Intestinal signaling to GABAergic neurons regulates a rhythmic behavior in *Caenorhabditis elegans*

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The *Caenorhabditis elegans* defecation motor program (DMP) is a highly coordinated rhythmic behavior that requires two GABAergic neurons that synapse onto the enteric muscles. One class of DMP mutants, called anterior body wall muscle contraction and expulsion defective (*aex*) mutants, exhibits similar defects to those caused by the loss of these two neurons. Here, we demonstrate that *aex-2* encodes a G-protein–coupled receptor (GPCR) and *aex-4* encodes an exocytic SNAP25 homologue. We found that *aex-2* functions in the nervous system and activates a $G_s\alpha$ signaling pathway to regulate defecation. *aex-4*, on the other hand, functions in the intestinal epithelial cells. Furthermore, we show that *aex-5*, which encodes a pro-protein convertase, functions in the intestine to regulate the DMP and that its secretion from the intestine is impaired in *aex-4* mutants. Activation of the $G_s\alpha$ GPCR pathway in GABAergic neurons can suppress the defecation defect of the intestinal mutants *aex-4* and *aex-5*. Lastly, we demonstrate that activation of GABAergic neurons using the light-gated cation channel channelrhodopsin-2 is sufficient to suppress the behavioral defects of *aex-2*, *aex-4*, and *aex-5*. These results genetically place intestinal genes *aex-4* and *aex-5* upstream of GABAergic GPCR signaling. We propose a model whereby the intestinal genes *aex-4* and *aex-5* control the DMP by regulating the secretion of a signal, which activates the neuronal receptor *aex-2*.

defecation | receptors | secretion | peptides | channelrhodopsin

The *Caenorhabditis elegans* defecation motor program (DMP) is a highly coordinated series of three muscle contractions that are executed every 45 sec [Fig. 1*A* and supporting information (SI) Movie S1]. The cycle is initiated by a posterior body wall muscle contraction (pBoc), followed 2–3 sec later by an anterior body wall muscle contraction (aBoc). About 1 sec after the aBoc, enteric muscles contract, thus causing the expulsion (Exp) of intestinal contents. The process repeats itself ≈45 sec later with little variability in the timing of contractions (1). A genetic screen for mutants that displayed defects in the DMP isolated mutants defective in each of the three muscle contractions, known as *pbo*, *abo*, and *exp* (1). The screen also recovered mutants defective in the last two muscle contractions (aBoc and Exp [*aex*]) and mutants defective in the cycle periodicity (i.e., longer or shorter than normal DMP cycling times) (1). Molecular studies of these mutants have suggested that the behavior is orchestrated through the communication between the intestine, GABAergic neurons, and muscle.

The periodicity of the DMP is regulated by the *C. elegans* intestine, a single-cell layer tube of polarized epithelial cells joined by gap junctions (2, 3). Intestinal Ca^{2+} oscillations with ≈45-sec periodicity appear to play a central role in this timing. They consist of a posterior-to-anterior Ca^{2+} wave whose levels peak in the posterior and anterior intestinal cells just before the pBoc and aBoc contractions, respectively (3–5). Mutations in genes involved in the maintenance of Ca^{2+} oscillations or in the propagation of Ca^{2+} waves between cells affect the periodicity of the DMP (3–5). These studies suggest that the intestine may control the timing of the DMP via a Ca^{2+}-dependent process, such as Ca^{2+}-induced exocytosis.

Furthermore, recent work demonstrates that the intestine induces the pBoc by releasing protons (through a Na^+/H^+ exchanger) onto posterior body wall muscle cells (6). The posterior body wall muscle cells contract in response to the change in pH because they express a proton-gated cation channel (6). By contrast, the Exp step of the DMP is regulated by the GABAergic neurons AVL and DVB (7, 8). These neurons secrete GABA onto enteric muscles that express the excitatory GABA receptor EXP-1 and cause them to contract (9).

If the intestine is the cycle timer and initiates the pBoc step and neurons initiate the Exp step, then how are the intestinal- and neuronal-mediated behaviors synchronized? It seems likely that studies of *aex* genes will give some insight into how the AVL and DVB neurons are activated, because the behavioral defects of *aex* mutants are reminiscent of animals whose AVL and DVB GABAergic neurons are laser ablated (7, 8). *aex-3* and *aex-6* regulate synaptic transmission, probably by regulating exocytosis of neurotransmitter: *aex-3* is a guanine nucleotide exchange factor that regulates Rab small guanosine triphosphatase function, and *aex-6* (also known as *rab-27*) is a Rab small guanosine triphosphatase that regulates secretory vesicle exocytosis (10, 11). *aex-5* encodes a pro-protein convertase, an enzyme that is copackaged with propeptides and processes them to make mature secretory molecules (12, 13). Lastly, Doi and Iwasaki (12) demonstrated that *aex-1* is a distant homologue of the synaptic gene *unc-13* (or Munc13), which acts in the intestine to regulate the DMP. Thus, prior molecular characterization of *aex* genes implicates that a secretory event is in control of aBoc and Exp.

Here, we uncover how the intestinal cells regulate the activity of GABAergic neuronal function during the DMP. We cloned *aex-4*, which encodes a SNAP25 soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) homologue, and *aex-2*, which encodes a G-protein–coupled receptor (GPCR). We demonstrate that whereas *aex-4* and *aex-5* act in the intestine to regulate defecation, *aex-2* functions in GABAergic neurons to control this behavior. Disruption of *aex-4* function blocks AEX-5 secretion from the intestine. Moreover, GABAergic expression of activated adenylyl cyclase or photoactivatable channelrho-

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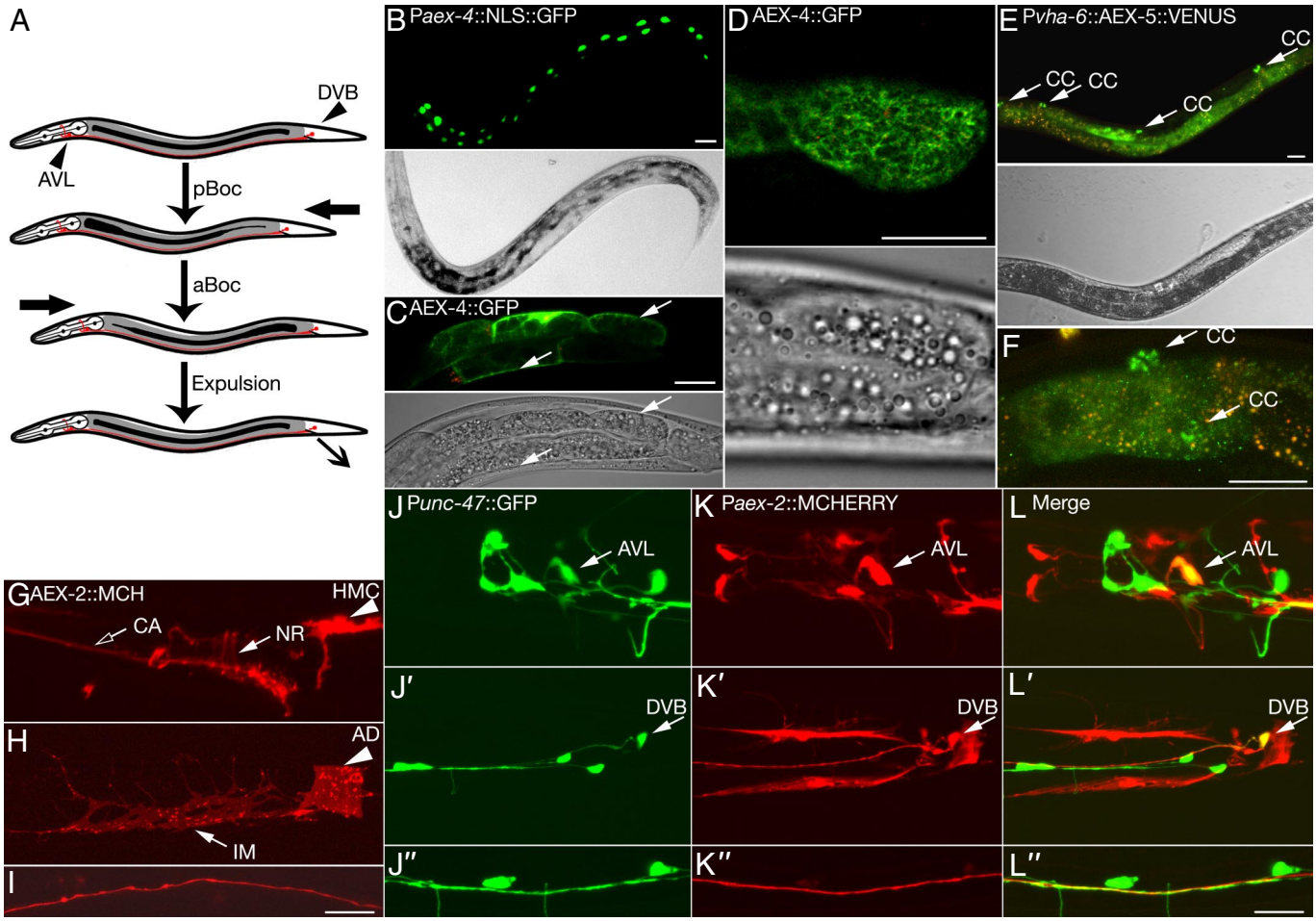

Fig. 1. *aex* genes are expressed in neuronal and nonneuronal tissues of *C. elegans*. (*A*) Diagram of the *C. elegans* DMP. First, a posterior contraction (pBoc) forces the intestinal contents to the anterior end of the worm. About 3 sec later, an anterior contraction (aBoc) forces intestinal contents to the posterior end. Within about 1 sec of the aBoc, an enteric muscle contraction in the tail leads to excretion of intestinal contents (Exp). Arrowheads indicate the location of AVL and DVB GABAergic neurons. (*B–D*) *aex-4* is expressed in the intestine. (*B*) GFP is detected solely in the intestinal nuclei in a transgenic animal that expresses nuclear localized GFP under the *aex-4* promoter. (*C*, *D*) Functional AEX-4::GFP fusion is expressed in intestinal cells and enriched at the cell surface (arrows; red indicates autofluorescence). (*C*) Confocal slice through the middle of the intestine. (*D*) Cell surface view of the posterior intestinal cell. Bright field images are provided for orientation. (*E*, *F*) Intestinal AEX-5::VENUS (which is driven by the intestinal promoter P*vha-6*) is secreted from the intestine and taken up by coelomocytes (CC, arrows; red indicates autofluorescence). (*G″–L″*) *aex-2* is expressed in the GABAergic neurons AVL and DVB as well as in enteric muscles. (*G* and *I*) AEX-2::mCherry is detected in the nerve ring (NR, arrow), ciliary sensory processes (CA, open arrow), nerve cord (I), and head mesodermal cell (HMC, arrowhead). (*H*) AEX-2::mCherry is expressed in the intestinal muscle (IM, arrow) and anal depressor (AD, arrowhead). (*J″–L″*) mCherry expressed under the *aex-2* promoter is detected in AVL (*J–L*, arrow) and DVB (*J′–L′*, arrow) GABAergic neurons. AEX-2::mCherry signal does not significantly overlap with GABAergic GFP in the ventral nerve cord (*J″–L″*). (Scale bar, 20 μm.) See *SI Text* for transgenes and clones.

dopsin (ChR2) rescues the defecation defects of *aex-2*, *aex-4*, and *aex-5*. We propose a model in which intestinal *aex* genes, *aex-4* and *aex-5*, regulate secretion of a signal that activates the GPCR *aex-2* in AVL and DVB, which, in turn, activates these neurons to complete the DMP.

Results

***aex* Mutants Are Primarily Defective in Exp and Only Mildly Defective in aBoc.** To understand better how the DMP operates, we carefully characterized the defecation defects of each of the *aex* mutants. *aex* mutants are primarily defective in the Exp step (Figs. S1 and S2). Surprisingly, *aex* mutants have only slightly fewer aBoc contractions per defecation cycle than WT; however, those aBoc contractions are usually significantly later in the cycle than in a WT DMP (Figs. S1 and S2). Therefore, the aBoc defects of most *aex* mutants are relatively mild when compared with the Exp defects. Most *aex* mutants have a relatively normal cycle length (or period), although *aex-5* did exhibit a slightly longer defecation cycle period (Fig. S1). The normal cycle periodicity suggests that these mutants do not have a defect in cycle time generation. Taken together, these results suggest that *aex* mutants are primarily defective in the Exp step.

***aex-4* Encodes a SNARE Protein.** We predicted that *aex-4* would encode a protein involved in exocytosis, neuropeptide production, or neuropeptide signaling, because all other *aex* strains have mutations in genes regulating these pathways (10, 12, 14). By searching the genomic interval in which *aex-4* had been mapped (1), we identified a candidate gene for the *aex-4* locus. All *aex-4* alleles sequenced have mutations in the gene T14G12.2, and a 4-kb genomic clone of this gene is sufficient to rescue the defecation defect of *aex-4* (Fig. S3 and S4*A*; *SI Text*, section V.B). Table S1 describes all transgenic strains used in this work. T14G12.2 encodes a homologue of the SNARE protein SNAP25 (Fig. S3) (15, 16). These findings implicate *aex-4* in exocytosis.

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AEX-4 Regulates the DMP from the Intestine. To dissect how AEX-4 regulates defecation behavior, we first determined the expression pattern of *aex-4* by building a transgenic animal that expresses GFP fused to a nuclear localization signal under the *aex-4* promoter (*SI Text*, section V.1). Surprisingly, *aex-4* expresses only in intestinal cells (Fig. 1*B*). To determine the subcellular localization of AEX-4, we built a transgene expressing a functional GFP-tagged AEX-4 fusion under its native promoter (*SI Text*, section V.C). GFP-tagged AEX-4 localizes along the plasma membrane of intestinal cells (Fig. 1 *C* and *D*). Therefore, AEX-4 likely acts at the surface of intestinal cells to regulate the DMP.

We expressed the *aex-4* gene under an intestinal-specific promoter, a muscle-specific promoter, and a neuronal-specific promoter to determine in what tissue *aex*-4 regulates the DMP (*SI Text*, section V.D–F). Intestinal expression of *aex-4* fully rescues the defecation defects of *aex-4* mutant animals (Fig. 2*A* and Fig. S4 *B* and *C*), whereas muscle- and neuronal-specific promoters only partially rescue the defecation defect of *aex-4* mutants (Fig. 2*A*).

To verify our tissue-specific rescue experiments, we tested whether RNAi of *aex-4* in a strain that is only sensitive to RNAi in the intestine would result in a defecation defect (17). Indeed, intestinal-specific RNAi of *aex-4* results in a strong Exp defect consistent with *aex-4* functioning in the intestine (Fig. 2*C*). In addition to *aex-4*, RNAi of *aex-5* and *aex-6* in the intestinal-specific RNAi strain causes a strong Exp defect. RNAi of *aex-3* causes a moderate Exp defect similar to that of the *aex-3* loss of function (Fig. S1). These results are consistent with *aex-3*, *aex-4*, *aex-5*, and *aex-6* functioning in the intestine. RNAi of *aex-1*, in contrast, causes only a mild defect in Exp, although tissue-specific rescue experiments suggest that *aex-1* functions in the intestine (12). We suspect that *aex-1* levels were not reduced enough in the intestinal-specific RNAi strain, because RNAi of *aex-1* in WT strains causes a strong Exp defect (data not shown). Combined together, these results strongly suggest that a cohort of exocytic *aex* genes, including *aex-4*, all function in the intestine to regulate the DMP.

AEX-5 Regulates the DMP from the Intestine. *aex-5* encodes a pro-protein convertase and was identified in the same screen that isolated *aex-4* (1, 12, 13). We confirmed that the pro-protein convertase gene is mutated in *aex-5(sa23)* by sequencing (lesion is C443W) and by genomic fosmid rescue (Fig. S4*A*). Because *aex-4* likely regulates secretion of a signal from the intestine and pro-protein convertases are typically packaged into secretory vesicles (14), we wished to determine whether AEX-5 is secreted from the intestine. A transgene in which intestinal-specific expression of AEX-5, fused to the VENUS variant of GFP (18), was driven fully rescues the defecation defects of *aex-5* mutant animals, whereas muscle- and neuronal-specific expression only partially rescues the defecation defects (Fig. 2*A*; Fig. S4 *B* and *C*; and *SI Text*, section V.G–I). AEX-5::VENUS is secreted from the intestine and subsequently endocytosed by coelomocytes, specialized endocytic cells in *C. elegans* (Fig. 1 *E–F*). These findings suggest AEX-5 is secreted from the intestine, where it regulates the DMP.

AEX-4 Regulates the Secretion of AEX-5 from the Intestine. We hypothesized that AEX-4 regulates the secretion of AEX-5 from the intestine. To test this, we determined if *aex-4* mutants are defective in AEX-5::VENUS secretion. WT animals that express AEX-5::VENUS in the intestine (*SI Text*, section V.T) secrete AEX-5::VENUS, which accumulates in coelomocytes. In contrast, *aex-4* mutants accumulate AEX-5::VENUS in intestinal cells and accumulate significantly less AEX-5::VENUS in coelomocytes (Fig. 3 and Fig. S5). There is no defect in the secretion of AEX-5::VENUS in WT or *aex-2* mutants. Therefore, AEX-4

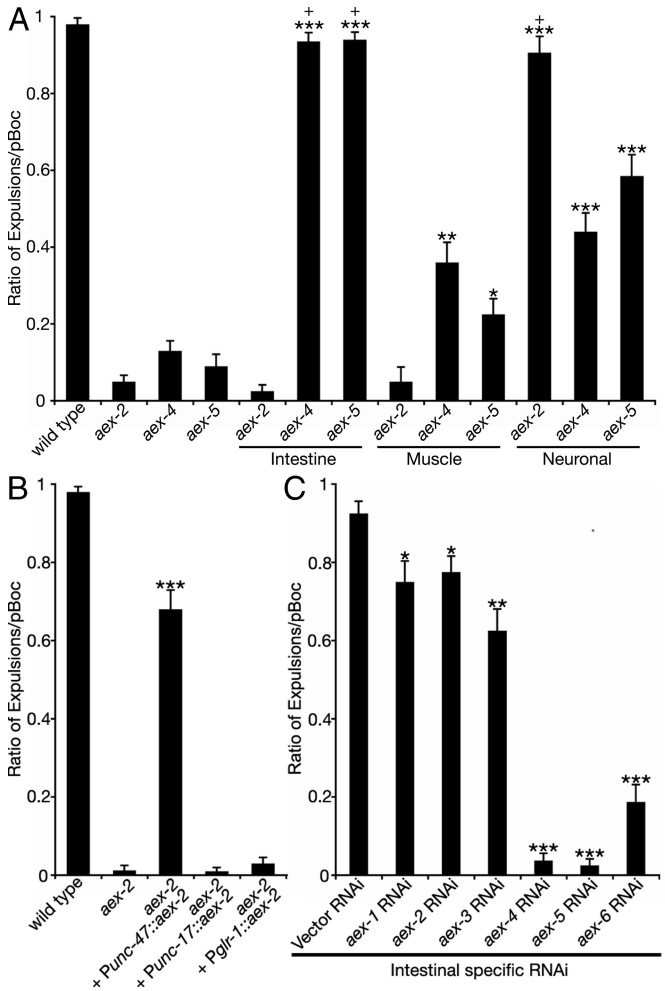

Fig. 2. *aex-4* and *aex-5* function in the intestine, whereas *aex-2* acts in GABAergic neurons to regulate defecation. (*A*) Neuronal promoter (P*rab-3*), muscular promoter (P*myo-3*), and intestinal promoter (P*ges-1* or P*vha-6*) were used to drive the expression of individual *aex* genes. Intestinal *aex-4* and *aex-5* fully rescue the *aex-4* and *aex-5* Exp defect, respectively, and neuronal *aex-2* rescues the *aex-2* Exp defect. Muscular and neuronal expressions of *aex-4* and *aex-5* only partially rescue the *aex-4* and *aex-5* Exp defect. (*B*) P*unc-47* (GABAergic neurons) but not P*unc-17* (cholinergic neurons) or P*glr-1* (subset of interneurons) expression of *aex-2* rescues the *aex-2* Exp defect. DMP function was assayed by observation of 8–20 animals for 10 cycles and plotted as the ratio of Exps to pBocs. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$ significantly different from the respective mutant. $+P > 0.05$ not significantly different from WT. (*C*) Intestinal RNAi of *aex-4*, *aex-5*, and *aex-6* induces strong Exp defects. RNAi of *aex-3* induces a moderate defecation defect, whereas the RNAi of *aex-1* and *aex-2* induces only mild defecation defects. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ significantly different from the vector control RNAi. Error bars represent SEM. See *SI Text* for transgenes and clones.

likely regulates the secretion of AEX-5 from the intestine during the DMP. These results lead us to the question of what receives this signal.

***aex-2* Encodes a GPCR.** We mapped *aex-2* to a region on the X chromosome and revealed that the gene T14B1.2 encodes *aex-2*. All sequenced *aex-2* alleles have mutations in T14B1.2, and a transgene that contains the T14B1.2 gene rescues the *aex-2* mutant phenotype (*SI Text*, section V.10–11 and Figs. S4*A* and S6). T14B1.2 encodes a protein that shares homology with the A class of GPCRs, some of which mediate peptide signaling (Fig. S6) (19). When expressing an *mCherry*-tagged *aex-2* genomic fusion construct (which fully rescues the *aex-2* defecation de-

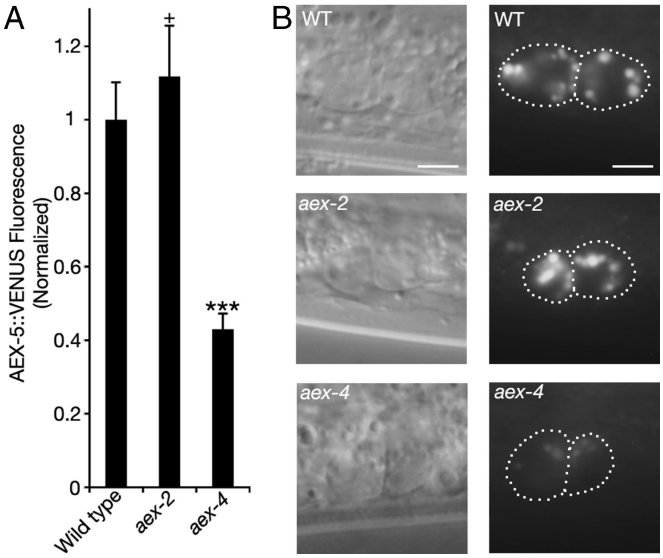

Fig. 3. Intestinal *aex-4* regulates the secretion of AEX-5. (*A*) In *aex-4* mutants, the total fluorescence, normalized to WT, of intestinal AEX-5::VENUS in coelomocytes is significantly less than that of WT. There is no significant change in coelomocyte fluorescence in *aex-2* mutants. ***, $P < 0.0005$ significantly different from the respective mutant. +, $P > 0.05$ not significantly different from WT. Error bars represent SEM. (*B*) Representative photographs of anterior coelomocytes in WT, *aex-2*, and *aex-4*. The left image is a Nomarski image. The right image is AEX-5::VENUS fluorescence. (Scale bars, 5 μm.)

fects), we detected AEX-2::mCherry signal in the nerve ring, ventral nerve cord, and enteric muscles (Fig. 1 *G–I*; *SI Text*, section V.J–K). So, unlike *aex-4*, which is exclusively expressed in the intestine, *aex-2* is expressed in neuronal and nonneuronal tissues.

***aex-2* Regulates the DMP from the Nervous System.** To determine what tissue *aex-2* functions in, we expressed an *aex-2* cDNA::GFP fusion under a neuronal promoter, a muscular promoter, and an intestinal promoter (*SI Text*, section V.L–N). Although neuronal expression of AEX-2::GFP fully rescues the defecation defects of *aex-2* mutants, muscular and intestinal *aex-2* does not (Fig. 2*A*). Consistent with this observation, intestinal-specific RNAi of *aex-2* does not cause a robust defecation defect (Fig. 2*C*). Thus, unlike *aex-4* and *aex-5*, *aex-2* acts in neurons to regulate the DMP.

Because previous studies have indicated that the GABAergic neurons AVL and DVB are required for Exp (7), we analyzed the expression pattern of our *aex-2* in various neuronal subtypes. We coexpressed mCherry protein under the *aex-2* promoter with the GFP under a *unc-17* (drives expression in cholinergic neurons), a *glr-1* (drives expression in subsets of interneurons), or a *unc-47* (drives expression in GABAergic neurons) promoter to assess if any of the *aex-2*–positive neurons are colabeled by these neuronal subtype-specific markers (*SI Text*, section V.O). We found that the *aex-2* reporter is detected in all three neuronal cell types (Fig. 1 *J–L* and Fig. S7). Briefly, at least one glutamatergic interneuron (likely AVD), several head and pharyngeal cholinergic neurons, and two GABAergic neurons (AVL and DVB) express mCherry under the control of the *aex-2* promoter (Fig. 1 *J–L* and Fig. S7). None of the ventral nerve cord motor neurons (neither cholinergic nor GABAergic) are labeled by P*aex-2*::mCherry. Therefore, *aex-2* is expressed in the defecation regulating GABAergic neurons (AVL and DVB) as well as in other types of nonmotor neurons.

To determine which neuronal subtype *aex-2* functions in, we expressed an *aex-2*::GFP cDNA fusion under neuronal subtype-specific promoters. GABAergic expression of *aex-2* significantly rescues the Exp defect and partially rescues the aBoc defect of *aex-2* mutants, whereas the other two promoters do not (Fig. 2*B* and Fig. S4 *B* and *C* and *SI Text*, section V.P–R). Taken together, these results suggest that *aex-2* acts in GABAergic neurons (i.e., AVL and DVB) to control the Exp, and perhaps the aBoc.

***aex-2* Functions Through a $G_s\alpha$ Signaling Pathway.** Because *aex-2* encodes a GPCR, we examined which Gα subunit might act downstream of the *aex-2* receptor signaling. To determine this, we genetically tested a variety of candidate Gα subunits for their ability to suppress *aex-2* mutant phenotype. We built double mutants between an *aex-2* loss-of-function mutant and a *gsa-1* ($G_s\alpha$) gain-of-function mutant (20) and an *egl-30* ($G_q\alpha$) gain-of-function mutant (21–23). We also built an *aex-2* and *dgk-1* (which encodes a diacylglycerol kinase) double loss-of-function mutant, because *dgk-1* normally antagonizes *egl-30* signaling and its loss-of-function phenocopies *egl-30* gain of function (21–23). $G_s\alpha$ gain-of-function mutation causes ectopic Exp in WT and *aex-2* mutant animals, and it suppresses the Exp defect in *aex-2* mutants (Fig. 4*A*). On the other hand, the $G_q\alpha$ pathway mutants do not have a strong effect on the *aex-2* phenotype (Fig. 4*A*). Interestingly, gain of function in *gsa-1* does not rescue the aBoc defect (Fig. S4 *B* and *C*). The irregular timing of Exps in *gsa-1* mutants (i.e., some Exps do not occur at the proper time, which is 3 sec after pBoc) may account for the lack of rescue of aBoc in *aex-2* mutants. To assess if the $G_s\alpha$ rescue is specifically mediated by GABAergic neurons, we tested whether the GABAergic expression of a gain-of-function adenylyl cyclase (*acy-1*) (24), a downstream effector of $G_s\alpha$, would suppress the defecation defects in *aex-2* mutants (*SI Text*, section V.S). Similar to the *gsa-1* gain-of-function mutant, overexpression of a gain-of-function *acy-1* gene in *aex-2* mutants causes ectopic Exps, and this expression significantly rescues the Exp defects in *aex-2* mutants (Fig. 4*A*). Taken together, these results support a model in which the *aex-2* GPCR acts in GABAergic neurons to regulate the DMP via a downstream $G_s\alpha$ and adenylyl cyclase pathway.

GABAergic Neurons Act Downstream of AEX-4 and AEX-5 to Control Defecation. Although *aex-2* acts in AVL and DVB GABAergic neurons, *aex-4* and *aex-5* function in the intestine to regulate Exp. We wished to determine if *aex-2* signaling acts downstream of intestinal *aex-4* and *aex-5*. Because we have not identified the ligand for AEX-2, we asked whether gain-of-function mutations in the *aex-2* pathway could suppress the *aex-4* and *aex-5* Exp defects. GABAergic expression of the gain-of-function *acy-1* gene is sufficient to rescue the Exp defects seen in *aex-4* and *aex-5* mutants (Fig. 4*A*). As seen in *aex-2* mutants, overexpression of gain-of-function *acy-1* in *aex-4* and *aex-5* mutants also causes ectopic Exps. These data suggest that GABAergic signaling of *aex-2* acts downstream of intestinal signaling of *aex-4* and *aex-5*.

Activation of GABAergic Neurons by a Light-Activatable Channel Can Bypass the Requirement of AEX-2, AEX-4, and AEX-5 for Defecation. We wished to demonstrate that the role of *aex-2*, *aex-4*, and *aex-5* is specifically to activate GABAergic neurons controlling the Exp step. We used YFP-tagged ChR2 under a GABAergic promoter to bypass the loss of these *aex* genes (*SI Text*, section VI). ChR2, from the green alga *Chlamydomonas reinhardtii*, is a light-activatable nonselective cation channel that, in the presence of all-*trans*-retinal, will depolarize excitable cells (25). We hypothesized that the activation of GABAergic neurons using the ChR2 transgene would suppress the behavioral defect of each of the *aex* mutants. Indeed, activation of ChR2 in GABAergic neurons by brief ($\approx$1 sec) pulses of blue light $\approx$2 sec after pBoc is sufficient to rescue the Exp defects of *aex-2*, *aex-4*, and

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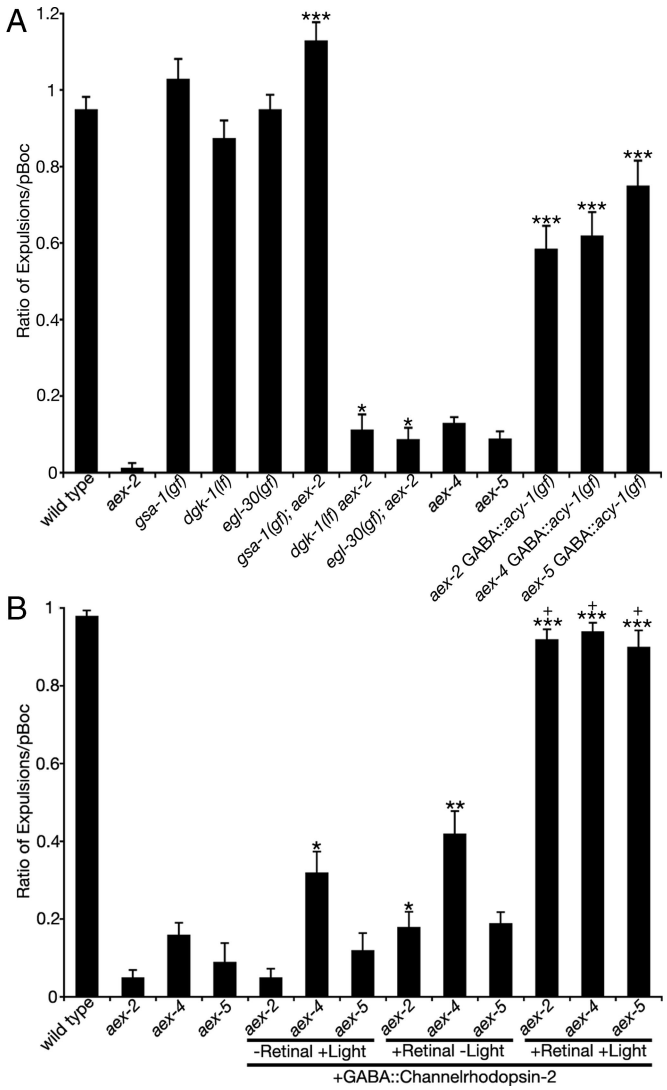

Fig. 4. *aex* genes likely regulate the defecation through downstream $G_s\alpha$ and adenylyl cyclase signaling and GABAergic neuron activation. (*A*) Gain-of-function allele of $G_s\alpha$ [*gsa-1(ce81)*] completely suppresses the Exp defects in *aex-2* mutants. In contrast, a gain-of-function in $G_q\alpha$ [*egl-30(js126)*] and a loss-of-function [*dgk-1(sy428)*] that phenocopies *egl-30* gain-of-function have only mild effects on the *aex-2* Exp defects. When an activated adenylyl cyclase gene [*acy-1(js127)*] is specifically expressed in the GABAergic neurons, it significantly suppresses the Exp defects of *aex-2*, *aex-4*, and *aex-5* mutants. (*B*) Activation of GABAergic neurons by photoactivatable ChR2 suppresses the Exp defects in *aex* mutants. The photoactivatable cation channel ChR2 was expressed specifically in GABAergic neurons under the *Punc-47* promoter in *aex-2*, *aex-4*, and *aex-5* mutants. In the presence of all-*trans* retinal and blue-light activation, the ChR2 fully suppresses the Exp defects in all the *aex* mutants. In contrast, in the absence of all-*trans* retinal or blue light, the ChR2 transgene does not rescue the defecation mutant phenotypes to WT levels. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ significantly different from the respective mutant. +, $P > 0.05$ not significantly different from WT. Error bars represent SEM. See *SI Text* for transgenes and clones.

aex-5 mutants completely (Fig. 4*B* and Movie S2). These results strongly suggest that *aex-2*, *aex-4*, and *aex-5* regulate the activity of GABAergic neurons AVL and DVB to induce Exp during the DMP.

Discussion

Several lines of evidence suggest that a group of exocytic genes function in the *C. elegans* intestine to control secretion of a signal to regulate the aBoc and Exp steps of the DMP. *aex-1*, *aex-3*, *aex-4*, and *aex-6* are each homologous to the genes that regulate exocytosis in secretory cells (11, 12). Here, we present data that these genes regulate the secretion of a signal from the intestine to induce aBoc and Exp. This model is further supported by the observation that *aex-4* mutants prevent the secretion of an intestinal AEX-5::VENUS into the pseudocoelom and its subsequent endocytosis by coelomocytes. Therefore, *aex-4* (and likely *aex-1*, *aex-3*, and *aex-6*) regulates the secretion of AEX-5 and, arguably, its substrate from the intestine. Although our data indicate *aex-3* and *aex-6* function in intestine, they do not exclude the possibility that these genes also function in AVL and DVB to regulate the DMP (see Fig. S8 for model).

The model of the intestine secreting a signal to regulate Exp prompted us to search for the receptor for the signal. *aex-2* encodes a putative GPCR, and our data suggest that this receptor likely functions in AVL and DVB GABAergic neurons to regulate Exp. Interestingly, when using a gain-of-function $G_s\alpha$ or an activated adenylyl cyclase that is expressed in GABAergic neurons, we suppressed the Exp defect not only in *aex-2* but in the intestinal *aex-4* and *aex-5* mutants. These results are consistent with *aex-2* encoding the receptor of the intestinal signal and acting downstream of intestinal *aex* genes.

We speculate that $G_s\alpha$ and adenylyl cyclase act downstream of *aex-2* to excite AVL and DVB during the Exp. In support of this model, some *gsa-1* loss-of-function animals, with a mosaic rescuing transgene, were reported to exhibit a defecation defect (26). We also observed a robust defect in the Exp step in these animals (11% ± 3%, $P < 0.0005$). In contrast, *acy-1* loss-of-function mutants, with a rescuing transgene expressing in muscle (27), do not have a significant defect in the Exp step (81% ± 7%, $P > 0.05$). This may be attributable to redundancy of the three other adenylyl cyclase genes in *C. elegans* (28) and/or the action of other *gsa-1* effectors acting in parallel to *acy-1*.

Activation of ChR2 in GABAergic neurons is sufficient to suppress the Exp defects of *aex-2*, *aex-4*, and *aex-5* mutants. The aBoc defect of these *aex* mutants, however, was not rescued. Activation of GABAergic ChR2 causes the worm to become paralyzed as a result of muscle relaxation; therefore, it may be difficult to observe the aBoc contractions under these conditions. These findings indicate that the *aex* genes likely act to activate AVL and DVB GABAergic neurons through *aex-2*.

Although *aex-2* is likely involved in AVL and DVB activation, it remains unclear how *aex-2* is activated. Because *aex-5* encodes a pro-protein convertase and *aex-5* mutants are defective in neuropeptide production (13), we suspect that *aex-2* encodes a neuropeptide-like receptor. There are ≈100 genes in the *C. elegans* genome encoding more than 250 peptides (29, 30), many of which are expressed in the intestine. The identification of *aex-2* ligand(s) will shed new light on our understanding of this GPCR's signaling and the regulation of neuronal functions by nonneuronal tissues.

Several of our observations suggest that the Exp step may be regulated by more than one signal. First, we noticed that activation of ChR2 in WT or *aex* mutant animals does not induce ectopic Exps. Only temporally correct light activation of AVL and DVB rescues the Exp defect of *aex-2*, *aex-4*, and *aex-5*. This observation indicates that activation of AVL and DVB (at least via ChR2) is permissive, but not completely instructive, to drive the Exp step of the DMP. Second, although constitutive activation of the $G_s\alpha$ pathway causes ectopic Exps in *aex* mutants, there is a strong tendency for Exps to occur at the proper time (≈3 sec after pBoc). If *aex* genes were the sole connection between the intestinal pacemaker and activation of Exp, then one would expect to see a weaker tendency for Exps to occur at the proper time. These observations suggest that a second signal may function to regulate the Exp step. This signal could, for example,

consist of a permissive signal that allows enteric muscles to be excited at the right time point in the DMP.

On first glance, one cannot help but notice the similarities between the intestinal *aex* genes and the neuronal secretory apparatus. SNARE proteins (AEX-4) are thought to create a membrane fusion structure at the nerve terminal, with SNARE regulators (AEX-1) playing a critical role in exocytosis (31). Although the precise function of Rabs (AEX-6) in exocytosis, *per se*, is unclear, they play an important role in synaptic transmission (11). Interestingly, although the regulator for AEX-6, AEX-3, has a similar defecation phenotype when mutated, the effector of AEX-6, RBF-1, does not (11). Perhaps AEX-6, also known as Rab27, functions through a novel effector to regulate the DMP. Given the similarities between the genes involved in the DMP and those involved in synaptic transmission, the *C. elegans* intestine could be seen as an alternative means for investigating the mechanisms governing regulated exocytosis and, by analogy, synaptic transmission. When one adds on the observation that RNAi is highly ineffective in *C. elegans* neurons (32) but very effective in the intestine (17), the intestine becomes an attractive model for discovering genes and genetic pathways regulating exocytosis.

Our work shows that the intestine may secrete a signal to activate the AVL and DVB neurons to induce the DMP (Fig. S8). This might explain why the Ca^{2+} oscillations are necessary for the timing of the DMP, because excitable cells use increased Ca^{2+} to induce secretion. Our work provides the first explanation of how the intestine may regulate this behavior by activating the AVL and DVB neurons.

Materials and Methods

Refer to *SI Text* for detailed materials and methods and for a description of transgenes and clones.

Behavioral Assay. For detailed methods, refer to *SI Text*, section IX. Briefly, 8–20 1-day-old adults were scored for 10 defecation cycles (i.e., 10 pBocs). Statistical significance was determined by using unpaired two-tailed Student *t* tests, with unequal variance.

ChR2 Experiments. For detailed methods, refer to *SI Text*, section X. Briefly, L4 larval-staged animals were grown in the presence or absence of 500 μM all-*trans* retinal (Sigma) overnight at 22°C. The next day, defecation was scored on a Leica MZ16F fluorescent stereomicroscope with an x-Cite 120 excitation light source (EXFO) and a standard GFP filter. During the DMP, animals were stimulated with a brief $\approx$1-sec pulse of blue light, $\approx$2 sec after pBoc.

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