *C. elegans* mutations affecting the GABA biosynthesis and uptake, this approach provides a valuable methodology for the study of GABA uptake.

Cells derived from *unc-25(e156)* (GAD) mutants expressing an integrated *Punc-25*::GFP transcriptional fusion were prepared using the methods described by Christensen *et al.* (2002). *unc-25(e156)* mutants do not synthesize detectable levels of GABA; consequently, it is possible to examine the uptake of GABA without the confounding presence of endogenous GABA. These cells were exposed to 840 μM GABA for 1 h, rinsed briefly, and then stained with an α-GABA antibody. As noted in *Materials and Methods*, our *Punc-25*::GFP fusion is robustly expressed in the RMEs and ventral cord GABAergic cells but not detectably in the AVL, DVB, and RIS cells. In addition, the DVB and VD cells arise postembryonically and should not be present in our preparation. Strong α-GABA staining was noted in several cell types (Figure 8), which fell into three categories: 1) GFP-positive neurons, 2) GFP-negative neurons, and 3) presumptive muscle cells. Although we did not make precise cell counts, α-GABA staining was absent from approximately

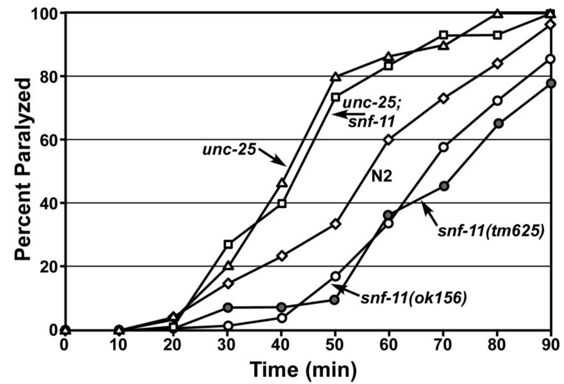

Figure 6. *snf-11* mutants are resistant to the acetylcholinesterase inhibitor aldicarb in an acute assay. Paralysis of N2, *unc-25(e156)*, *snf-11(ok156)*, *snf-11(tm625)*, and *unc-25(e156)*; *snf-11(ok156)* animals was monitored over time on 2 mM aldicarb plates. Paralysis was defined as the complete absence of spontaneous and provoked (prodded with a worm pick) locomotion.

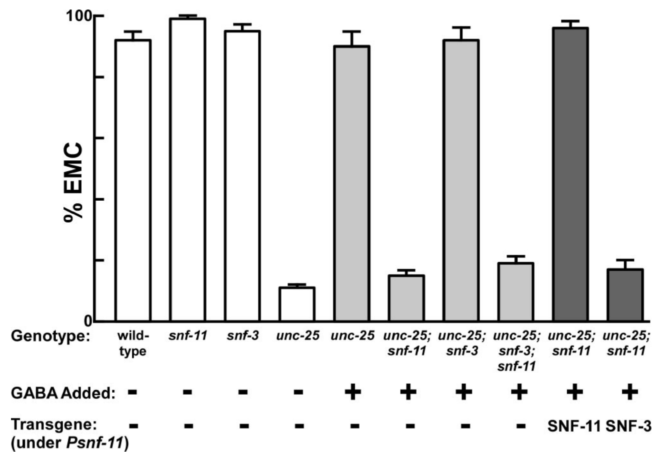

Figure 7. GABA transport dependent defecation phenotype. Exogenous GABA rescues the defecation defect in *unc-25* (GAD) mutants. GABA uptake and behavioral rescue is dependent on GABA transporters (blocked by the GAT inhibitor nipecotic acid; McIntire *et al.*, 1993b). We constructed *snf-11; unc-25* double mutants and asked whether rescue of the *unc-25* defecation defect with exogenous GABA is blocked by the transporter mutation. The *snf-11(ok156)* mutation blocks rescue of the defecation defect with exogenous GABA. In contrast, the *snf-3(ok293)* mutation has no effect, either singly, or in combination with *snf-11*. Expression of the *snf-11* cDNA under control of the *snf-11* promoter (*Psnf-11::snf-11)* fully rescues the GABA transport dependent phenotype; this phenotype is not rescued by expressing the *snf-3* cDNA (*Psnf-11::snf-3)*.

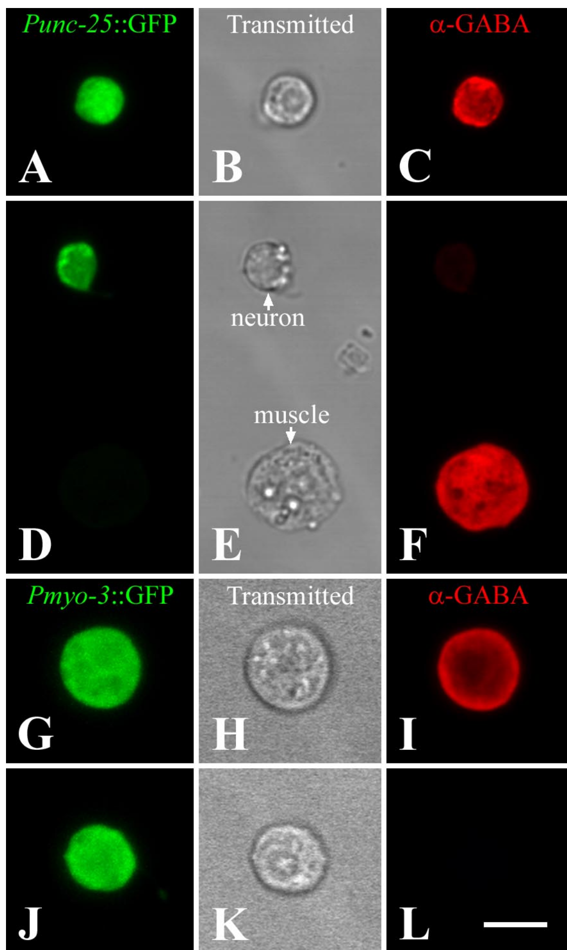

Figure 8. GABA uptake in neurons and muscle cells in *C. elegans* embryonic cell culture. (A–F) Cells prepared from an *unc-25(e156)* transgenic strain with an integrated *Punc-25*::GFP transgene, incubated with 840 μM GABA, and stained with α-GFP (green) and α-GABA (red) antibodies. GFP-positive neurons fall into two categories, cells with GABA uptake activity (A–C), and cells lacking GABA uptake activity (D–F). Note the GABA-positive muscle cell. (G–L) Cells prepared from *unc-25(e156)* (G–I) and *unc-25(e156); snf-11(ok156)* (J–L) transgenic strains carrying a *Pmyo-3*::GFP transgene, incubated with 840 μM GABA, and stained with α-GFP (green) and α-GABA (red) antibodies. GFP-positive muscle cells possess GABA uptake activity (G–I) and this uptake activity is dependent on *snf-11* (J–L). The same results were observed in muscle cells with a more differentiated appearance (see Supplemental Figure 2). Bar, 5 μm.

one-half of the GFP-positive neurons. We believe that the GABA-positive GFP-positive neurons are RMEs, based on the expression patterns described above for the *Psnf-11* and *Punc-25*::GFP reporters in vivo. The GABA-positive GFP-negative neurons are presumably AVL, RIS, and non-GABAergic neurons (i.e., RID) that express *snf-11*. The GFP-positive GABA-negative cells are presumably ventral cord DD GABAergic cells, based on the absence of *snf-11* expression in these cells in vivo.

To confirm the *snf-11* dependence of GABA uptake, we cultured embryonic cells derived from *unc-25* single and *unc-25; snf-11* double mutants, incubated with GABA, and stained with α-GABA antibodies. These cells also carry a *Pmyo-3*::GFP reporter (as an extrachromosomal array; *ceEx64)* that is expressed in all body wall muscle cells, and α-GFP antibodies were included as staining controls. We found that the *snf-11* mutation blocks all significant GABA uptake activity in the cultured embryonic cells, including body wall muscle cells (Figure 8). Therefore, the *snf-11* gene seems to encode the sole high-affinity GABA transporter in *C. elegans*. Furthermore, our observations that muscles cells possess GABA uptake activity and that a *snf-11* mutation blocks this uptake, confirm our observation of *snf-11* expression in these cells.

DISCUSSION

snf-11 Encodes the Only High-Affinity GABA Transporter in C. elegans

We report here that the *snf-11* gene in *C. elegans* encodes a functional GAT with properties similar to those of the mammalian high-affinity GABA transporters. Four distinct subtypes of GABA transporters, GAT1, GAT2, GAT3, and BGT1, have been described in rats (Borden, 1996). These GATs are expressed differentially in the rat nervous system and have distinct physiological and pharmacological properties. We found that SNF-11 is inhibited by the GAT1-specific antagonist SKF89976A and is not inhibited by GAT2/3-specific antagonist β-alanine, indicating that SNF-11 belongs to the GAT1-subtype of GABA transporters.

We also present evidence that *snf-11* encodes the only high-affinity GABA transporter in *C. elegans*. There are two genes in *C. elegans*, *snf-3* and *snf-11*, that encode proteins with significant similarity to mammalian plasma membrane GABA transporters. SNF-3 is 38% identical to GAT1, whereas SNF-11 is 46% identical to GAT1. In this study, we demonstrate that SNF-11 is expressed in all cells that possess significant GABA uptake activity. In contrast, the SNF-3 protein is not expressed in cells possessing GABA uptake activity; SNF-3 is primarily expressed in sheath and socket cells in the head, and epithelial cells surrounding the vulva (our unpublished data). Furthermore, a *snf-11* null mutation seems to block all significant GABA uptake in a *C. elegans* primary cell culture paradigm. Finally, expression of the *snf-3* cDNA under control of the *snf-11* promoter does not rescue the *snf-11* mutant phenotypes.

As noted above, there are four subtypes of GABA transporters in rats; these subtypes are each encoded by separate genes. In contrast, both *C. elegans* and *Drosophila melanogaster* possess a single GABA transporter gene. In *C. elegans*, the GABAergic nervous system is very simple, consisting of 26 cells capable of synthesizing GABA (Schuske *et al*., 2004), and mediates a simple repertoire of behaviors. Presumably, the existence of multiple GAT subtypes in mammals reflects the complexity of the GABAergic nervous system in these organisms.

Comparison of the SNF-11 sequence to mammalian GAT1 sequences (Supplemental Figure 1) reveals similarities and differences that may be relevant to transporter function and regulation. The N-terminal cytoplasmic domain of GAT1 is known to regulate transporter function through interaction with syntaxin 1A (Beckman *et al*., 1998; Deken *et al*., 2000), and by interaction with the GAT1 fourth intracellular loop (Hansra *et al*., 2004). The SNF-11 N-terminal cytoplasmic domain is much shorter than that of GAT1 (18 versus 52 amino acids), yet it shares some features with the region proximal to the GAT1 first transmembrane domain (TM I). This region of GAT1 binds to syntaxin, and a cluster of three aspartic acid residues mediates this binding (Deken *et al*., 2000). Alignment of SNF-11 and GAT1 indicates that SNF-11 does not have the three aspartic acids, but rather two glutamic acid residues at comparable positions (Supplemental Figure 1), which may be sufficient to mediate syntaxin binding.

The fourth intracellular loop of GAT1 (between TM VIII and TM IX) is involved in intramolecular interactions and regulation of transport kinetics (Hansra *et al.*, 2004). In this region of the protein, SNF-11 is 17/29 identical (23/29 similar) to GAT1. In particular, there is a cluster of basic amino acids, just proximal to the ninth transmembrane domain, that is required for the interaction with the GAT1 N-terminal domain (Hansra *et al.*, 2004); in this region, there are four arginine residues in GAT1, versus three arginines and two lysines in SNF-11. Therefore, it is likely that regulatory interactions ascribed to the GAT1 fourth intracellular loop are conserved in SNF-11.

The C-terminal cytoplasmic tail of rat GAT1 contains two important localization motifs: a hydrophobic VMI tripeptide (VMV in the human protein), and the C-terminal AYI, which is a type II PDZ domain interaction motif (Farhan *et al.*, 2004). We note that SNF-11 contains neither of these motifs.

snf-11–*deficient Phenotypes*

The loss of *snf-11* function in *C. elegans* does not result in a dramatic mutant phenotype. This observation is consistent with previous observations made on worms treated with the GAT inhibitor nipecotic acid (McIntire *et al.*, 1993b), which likewise exhibited no obvious behavioral abnormalities. However, we were able to demonstrate phenotypic differences between wild-type and *snf-11* mutant animals using two pharmacobehavioral assays: 1) a GABA-dependent defecation assay and 2) an acute aldicarb assay.

The GABA-dependent defecation assay provides a means of detecting GABA uptake activity in the defecation neurons, AVL and DVB. Foraging behavior, which is modulated by the RMEs, can be assayed simultaneously, but is less amenable to quantification. We found that GABA uptake in both defecation and foraging neurons is completely dependent on *snf-11* activity. The defecation assay, in particular, could provide a convenient means of identifying additional *snf-11* mutations.

Aldicarb resistance is usually interpreted as a measure of ACh release (presynaptic) or response (postsynaptic); however, because GABA antagonizes the action of ACh, GABA levels may contribute to resistant or sensitive phenotypes. This is clearly seen in the case of *unc-25* (GAD) mutants, which are hypersensitive to aldicarb. GAT1-deficient mice have elevated levels of extracellular GABA, resulting in increased activation of post-synaptic $GABA_A$ receptors (Jensen *et al.*, 2003). Based on this observation, we expected that *snf-11* mutants also would have elevated levels of extracellular GABA. We found that *snf-11* mutants are resistant to aldicarb in an acute assay (Figure 6). This resistance requires GABA synthesis and release, however, because *unc-25; snf-11* double mutants are as hypersensitive as *unc-25* single mutants (Figure 6).

The loss of GAT1 function in mice results in significant neurological defects, including tremors, ataxia, and anxiety (Chiu *et al.*, 2005). The reduced GABA uptake activity results in elevated extracellular GABA levels, which in turn result in an increased $GABA_A$ receptor-mediated tonic conductance in cerebellar granule and Purkinje cells (Chiu *et al.*, 2005; also Jensen *et al.*, 2003). Jensen *et al.* (2003) also report that GAT1-deficient mice lack a presynaptic $GABA_B$-mediated conductance. Clearly, the loss of GAT1 activity in mice has significant effects on GABAergic signaling and, consequently, on GABAergic behaviors.

In contrast, the loss of GAT activity in worms has no major behavioral consequences, suggesting that these animals are not dramatically hyper-GABAergic. Perhaps because of the structure and geometry of GABAergic synapses in *C. elegans*, diffusion seems to be adequate to reduce extracellular GABA to tolerable levels. In addition, GABA is the most abundant inhibitory neurotransmitter in the mouse central nervous system and modulates a wide range of behaviors. In contrast, GABA is primarily used at neuromuscular synapses in *C. elegans*, and its action is counteracted by excitatory cholinergic input. These differences may also account for the relative subtlety of the *snf-11* mutant phenotypes compared with those of the mouse GAT1 knockout.

Expression and Localization of SNF-11 in C. elegans

Our results indicate that all GABAergic synapses are associated with either presynaptic or postsynaptic (or both) expression of SNF-11. The SNF-11 protein was detected in both neuronal cell bodies and processes and was concentrated at presumptive synapses in some cells (RMED and RMEV). In mammals, the four GAT subtypes are differentially expressed and localized, although there is more overlap in expression than previously realized. Until recently, expression studies suggested that GAT1 is exclusively expressed in neurons. However, recent studies show that GAT1 is expressed in both neurons and glial cells. There is strong evidence for expression of GAT3, GAT1, and GAT2 (in order of abundance) in glial cells in the mature neocortex, and GAT1 and GAT2 in neurons. However, only GAT1 seems to have a synaptic localization pattern (Conti *et al.*, 1998; Minelli *et al.*, 2003; Chiu *et al.*, 2002). The apparent localization of SNF-11 to synapses in a subset of neurons is consistent with the localization of the GAT1 subtype of GABA transporter.

The absence of *snf-11* expression in the ventral cord inhibitory (DD and VD) motor neurons is puzzling. The ventral cord inhibitory motor neurons are required for coordinated movement, and they function in opposition to the cholinergic excitatory motor neurons. SNF-11 is expressed in the postsynaptic muscle cells, and this muscle expression presumably provides the GABA clearance function at these neuromuscular synapses. We suggest that the expression of plasma membrane GABA transporters in the ventral cord inhibitory motor neurons either may confer no energetic advantage, or it may be incompatible with their function. Recent studies on the dopamine transporter in *C. elegans* suggest that the channel characteristics of neurotransmitter transporters may contribute significantly to the electrical properties of cells (Carvelli *et al.*, 2004). Possibly, the channel properties of SNF-11 would compromise the conductive properties of the ventral cord inhibitory motor neurons.

Discrepancies with a Previous Study on snf-11

We noted several discrepancies between our results and those reported recently by Jiang *et al*. (2005). We found that *snf-11* is expressed in a subset of GABAergic neurons as well as the body wall, anal, and uterine muscles. In contrast to Jiang *et al*. (2005), we did not observe expression in the ventral nerve cord inhibitory motor neurons. However, we note that the reporter construct described by Jiang *et al*. (2005) is missing the 752-base pair proximal-most portion of the regulatory region described in this study, which may account for the observed differences in expression. Several additional lines of evidence also support our observations. We and other investigators (McIntire *et al.*, 1993b) have been unable to demonstrate uptake of exogenous GABA into the ventral cord inhibitory (DD and VD) motor neurons. The homologous neurons in *Ascaris* (DI and VI) also lack GABA uptake activity (Guastella *et al.*, 1991). Although previous studies (McIntire *et al.*, 1993b) found no evidence of GABA uptake into muscles in *C. elegans*, our observations are con-

sistent with the results of GABA uptake experiments in *Ascaris* (Guastella *et al*., 1991).

We also report phenotypic difference between our *snf-11* null mutants and the partial RNAi knockdown reported by Jiang *et al*. (2005). The *snf-11* null mutants have no obvious phenotype, and the absence of GABA uptake activity seems to have little effect on GABAergic behaviors. In contrast, Jiang *et al*. (2005) reported that RNA interference (RNAi) knockdown leads to movement and defecation defects. Although it is possible that *snf-11* mutants compensate during their development for the absence of *snf-11* function, it should be noted that exposure to GAT inhibitors does not result in behavioral phenotypes such as those observed by Jiang *et al*. (2005) but rather results in phenotypes identical to those of the *snf-11* null mutants. Furthermore, the RNAi phenotype reported by Jiang *et al*. (2005) is precisely the opposite of what one would expect from an excess of GABAergic signaling. Because eliminating GABAergic function results in constipation (McIntire *et al*., 1993a,b), it is surprising that Jiang *et al*. (2005) observed constipation in *snf-11* knockdown animals, which ought to have excess GABAergic signaling. Likewise, Jiang *et al*. (2005) report that *snf-11* RNAi leads to aldicarb hypersensitivity, which is a phenotype associated with reduced GABAergic signaling. In contrast, we observed that *snf-11* null mutants are aldicarb resistant, which is consistent with increased GABA function. Clearly, the *snf-11* knockouts provide a better means of assessing the phenotypic effects of eliminating GABA uptake activity.

Neurotransmitter transporters potentially function in both the clearance and recycling of neurotransmitters from synaptic clefts. The results described by Jiang *et al*. (2005) strongly imply that the recycling function is more critical for GABAergic neurotransmission in *C. elegans* because *snf-11* RNAi results in a GABAergic "loss-of-function" phenotype. Alternatively, Jiang *et al*. (2005) suggest that GABA receptors have been down-regulated; however, other studies fail to support this view (Gally and Bessereau, 2003). In contrast, our results clearly indicate that the GABA transporter in *C. elegans* functions chiefly in the clearance of GABA. Not only do *snf-11* null mutants lack a discernible GABA loss-of-function phenotype, but our results also demonstrate that these mutants are, indeed, hyper-GABAergic.

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