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# Gut neuroendocrine signaling regulates synaptic assembly in *C. elegans*

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## Abstract

**Synaptic connections are essential to build a functional brain. How synapses are formed during development is a fundamental question in neuroscience. Recent studies provided evidence that the gut plays an important role in neuronal development through processing signals derived from gut microbes or nutrients. Defects in gut–brain communication can lead to various neurological disorders. Although the roles of the gut in communicating signals from its internal environment to the brain are well known, it remains unclear whether the gut plays a genetically encoded role in neuronal development. Using *C. elegans* as a model, we uncover that a Wnt-endocrine signaling pathway in the gut regulates synaptic development in the brain. A canonical Wnt signaling pathway promotes synapse formation through regulating the expression of the neuropeptides encoding gene *nlp-40* in the gut, which functions through the neuronally expressed GPCR/AEX-2 receptor during development. Wnt-NLP-40-AEX-2 signaling likely acts to modulate neuronal activity. Our study reveals a genetic role of the gut in synaptic development and identifies a novel contribution of the gut–brain axis.**

**Keywords** gut–brain axis; neuropeptide; NLP-40; synaptic development; Wnt
**Subject Categories** Neuroscience; Signal Transduction
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## Introduction

The human brain contains billions of neurons wired through trillions of highly specific synaptic connections. These connections provide the structural bases of brain functions. Synaptic structures are largely formed during the early development stage and precisely regulated by evolutionally conserved molecules and signaling pathways, among which is the well-known Wnt signaling (Salinas & Zou, 2008; Farias *et al*, 2010; Dickins & Salinas, 2013; Zwamborn *et al*, 2018; Ohkawara *et al*, 2021; Teo & Salinas, 2021).

Wnt signaling is highly conserved cross animal kingdom and plays critical roles in various steps of neuronal development including synaptogenesis (Zacharias *et al*, 1999; Packard *et al*, 2002; Klassen & Shen, 2007; Jing *et al*, 2009; Oliva & Inestrosa, 2015). In this process, the Wnt ligands secreted either from the nervous system or from the neighbor tissues bind to the receptors on neurons or the postsynaptic cells (Hall *et al*, 2000; Packard *et al*, 2002; Klassen & Shen, 2007), which activate the downstream signaling cascades. Wnts function through either the β-catenin-dependent canonical or the independent noncanonical pathway (Moon *et al*, 2002; Veeman *et al*, 2003). However, the detail mechanisms underlying synaptic development regulated by Wnts are not well understood. Interestingly, recent studies showed that Wnt signaling participates in brain–gut communication (Zhang *et al*, 2018). Metagenomic analysis also shows that Wnt signaling is upregulated in psoriasis patients' gut microbiota (Xiao *et al*, 2021), indicating that it may be involved in gut–brain communication. However, direct evidence regarding whether and how the intestinal Wnt signaling is involved in regulating brain development or functions is still missing.

The gastrointestinal (GI) tract plays a critical role in regulating brain development and function. It not only provides the nutrients, but also directly senses the gut environmental stimuli. Through the gut–brain axis, diets and gut microbes regulate neurogenesis, microglia and astrocyte activation, myelination, blood brain barrier permeability, synaptic pruning and plasticity (Cryan *et al*, 2019). Dysregulation of the gut–brain axis is highly correlated with various neurodevelopmental or neurodegenerative disorders including autism spectrum disorders, Alzheimer's disease, and Parkinson's disease (Mulak & Bonaz, 2015; Li & Zhou, 2016; Quigley, 2017; Kowalski & Mulak, 2019; Chidambaram *et al*, 2020). Although accumulative evidence indicates that gut microbiota regulates neuronal development and function in the brain, it remains largely unknown whether the gut plays any environment-independent, genetically encoded role in regulating brain development.

*C. elegans* is an excellent model for addressing the mechanisms underlying synaptic formation due to its simple and amendable nerve system (Zhen *et al*, 2000; Jin, 2005). For example, the presynaptic structure can be labeled and visualized with fluorescence-tagged synaptic vesicles or active zone proteins at the single cellular level (Baum *et al*, 1999; Colon-Ramos *et al*, 2007; Mizumoto & Shen, 2013). Similarly, postsynaptic receptors can be fluorescently tagged (Brockie & Maricq, 2003; Mizumoto & Shen, 2013). With

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those tools, many conserved synaptic regulators have been identified including IgG family members, Netrin/DCC, and Wnts (Hendi *et al*, 2019). However, the underlying molecular mechanisms for most regulators remain unidentified.

In this study, we uncovered that a Wnt-neuroendocrine pathway in the gut regulates presynaptic formation in the nerve ring. Wnt signaling functions by upregulating the NLP-40 neuropeptides in the gut. NLP-40 peptides are secreted and then promote synaptic formation most likely through GPCR/AEX-2 mediated neuronal activity. Therefore, we uncovered a genetically encoded role of gut in regulating synaptogenesis in the nerve ring, which reveals a novel mode of gut–brain interaction.

## Results

### Wnt signaling in the gut regulates AIY presynaptic formation

*C. elegans* AIY neurons are a pair of bilateral and symmetric interneurons located in the nerve ring, which is a structure analogous to vertebrate's brain (Fig 1A) (White *et al*, 1986). AIY neurites form presynaptic structures with a stereotypic and highly reproducible pattern (Fig 1A') (White *et al*, 1986; Colon-Ramos *et al*, 2007).

Wnt signaling pathways are highly conserved and play diverse roles in regulating synaptic formation, plasticity, and maintenance (Dickins & Salinas, 2013; McLeod & Salinas, 2018; Yang & Zhang, 2020). We previously found that components in a canonical Wnt signaling pathway including Wnt/CWN-2, Frizzled/CFZ-2, Disheveled/DSH-2, β-catenin/SYS-1, and TCF/POP-1 were required for Zone 2 presynaptic clustering (Shi *et al*, 2018). This Wnt signaling pathway acts both in the nervous system and in the intestine to regulate AIY presynaptic morphology (Shi *et al*, 2018). To further address the role of Wnt signaling in AIY presynaptic formation, we first quantified the fluorescence intensity of the AIY synaptic vesicle marker GFP::RAB-3 in *cwn-2(ok895)*, *cfz-2(ok1201)*, *pop-1(hu9)* loss-of-function mutants and *sys-1* RNAi animals, and found that the GFP intensity was robustly reduced as compared to that in the wild-type animals (Fig 1B–H). To confirm the role of Wnt signaling in AIY presynaptic formation, we examined additional presynaptic makers including the active zone protein GFP::SYD-1, synaptic vesicle protein YFP::SNB-1. Similarly, both SYD-1 and SNB-1 reporters were reduced in the Wnt mutants (Figs 1B'–H' and EV1A–E). Furthermore, we also examined the endogenous synaptic marker using split GFP approaches to cell-specifically label AIY presynaptic sites. We knocked in GFP11 at the N-terminal of endogenous RAB-3 through Crispr/Cas9 and expressed GFP1-10 with AIY-specific *ttx-3* promoter. As we could not detect GFP signals when GFP::RAB-3 expression level is low (unpublished data), we knocked in 7xGFP11, which can significantly increase GFP level (Kamiyama *et al*, 2016). With this split GFP tagged marker, we observed the same results as with other presynaptic markers (Fig EV1F–K). To exclude the possibility that the reduction in the presynaptic marker is specific to the green fluorophore, we quantified the mCherry::RAB-3 and obtained similar results (Appendix Fig S1A–G), which suggests the reduction of synaptic signals in Wnt mutants is not fluorophore specific. Finally, to exclude the possibility that the decrease of synaptic marker in AIY is due to the effect of Wnt signaling on the *ttx-3* promoter or on AIY morphogenesis, we quantified the cytoplasmic mCherry driven by the same promoter and found that the fluorescence intensity and the AIY morphology did not change in the *cfz-2 (ok1201)* or *pop-1(hu9)* mutants (Fig EV2A–C and F). These results suggest that the reduction of AIY presynaptic marker is not due to the effect of Wnt signaling on *ttx-3* promoter or AIY morphogenesis. These data collectively support the following two conclusions. First, Wnt signaling is required for AIY synaptic formation. Second, all synaptic markers we examined are similarly regulated by the Wnt signaling pathway. Therefore, for convenience, unless specified, we used the integrated GFP::RAB-3 (*wyIs45)* for the rest of analysis.

To determine the site of action, we conducted tissue-specific RNAi. We used the RNA transport *sid-1* mutants with *sid-1* transgene specifically expressed in either the gut or the nervous system (Calixto *et al*, 2010; Melo & Ruvkun, 2012). We confirmed the specificity and the efficiency of RNAi by knocking down the cytoplasmic GFP reporters, and found that both efficiency and specificity of intestinal-specific RNAi was robust (no GFP changed in nervous system, 99.5% reduction in the intestine). Notably, although the efficiency for neuronal-specific RNAi was also robust, the specificity was not perfect (GFP was reduced by 70.24% in nervous system, 43.01% in the intestine) (Appendix Fig S2A–D). With this system, we found that the AIY presynaptic markers were reduced when Wnt signaling components were knocked down in the intestine (Fig 1I–N), but not in the nervous system (Fig 1I'–N'). These results suggest

**Figure 1. AIY presynaptic formation requires intestinal Wnt signaling.**

A A bright field image of a wild-type adult *C. elegans*. The position of the AIY interneurons in the head is indicated with green, and the gut is outlined with dashed lines.

A' A cartoon diagram of the *C. elegans* head modified from wormatlas with permission. The AIY processes and soma are indicated in gray, and the presynaptic sites are marked with green (Altun & Hall, 2021).

B–G' Confocal images of the AIY labeled with synaptic vesicle marker GFP::RAB-3 (B–G, green) and active zone marker GFP::SYD-1 (B'-G', pseudo-red) in wild-type (B, B'), *cwn-2(ok895)* (C, C'), *cfz-2(ok1201)* (D, D'), *pop-1(hu9)* (E, E') and *sys-1* RNAi (G, G') animals.

H–H' Quantification of the fluorescence intensity of AIY RAB-3 (H) and SYD-1 (H') for the indicated genotypes.

I–M' Confocal images of AIY labeled with mCherry::RAB-3 (pseudo-green) (I-M), or GFP::RAB-3 (I'-M') of tissue-specific RNAi treatment for control (I-I'), *cwn-2*(J-J'), *cfz-2* (K–K'), *sys-1*(L-L') and *pop-1*(M-M').

N–N' Quantification of the fluorescence intensity of AIY mCherry::RAB-3 or GFP::RAB-3 for the indicated RNAi treatment.

Data information: The scale bar in (B) and (I) are 10 μm and applies to (C–G') and (I'–M'), respectively. Asterisks indicate AIY soma. The AIY synaptic region was quantified for the synaptic intensity analysis. For (H–H', N–N'), each dot represents one animal, data were collected from at least three independent experiments. Statistical analyses are based on one-way ANOVA followed by Dunnett's test (for mutants) or two-tailed Student's *t*-test (for RNAi). ns: not significance ($P > 0.05$), ****$P < 0.0001$ as compared to the wild-type or control RNAi. Error bars represent SEM.
Source data are available online for this figure.

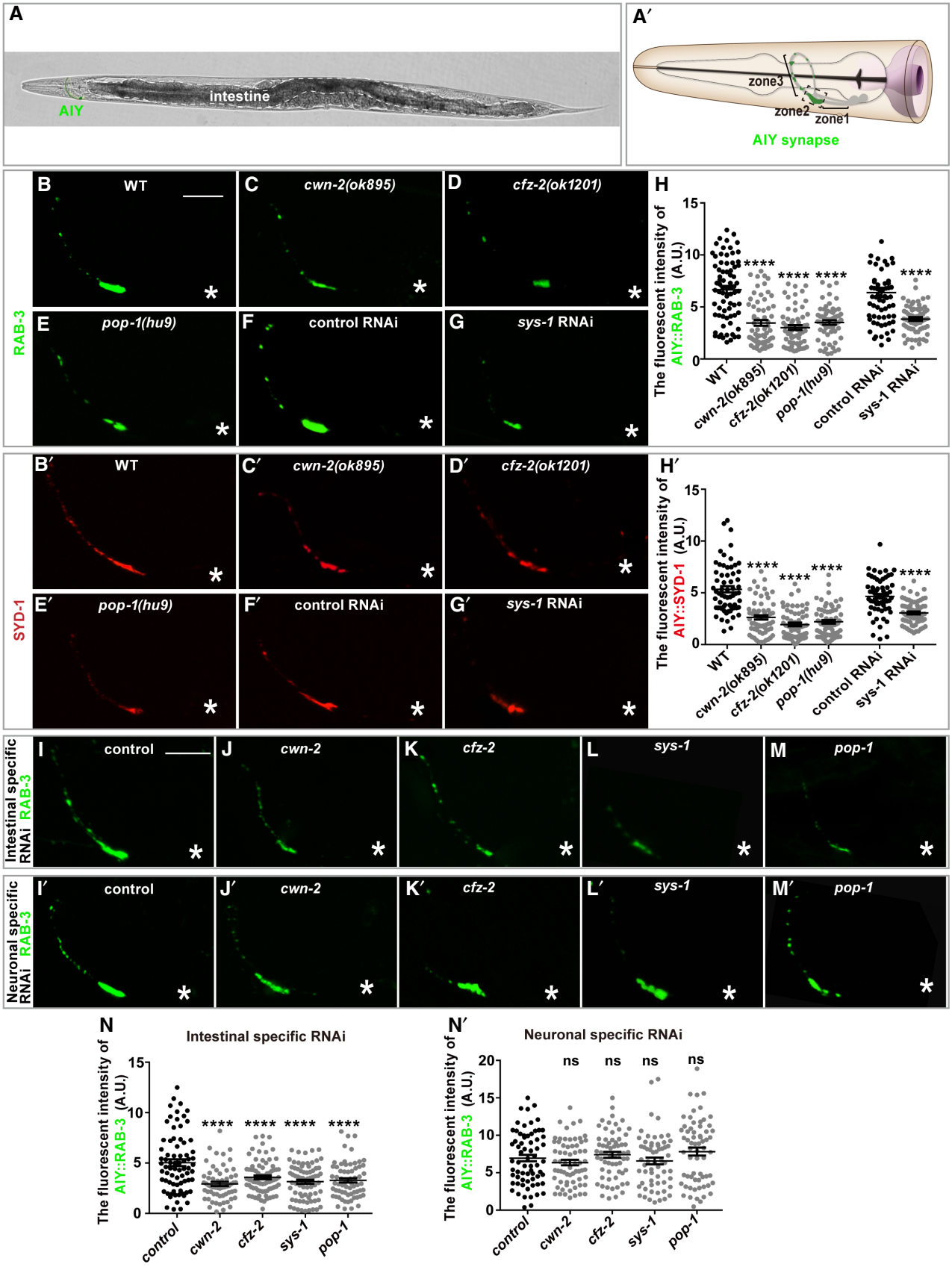
A
intestine
AIY
A′
zone3
zone2
zone1
AIY synapse
RAB-3
B WT
C cwn-2(ok895)
D cfz-2(ok1201)
E pop-1(hu9)
F control RNAi
G sys-1 RNAi
H
The fluorescent intensity of AIY::RAB-3 (A.U.)
WT
cwn-2(ok895)
cfz-2(ok1201)
pop-1(hu9)
control RNAi
sys-1 RNAi
SYD-1
B′ WT
C′ cwn-2(ok895)
D′ cfz-2(ok1201)
E′ pop-1(hu9)
F′ control RNAi
G′ sys-1 RNAi
H′
The fluorescent intensity of AIY::SYD-1 (A.U.)
Intestinal specific RNAi RAB-3
I control
J cwn-2
K cfz-2
L sys-1
M pop-1
Neuronal specific RNAi RAB-3
I′ control
J′ cwn-2
K′ cfz-2
L′ sys-1
M′ pop-1
N
Intestinal specific RNAi
The fluorescent intensity of AIY::RAB-3 (A.U.)
control
cwn-2
cfz-2
sys-1
pop-1
N′
Neuronal specific RNAi
ns

**Figure 1.**

that Wnt signaling is required in the intestine. It is interesting that neuronal-specific RNAi against Wnt components did not affect the synaptic phenotype even though it also knocked down the intestinal expression. One possible explanation is that the knockdown efficiency is not enough. To confirm the action site, we performed tissue-specific rescue experiments. Consistently, we found that *cfz-2 (ok1201)* mutants were only rescued by the intestinal-specific, but not by neuronal-specific, expression of *cfz-2* cDNA (Appendix Fig S3A–F), indicating that Wnt signaling in the intestine is sufficient for regulating AIY synaptic formation. Together, these data indicate that the canonical Wnt pathway acts in the intestine to regulate AIY presynaptic formation.

### Intestinal neuropeptide/NLP-40 is required for synaptogenesis

The intestine communicates with the brain *via* neuroendocrine signaling (Dimaline & Dockray, 1994; Campbell *et al*, 2004; Nassel *et al*, 2019). To test whether the Wnt signaling regulates AIY synaptogenesis through intestine-expressed neuropeptides, we first examined if EGL-3/KC2, a key proprotein convertase for neuropeptide synthesis (Li & Kim, 2008; Hung *et al*, 2014), was required for AIY synaptic formation. Indeed, the fluorescence intensity of AIY presynaptic markers was robustly reduced in *egl-3(n589)* mutants ($P < 0.0001$, Fig 2A). To ask whether *egl-3* acts in the intestine, we performed tissue-specific rescue experiments. We found that *egl-3* rescued the presynaptic defects when expressed either in the intestine or in the nervous system (Fig EV3A–E), suggesting that *egl-3* functions in both intestine and nervous system. Furthermore, we examined whether the intestine-specific proprotein convertase AEX-5/KC3 (Pauli *et al*, 2006; Li & Kim, 2008) is required for AIY presynaptic formation. We found similar presynaptic defects in *aex-5(sa23)* mutants ($P < 0.0001$, Fig 2A). These data suggest that intestinal neuropeptides are required for the AIY presynaptic formation.

There are 131 neuropeptide encoding genes identified in *C. elegans* genome (Nathoo *et al*, 2001; Van Bael *et al*, 2018). We screened intestine-expressed NLP and FLP family neuropeptides encoding genes through RNAi (Fig 2B) (Pauli *et al*, 2006; Li & Kim, 2008; Spencer *et al*, 2011; Wang *et al*, 2013), and found that knockdown of *nlp-40* resulted in a dramatic reduction of the synaptic marker. These data suggest that NLP-40 peptides are required for the AIY presynaptic formation (Fig 2C).

*nlp-40* encodes a neuropeptide precursor protein, which contains a N-terminal signal peptide sequence and four predicted mature peptides (Wang *et al*, 2013). To validate the *nlp-40* RNAi result, we examined two independently isolated *nlp-40* mutant alleles, *tm4085* and *vj3*. *nlp-40(tm4085)* harbors an in-frame deletion that deletes the neuropeptide P1, part of P2, and introduces a mutation from A70 to V in P3 and an early stop deleting the last three amino acid at the C-terminus of P4 (Fig 2D and Appendix Fig S4). *vj3* deletes the *nlp-40* promoter, the first exon and first intron (Fig 2D) (Wang *et al*, 2013). Similar to the RNAi results, we observed a robust reduction of the AIY synaptic vesicles and active zone markers in both *nlp-40* mutants (Fig 2E–G and J, Appendix Fig S5A–D, A'–C'). Furthermore, the AIY presynaptic defects in *nlp-40(tm4085)* and *nlp-40(vj3)* mutants were rescued by a wild-type *nlp-40* transgene (Fig 2H–J). Because the *vj3* allele also deletes part of a neighboring gene (Wang *et al*, 2013), all further analyses of *nlp-40* mutants were made with the *tm4085* allele.

To confirm the role of *nlp*-40 in synaptic formation, we examined additional presynaptic markers including YFP::SNB-1 and the endogenous RAB-3 GFP11 tag. We found a similar reduction of fluorescence intensity with both synaptic markers (Appendix Fig S6A, B, D, A', B′, and D′). To exclude the possibility that the reduction of AIY presynaptic marker is due to the effect of *nlp-40* on AIY morphology or the *ttx-3* promoter activity, we quantified the intensity of the transcription reporter P*ttx-3*::mCherry and found it was not affected in the mutants (Fig EV2A, D and F). These data collectively indicate that *nlp-40* is required for AIY synaptogenesis.

The above data so far suggest a model in which *nlp-40* functions in the intestine. To test this model, we built P*nlp-40*::NLP-40::mNeonGreen fusion reporter and found that *nlp-40* was highly expressed in the gut beginning at an early embryonic stage and observed NLP-40 peptides in the intestine and coelomocytes, which is consistent with the previous report (Appendix Fig S7A–D) (Wang *et al*, 2013). We then performed tissue-specific RNAi and observed a robust reduction of AIY presynaptic marker GFP::RAB-3 when *nlp-40* were knocked down in the intestine (GFP::RAB-3 expression was reduced by 54.48%. $P < 0.0001$; Fig 2K–M). Finally, we performed tissue-specific rescue and found the AIY presynaptic defects were rescued by the intestine-, but not neuron-specific expression of *nlp-40* (Fig EV4A–F). These data collectively support a model that intestinal expressed *nlp-40* is required for AIY synaptic formation. We posit that NLP-40 peptides secreted

**Figure 2. AIY presynaptic formation requires intestinal expressed *nlp-40*.**

A Quantification of the fluorescence intensity of AIY GFP::RAB-3 in wild-type, *egl-3(n589)* and *aex-5(sa23)* mutants.
B A Schematic diagram describes the RNAi screen strategy to identify intestinal neuropeptides required for AIY synaptogenesis.
C Quantification of the fluorescence intensity of AIY GFP::RAB-3 for the indicated RNAi treatment. Candidates are intestinal expressed *nlp* or *flp*.
D Diagram of *nlp-40* genomic structure. P1 to P4 indicates the predicted peptides encoded by *nlp-40*. The boxes and lines represent exons and introns. Black and gray indicate translating and untranslating regions. The lines beneath indicate the deletion region.
E–I Confocal images of AIY labeled with GFP::RAB-3 in wild-type (E), *nlp-40(tm4085)* (F), *nlp-40(vj3)* (G), *nlp-40(tm4085)* (H) or *nlp-40(vj3)* with a wild-type *nlp-40* transgene (I).
J Quantification of the fluorescence intensity of AIY GFP::RAB-3 for the indicated genotypes. Transgenic data are averaged from at least two independent lines.
K–L Confocal images of AIY synaptic marker GFP::RAB-3 for the indicated intestinal-specific RNAi treatment.
M Quantification for (K) and (L).

Data information: The scale bar in (E) and (K) are 10 μm and applies to (F–I) and (L), respectively. Asterisks indicate AIY soma. The AIY synaptic region was quantified for the synaptic intensity analysis. For (A, C, J, and M), each dot represents one animal. Data were collected from at least three independent experiments. Statistics are based on one-way ANOVA followed by Dunnett's test (C and J) or two-tailed Student's *t*-test (A and M). $^{**}P < 0.01$, $^{****}P < 0.0001$. Error bars represent SEM.
Source data are available online for this figure.

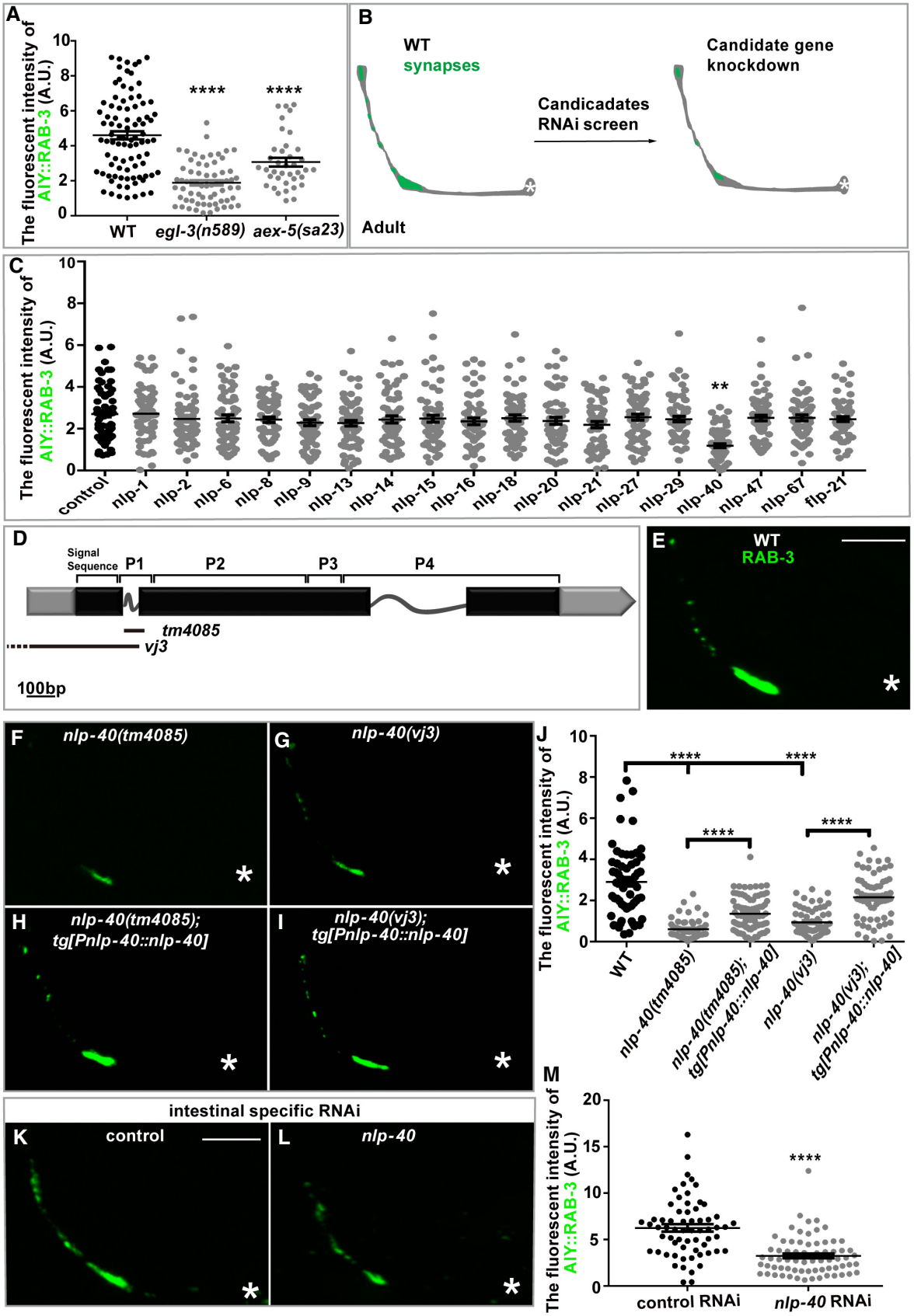
A
The fluorescent intensity of AIY::RAB-3 (A.U.)
WT
egl-3(n589)
aex-5(sa23)
****
B
WT synapses
Candicadates RNAi screen
Candidate gene knockdown
Adult
C
The fluorescent intensity of AIY::RAB-3 (A.U.)
control
nlp-1
nlp-2
nlp-6
nlp-8
nlp-9
nlp-13
nlp-14
nlp-15
nlp-16
nlp-18
nlp-20
nlp-21
nlp-27
nlp-29
nlp-40
nlp-47
nlp-67
flp-21
**
D
Signal Sequence
P1
P2
P3
P4
tm4085
vj3
100bp
E
WT RAB-3
F
nlp-40(tm4085)
G
nlp-40(vj3)
H
nlp-40(tm4085); tg[Pnlp-40::nlp-40]
I
nlp-40(vj3); tg[Pnlp-40::nlp-40]
J
The fluorescent intensity of AIY::RAB-3 (A.U.)
WT
nlp-40(tm4085)
nlp-40(tm4085); tg[Pnlp-40::nlp-40]
nlp-40(vj3)
nlp-40(vj3); tg[Pnlp-40::nlp-40]
intestinal specific RNAi
K
control
L
nlp-40
M
The fluorescent intensity of AIY::RAB-3 (A.U.)
control RNAi
nlp-40 RNAi

**Figure 2.**

from the gut likely travel to the nervous system and promote AIY presynaptic assembly.

To address when *nlp-40* is required for the AIY presynaptic formation, we quantified the fluorescence intensity of AIY GFP::RAB-3 in *nlp-40(tm4085)* mutants at the larval L1, L4 and adult Day 1 stage. We found that the fluorescent signal was dramatically reduced in *nlp-40(tm4085)* mutants from newly hatched L1 stage throughout the adult stage (Appendix Fig S7E–G, E'–G', and H). These data suggest that intestinal *nlp-40* acts prior to hatching and is required for AIY presynaptic formation during embryonic development.

### *nlp-40* acts downstream of the Wnt signaling pathway to promote AIY presynaptic formation

Thus far, we demonstrated that AIY presynaptic assembly requires both Wnt and NLP-40 neuropeptide signaling from the gut. To determine whether Wnt signaling and neuropeptides act in the same pathway, we examined the AIY presynaptic phenotype in three sets of double mutants: *cwn-2(ok895);nlp-40(tm4085)*, *cfz-2(ok1201);nlp-40(tm4085)*, and *pop-1(hu9);nlp-40(tm4085)* mutants. We found that the fluorescence intensity of the AIY GFP::RAB-3 in all double mutants was similar to that in *nlp-40(tm4085)* single mutants, but slightly weaker than in Wnt signaling single mutants (Fig 3A). Furthermore, we also found that the reduction of AIY presynaptic reporters in *cwn-2(ok895)* or *cfz-2(ok1201)* mutants was partially restored by expressing a wild-type *nlp-40* transgene (Fig 3B). However, expressing *nlp-40* transgene in wild-type animals did not increase the AIY presynaptic marker (Fig 3B), indicating that the rescue of AIY presynaptic marker is due to restoring *nlp-40* expression in Wnt mutants, not *nlp-40* overexpression. Considering that *nlp-40* could be regulated by Wnt signaling pathway (as discussed below), we tried to restore the AIY synaptic defects by expressing *nlp-40* with intestinal-specific, Wnt-independent promoter *vha-6*. While the suppression efficiency was improved as compared with *nlp-40* promoter, it was not fully restored to the wild-type levels (Fig 3B), suggesting that Wnt signaling may also acts through an *nlp-40*-independent pathway. These data are consistent with a model, in which *nlp-40* acts downstream of the Wnt signaling pathway to regulate AIY presynaptic assembly.

A simple model to explain the genetic interaction between *nlp-40* and Wnt signaling is that Wnt signaling regulates *nlp-40* expression. To test this, we first quantified the *nlp-40* mRNA levels by quantitative reverse transcription PCR, and found that the mRNA levels were significantly reduced in *cwn-2(ok895)*, *cfz-2(ok1201)* or *pop-1(hu9)* mutants compared to that in wild-type animals (Fig 3C). Second, we examined the *nlp-40* transcriptional reporter P*nlp-40::GFP* and observed a significant reduction in Wnt signaling mutants or knockdown animals (Fig 3D–J). Lastly, to exclude the possibility that Wnt signaling affects the overall expression of intestinal genes in general, we examined the expression of intestinal-specific *erm-1* and *vha-6* reporters (Oka *et al*, 2001; Gobel *et al*, 2004). We found that the expression of both reporters was not affected by mutations or RNAi knockdown of the same set of Wnt components (Fig 3K–Q and Appendix Fig S8A–F). Together, our data demonstrate that the Wnt signaling pathway promotes AIY presynaptic assembly by upregulating *nlp-40* in the intestine.

### GPCR/AEX-2 acts in the nervous system as the NLP-40 receptor to regulate synaptic formation

AEX-2 serves as the NLP-40 peptides receptor that regulates rhythmic behavior and anoxic survival (Wang *et al*, 2013; Doshi *et al*, 2019). To test whether AEX-2 also acts as the NLP-40 peptides receptor to regulate AIY presynaptic formation, we examined the fluorescence intensity of AIY GFP::RAB-3 in *aex-2(sa3)* mutants (Fig 4A), and found a robust GFP::RAB-3 reduction in the mutants (76.84% reduction as compared to that in wild-type animals, $P < 0.0001$; Fig 4B, D and L). The reduction of the AIY GFP::RAB-3 in *aex-2(sa3)* mutants was rescued by a wild-type *aex-2* transgene (Fig 4G and M). We also observed similar reduction in *aex-2* mutants with YFP::SNB-1 and endogenous tagged RAB-3 (Appendix Fig S6A, C, D, A', C′, and D′). In addition, we noticed that *aex-2* did not affect the expression of P*ttx-3::*mCherry (Fig EV2A, E, and F), indicating that the reduction of fluorescence intensity is specific to synaptic markers. The data suggest that *aex-2* is required for AIY presynaptic formation.

If AEX-2 functions as the NLP-40 receptor, we would expect that they act in the same genetic pathway. To test this model, we built *nlp-40(tm4085);aex-2(sa3)* double mutants and found that AIY GFP::RAB-3 intensity in the double mutants was similar to that in either single mutants (Fig 4B–E and L), indicating that they indeed act in the same pathway. In addition, expressing NLP-40 in *aex-2(sa3)* did not suppress the AIY synaptic defect (Fig 4F and L). These data are consistent with the model in which AEX-2 acts as the receptor of NLP-40 peptides to regulate the AIY presynaptic formation.

We next sought to determine *aex-2* action site. First, we built an *aex-2* transcriptional reporter P*aex-2::mNeonGreen*, and found that

**Figure 3. Wnt signaling regulates AIY presynaptic formation by promoting *nlp-40* expression.**

A–C Quantification of the fluorescence intensity of AIY presynaptic marker GFP::RAB-3 (A, B) and *nlp-40* mRNA level (C) for the indicated genotypes. The AIY synaptic region was quantified for the synaptic intensity analysis.

D–I Confocal images of the transcriptional *nlp-40* reporter (*Pnlp-40::GFP*) in wild-type (D), *cwn-2(ok895)* (E), *cfz-2(ok1201)* (F), *pop-1(hu9)* (G), and control or *sys-1* RNAi (H and I) animals at adult Day 1 stage.

J Quantification of the P*nlp-40::GFP* fluorescence intensity for the indicated genotypes.

K–P Confocal images of an intestinal-specific reporter ERM-1::GFP in wild-type (K), *cwn-2(ok895)* (L), *cfz-2(ok1201)* (M), *pop-1(hu9)* (N), and control or *sys-1* RNAi (O and P) animals at the adult Day1 stage.

Q Quantification of the ERM-1::GFP fluorescence intensity for the indicated genotype.

Data information: The scale bar in (D) is 50 μm and applies to (E–I, K–P). For (A, B, C, J, and Q),each dot represents one animal. Data were collected from at least three independent experiments. Statistical analyses are based on one-way ANOVA followed by Dunnett's test (for mutants) and two-tailed Student's *t*-test (for RNAi).
**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns: not significant as compared to WT or control RNAi ($P > 0.05$). Error bars represent SEM.
Source data are available online for this figure.

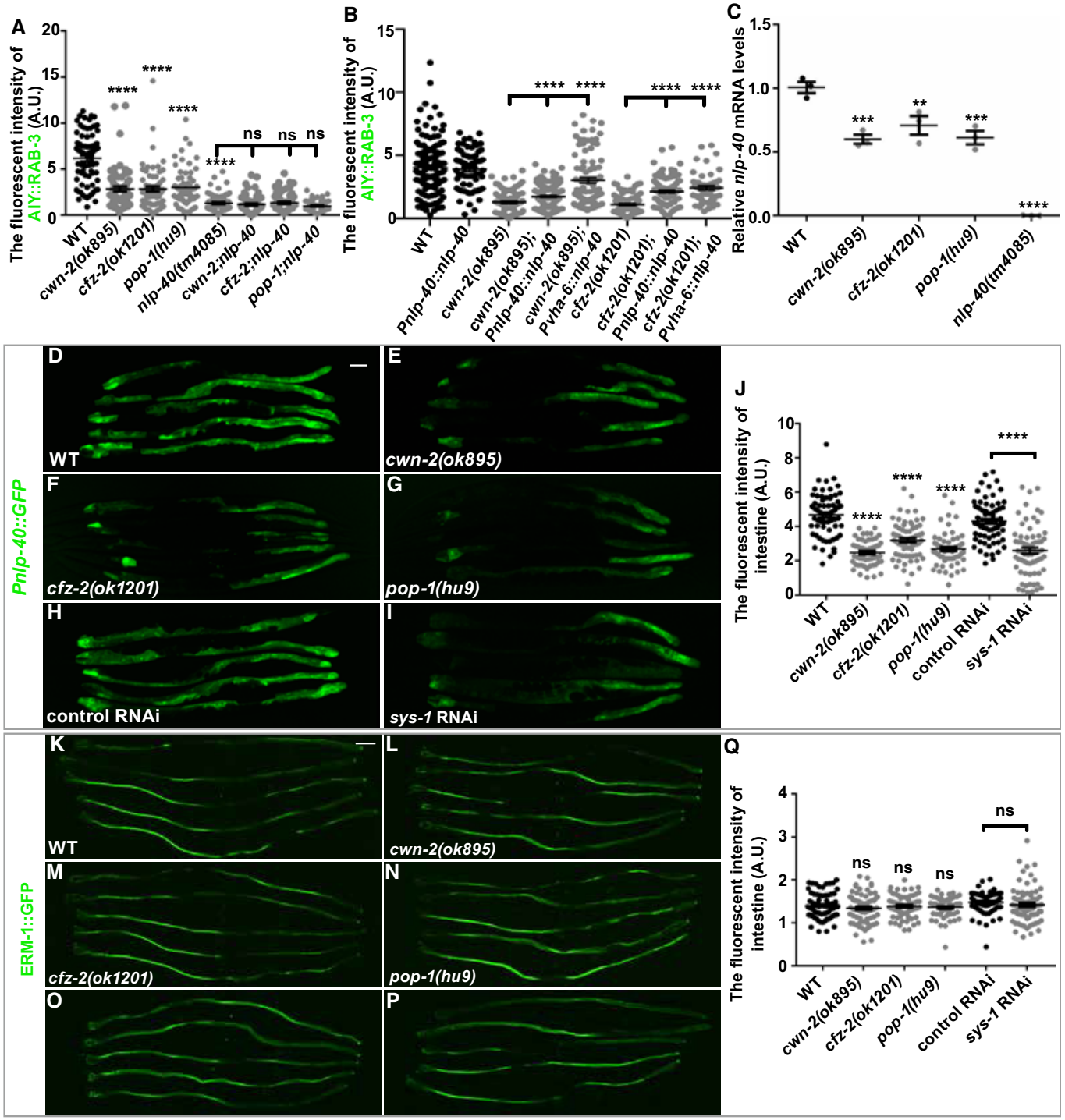


**Figure 3.**

*aex-2* was mainly expressed in the nervous system with some in the posterior gut (Fig 4N), which is consistent with the previous report (Wang *et al*, 2013). Second, we co-expressed P*aex-2*::mNeonGreen with the AIY-specific marker P*ttx-3*::mCherry, and found that the expression of those two reporters overlapped (Fig 4O–Q'), indicating that *aex-2* is expressed in AIY interneurons. Lastly, we performed tissue-specific *aex-2* rescue experiments and found that the AIY presynaptic defect was rescued only when *aex-2* was expressed in the nervous system or AIY, but not in the intestine or body-wall muscle (Fig 4G–K and M). We also noticed that the rescue was better with the pan-neuronal promoter than with AIY-specific ones (Fig 4M), indicating that *aex-2* functions both in AIY and other neurons. These data indicate that *aex-2* acts in the AIY and other neurons to regulate presynaptic formation.

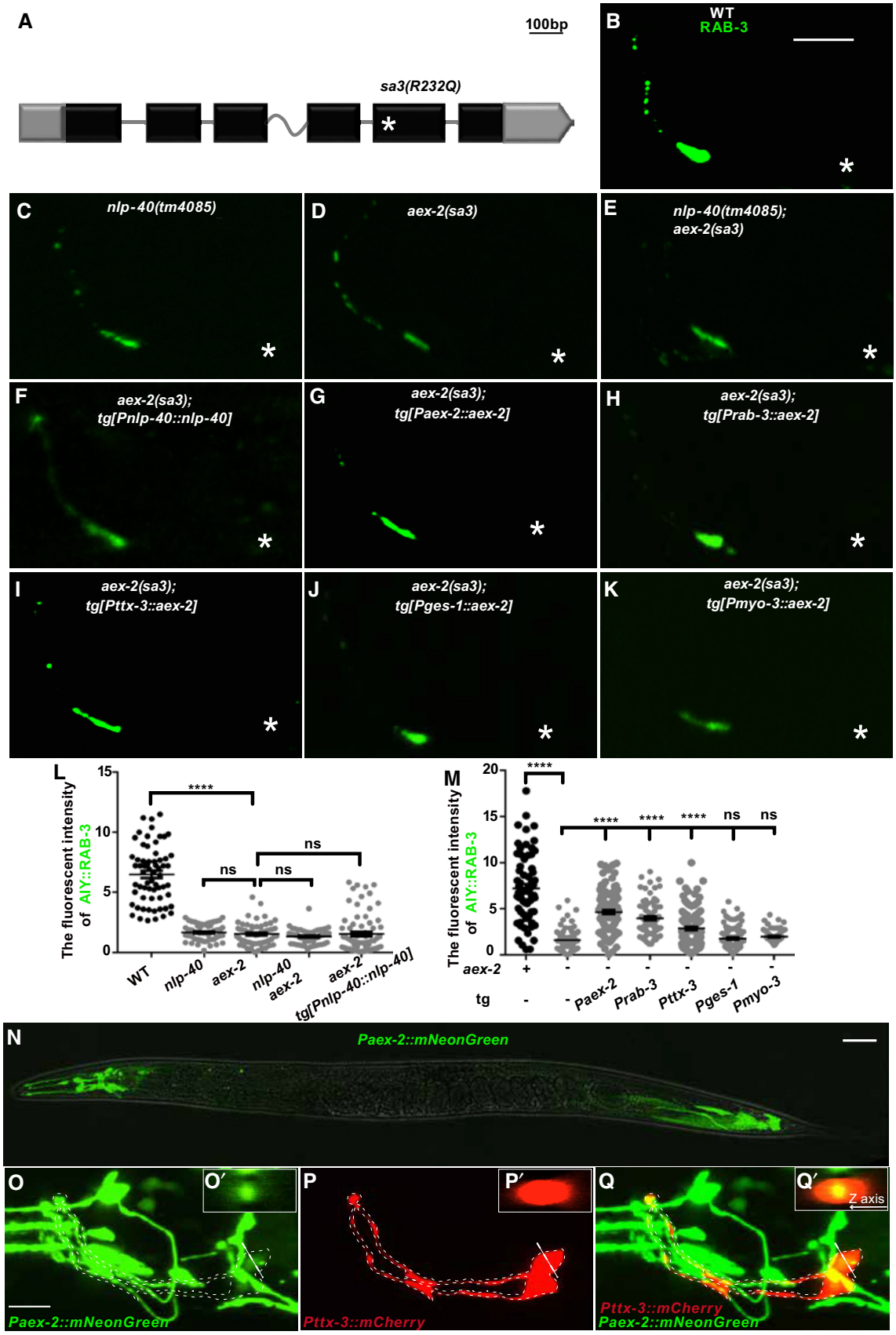


**Figure 4.**

**Figure 4. Neuropeptide/NLP-40 receptor GPCR/AEX-2 is required for AIY presynaptic formation.**

A Diagram of *aex-2* genomic structure. The boxes and lines represent exons and introns. Black and gray indicate translating and untranslating regions. The white asterisk marks the location of the R232Q mutation site of *sa3* allele.

B–K Confocal images of AIY presynaptic site labeled with GFP::RAB-3 in wild-type(B), *nlp-40(tm4085)*(C), *aex-2(sa3)*(D), *nlp-40(tm4085); aex-2(sa3)* (E) overexpression NLP-40 in *aex-2(sa3)* mutants(F) and *aex-2(sa3)* with a wild-type *aex-2* transgene driven by the endogenous promoter (*Paex-2*) (G), neuron-specific promoter (*Prab-3*) (H), AIY-specific promoter (*Pttx-3*) (I) intestinal-specific promoter (*Pges-1*) (J) or muscle-specific promoter (*Pmyo-3*)(K).

L–M Quantification of the fluorescence intensity of AIY GFP::RAB-3 for the indicated genotypes. Each dot represents one animal. Data were collected from at least three independent experiments. Transgenic data are averaged from at least two independent lines. tg: *Paex-2::aex-2*, *Pttx-3::aex-2* or *Pges-1::aex-2* transgene, +: wild-type; —: mutant or without tg. The AIY synaptic region was quantified for the synaptic intensity analysis.

N–Q' Confocal images of *Paex-2*::mNeonGreen of whole animal with bright field (N), the head region labeled with *Paex-2*::mNeonGreen and *Pttx-3*::mCherry with green channel (O), red channel (P) and the merged channels (Q). (O''–Q') are the cross sections corresponding to the dashed line sites.

Data information: The scale bar in (B) and (O) are 10 μm, and applies to (C–K) and (P–Q'), respectively; the scale bar in (N) is 50 μm. Asterisks indicate AIY soma. Statistical analyses are based on one-way ANOVA followed by Dunnett's test. ns: not significance ($P > 0.05$), ****$P < 0.0001$. Error bars represent SEM.
Source data are available online for this figure.

*nlp-40* and *aex-2* regulate enteric muscle activity, and the corresponding mutants show defects in alimentation (Wang *et al*, 2013). To address whether mutants with alimentation defects also affect AIY synaptic formation, we examined *egl-19* and *eat-2*, which regulate rhythmic defecation behavior and feeding behavior (Wang & Sieburth, 2013; Kumar *et al*, 2019). We found that the fluorescence intensity of AIY synaptic marker was not affected in either *egl-19* or *eat-2* mutants (Appendix Fig S9). These data suggest that the reduction of AIY synapses is not generally affected by alimentation defects.

### Wnt-endocrine signaling regulates AIY activity

Neuropeptides can modulate ion channel and therefore neuronal activity (Matsushita & Arikawa, 1997; Rogers *et al*, 2001; Davis & Ghosh, 2007). NLP-40 peptides activate GABAergic neurons (Wang *et al*, 2013; Oliva & Inestrosa, 2015). To determine whether Wnt/*nlp-40/aex-2* regulates AIY activity, we recorded AIY calcium signaling using GCaMP6, a genetically encoded calcium indicator (Fig 5A and A') (Luo *et al*, 2014). In wild-type animals, AIY spontaneously fires about six times per minute (Wang *et al*, 2021). In *cfz-2(ok1201)*, *nlp-40 (tm4085)* or *aex-2(sa3)* mutants, although the firing amplitude was not affected (Fig 5B and D), the frequency was significantly reduced (Fig 5B and C), and the activity defects were rescued by expressing the corresponding wild-type transgene (Fig 5B and C). These data collectively suggest that Wnt-NLP-40 signaling pathway promotes AIY synaptic assembly most likely by modulating the neuronal activity.

### Wnt-NLP-40 signaling pathway is required for the presynaptic formation in the nerve ring

So far, we have demonstrated that Wnt-NLP-40 signaling in the intestine is required for AIY presynaptic assembly. To determine whether this regulation is specific for AIY neurons or more general, we first quantified pan-neuronal synaptic GFP::RAB-3 (driven by pan-neuronal *rab-3* promoter) in the nerve ring region in Wnt signaling mutants. The GFP::RAB-3 was significantly reduced in *cwn-2(ok895)*, *cfz-2(ok1201)*, or *pop-1(hu9)* mutants (the intensity was reduced by 33.98, 45.59, and 52.64%, respectively, $P < 0.0001$ as compared to that in wild-type animals, respectively; Fig 6A–E and H). Then, we performed rescue experiments and found that the reduction of the GFP::RAB-3 in *cwn-2(ok895)* was rescued by a wild-type *cwn-2* transgene (Fig 6I). Lastly, to exclude the possibility that the GFP reduction is due to the effects of Wnt on *rab-3* promoter, we quantified the expression of *rab-3* by quantitative reverse transcriptional PCR and found that the *rab-3* mRNA levels were not affected by Wnt mutants (Fig EV5G). Furthermore, we quantified P*rab-3::*mCherry intensity and found the expression level was not affected by Wnt signaling either (Fig EV5A–D, H). These data collectively indicate that the Wnt signaling promotes the presynaptic formation in the nerve ring.

Next, we examined the presynaptic marker in *nlp-40* and *aex-2* mutants. We found that the GFP::RAB-3 intensity was dramatically reduced in either *nlp-40(tm4085)* or *aex-2(sa3)* mutants (the intensity was reduced by 44.89 and 49.65%, respectively, $P < 0.0001$ compared to WT; Fig 6F–H). The reduction of the presynaptic marker was rescued by the corresponding wild-type transgene (Fig 6I). We further showed that both the levels of *rab-3* mRNA and the *Prab-3::*mCherry were not affected by either *nlp*-40 or *aex*-2 mutations (Fig EV5A, E–H), which excludes the possibility that the P*rab-3::*GFP::RAB-3 reduction is due to the effects of *nlp*-40 or *aex*-2 on *rab-3* promoter. The results collectively indicate that *nlp-40* and *aex-2* are required for presynaptic formation in nerve ring.

## Discussion

We have demonstrated that a canonical Wnt signaling pathway that includes Wnt/CWN-2, Frizzled/CFZ-2, β-catenin/SYS-1, and TCF/POP-1 acts in the gut to promote synaptic assembly in the *C. elegans* nerve ring. Wnt signaling exerts this effect by upregulating the expression of *nlp-40*, which encodes a neuropeptide precursor. NLP-40 neuropeptides promote synaptic assembly through the neuronally expressed GPCR receptor AEX-2 (Fig 7). This gut Wnt-neuroendocrine signaling promotes synaptic formation likely through modulating neuronal activity. We therefore uncovered a previously unidentified molecular mechanism by which gut regulates synaptic formation in the nerve ring. These results highlight the genetic role of gut in the neurodevelopment, and reveal a novel function of gut–brain axis.

### Wnt-Neuropeptide axis regulates synaptic formation

The genes encoding Wnt signaling pathway components and their roles in synaptogenesis are highly conserved from *C. elegans* to mammals (Park & Shen, 2012; He *et al*, 2018). However, the underlying mechanisms are not completely understood. In this study, we

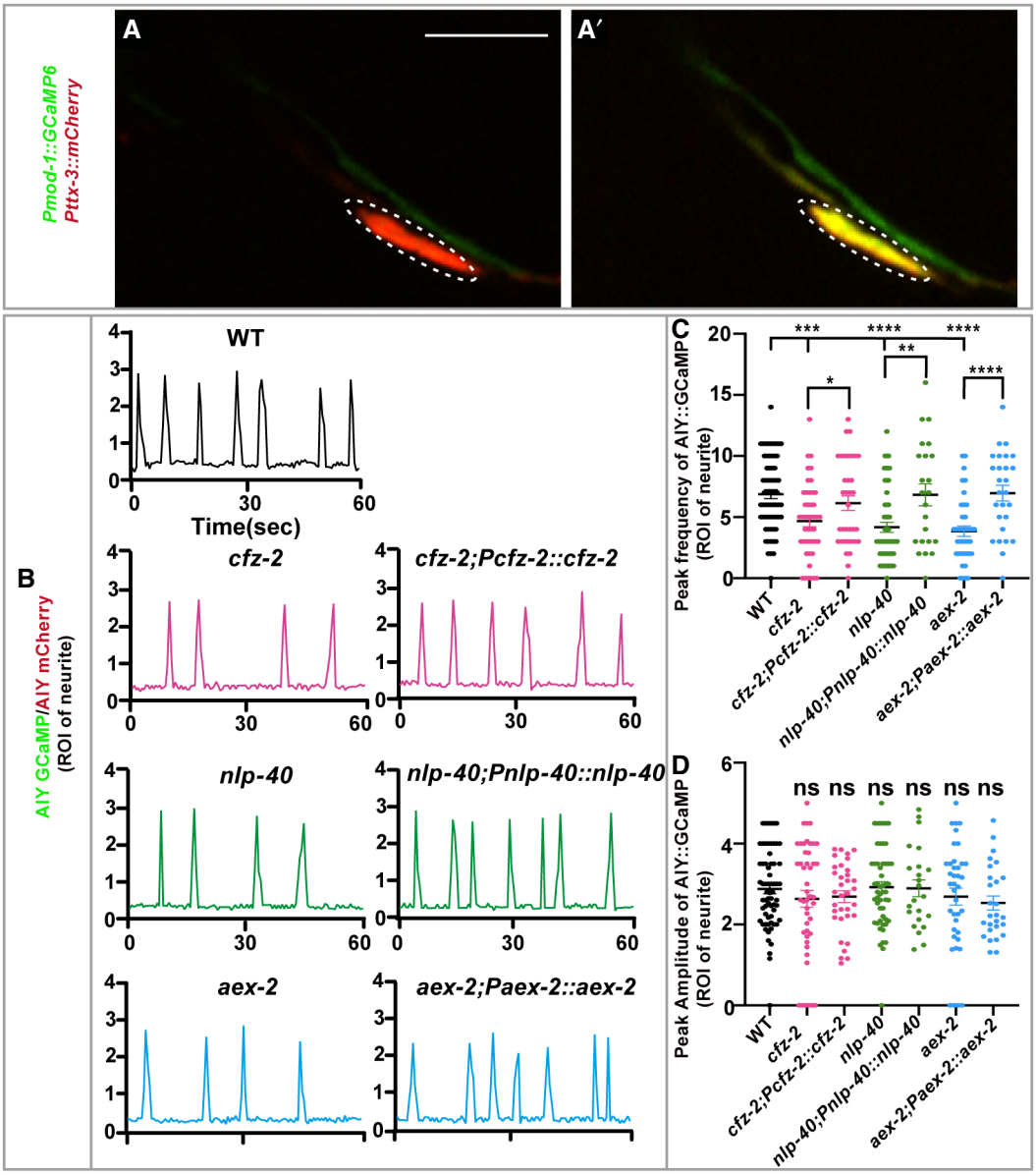


**Figure 5. Wnt/NLP-40 signaling pathway modulates AIY activity.**

A–A' Representative confocal images showing inactive (A) and active(A') state of AIY::GCaMP6s (with *mod-1* promoter) colabeled with mCherry (with *ttx-3* promoter) (red). Dashed ovals mark the AIY zone 2 region where GCaMP was quantified.

B Representative AIY calcium activity over 60 s for the indicated genotypes.

C, D Quantification of the peak frequency and amplitude of AIY::GCaMP6s in for the indicated genotypes.

Data information: The scale bar in (A) is 10 μm and applies to (A'). Each dot represents one animal. Data were collected from at least three independent experiments. Transgenic data were collected from at least two independent lines. Statistical analyses are based on one-way ANOVA followed by Dunnett's test. ns: not significance ($P > 0.05$); $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Error bars represent SEM.

Source data are available online for this figure.

demonstrated that intestinal Wnt signaling promotes synaptic formation in the nerve ring. This Wnt signaling promotes synaptic assembly by upregulating expression of the intestinal secreted NLP-40 peptides, which act through the neuronal expressed GPCR/AEX-2 receptor.

Wnt ligands are expressed either in the nervous system or in non-neuronal tissues to regulate synaptic assembly or plasticity. However, the receptors or downstream components have only been shown to act in neurons or the postsynaptic target cells. For example, the Wnt ligands, LIN-44 and EGL-20, expressed in the tail suppress presynaptic assembly through the Frizzled receptor LIN-17 or MIG-1 in DA8, DA9, and DB7 neurons (Klassen & Shen, 2007; Mizumoto & Shen, 2013). Presynaptic CWN-2 and LIN-44 regulate activity-dependent postsynaptic localization of the acetylcholine

 

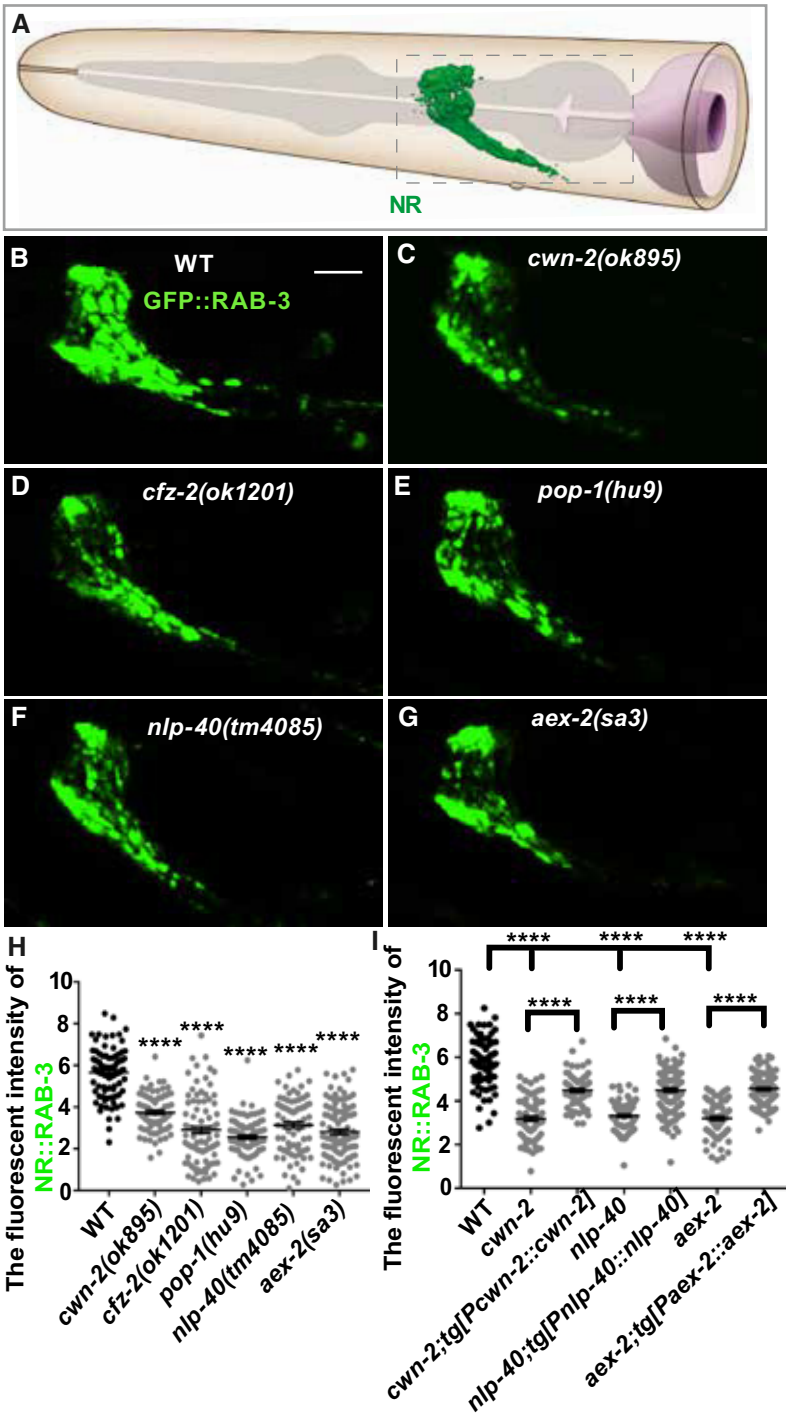


**Figure 6. Synaptic formation in the nerve ring requires Wnt-NLP-40 neuropeptide signaling.**

A A cartoon diagram of the *C. elegans* head modified from wormatlas with permission (Altun & Hall, 2021). Green marks the presynaptic region in the nerve ring.

B–G Confocal images of *Prab-3*::GFP::RAB-3 in the nerve region corresponding to the dashed box in (A) of wild-type (B), *cwn-2(ok895)* (C), *cfz-2(ok1201)* (D), *pop-1(hu9)* (E), *nlp-40(tm4085)* (F) and *aex-2(sa3)* (G) mutants.

H–I Quantification of the GFP::RAB-3 intensity in the nerve ring synaptic region for the indicated genotypes. Each dot represents one animal. Data were collected from at least three independent experiments. Transgenic data are averaged from at least two independent lines.

Data information: The scale bar in (B) is 10 μm and applies to (C–G). Statistical analyses are based on one-way ANOVA followed by Dunnett's test (H and I) and two-tailed Student's *t*-test (*cwn-2* vs. *cwn-2;tg*, *nlp-40* vs. *nlp-40;tg*, *aex-2* vs. *aex-2;tg*). ****$P < 0.0001$. Error bars represent SEM.
Source data are available online for this figure.

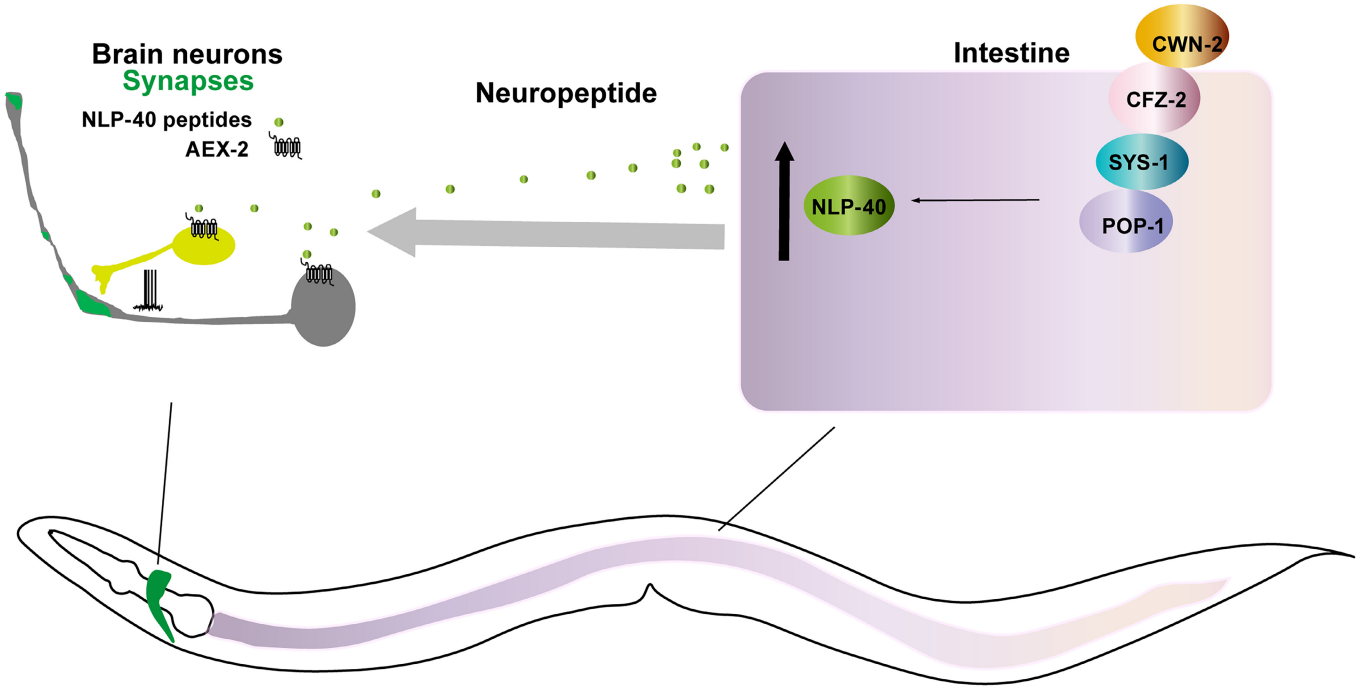


**Figure 7. A model describing synaptic assembly regulated by a Wnt-neuropeptide signaling from gut.**

A canonical Wnt signaling pathway including Wnt/CWN-2, Frizzled/CFZ-2, β-catenin/SYS-1 and TCF/POP-1 acts in the gut to promote the synaptic assembly in the *C. elegans* brain. The Wnt signaling does so by regulating *nlp-40*, which encodes neuropeptides. NLP-40 peptides secreted from gut promote the synaptic assemble by activating the neuronal activity through the neuronal expressed receptor GPCR/AEX-2.

receptor ACR-16 mediated by LIN-17 and CAM-1 receptors in the postsynaptic muscle cells (Jensen *et al*, 2012; Pandey *et al*, 2017). Through the β-catenin/BAR-1 and TCF/LEF/POP-1 transcription factor, unidentified Wnt signaling regulates the abundance of the postsynaptic glutamate receptor GLR-1 (Dreier *et al*, 2005). Here, we identified that a canonical Wnt signaling, including the ligand CWN-2, Frizzled receptor CFZ-2, β-catenin/SYS-1, and TCF/POP-1, in the gut promotes synaptic assembly in the *C. elegans* brain.

NLP-40 belongs to the neuropeptide-like proteins (NLPs) family and is required for rhythmic defecation, anoxia survival, and axon regeneration (Husson *et al*, 2005; Wang *et al*, 2013; Doshi *et al*, 2019; Lin-Moore *et al*, 2021). Neuropeptides are small bioactive signal peptides including insulin-like peptides (INS), FMRFamide-related peptides (FLPs), and neuropeptide-like proteins (NLPs) (Li & Kim, 2008). Those peptides are expressed both in the nervous system and in the non-neuronal tissues with a broad spectrum of biological functions (Li & Kim, 2008; Hu *et al*, 2011; Hu *et al*, 2015; Chew *et al*, 2018; De Fruyt *et al*, 2020; Chai *et al*, 2021; Ramachandran *et al*, 2021; Sun & Hobert, 2021). For example, INSs regulate aversive learning behavior (Lee & Mylonakis, 2017) and feeding state-dependent gene expression in ADL neurons (Gruner *et al*, 2016). FLPs modulate the locomotion and body wave and head search behaviors (Reinitz *et al*, 2000; Li, 2005; Chikka *et al*, 2016). Similarly, NLPs regulate locomotion, body posture, and rhythmic behaviors (Couillault *et al*, 2004).

The role of Wnt in synaptic development is highly conserved in vertebrates (Budnik & Salinas, 2011; Park & Shen, 2012; He *et al*, 2018). For example, Wnt7a and Wnt5a play critical roles in pre- or postsynaptic development in mammals (He *et al*, 2018). Although the role of Wnt-neuropeptide in synaptic development was previously unknown, Wnts can regulate the expression of gut hormone/neuropeptides (Garcia-Martinez *et al*, 2009; Garcia-Jimenez, 2010; Kim *et al*, 2015). This suggests that Wnt-neuropeptide interactions are evolutionally conserved. In addition, hormones/neuropeptides are also involved in synaptic assembly and function in mammals (D'Ercole *et al*, 2002; Garcia *et al*, 2014; Corvino *et al*, 2015; Wang *et al*, 2015). Collectively, these studies suggest that the function of Wnt-neuropeptide axis in synaptic formation is most likely conserved in vertebrates.

### A genetic role of gut in synaptic development

Through tissue-specific RNAi or tissue-specific rescue experiments, we demonstrated that the Wnt-NLP-40 axis acts in the gut to regulate presynaptic assembly of nerve ring neurons. Furthermore, we showed that the effect of the Wnt-NLP-40 axis on synaptic formation starts at the newly hatched L1 stage, suggesting this pathway acts during embryogenesis before feeding. We propose that the Wnt-NLP-40 axis acts as an intrinsic genetically encoded program.

Gut–brain communication plays an important role in coping with unfavorite environmental conditions. For example, under stress conditions, intestinal signaling regulates locomotion by modulating synaptic transmission at the neuromuscular junctions (D'Ercole *et al*, 2002; Staab *et al*, 2013; Zheng *et al*, 2021). Intestinal infections can affect the host's learning and behavior by neuroendocrine

 

signaling pathways (Singh & Aballay, 2019). Intestinal immune responses can also protect neurons from degeneration (Chikka *et al*, 2016). However, the genetic role of gut in the brain development is largely unexplored. Our finding provides a molecular mechanism underlying this novel gut–brain interaction.

In vertebrates, the gut–brain–microbiota axis plays a vital role in mediating brain development and brain function, such as locomotion, exploratory and risk-taking behaviors and spatial memory (Bercik *et al*, 2011; Diaz Heijtz *et al*, 2011; Gareau *et al*, 2011; Neufeld *et al*, 2011). Endocrine is a highly conserved signaling pathway mediated the gut–brain communication (Matafome *et al*, 2017; Farzi *et al*, 2018; Sun *et al*, 2018). Although it is unknown if gut–brain axis plays any intrinsic role in neural circuit formation in vertebrates, we posit that the signaling pathway regulating synaptic formation is probably conserved given the conservation of the gut–brain axis, Wnt signaling, and neuroendocrine signaling.

#### Neural activity regulates synaptic formation

The invertebrate neural circuits were initially thought to be genetically hardwired (Jin *et al*, 1999; Gally & Bessereau, 2003; Hiesinger *et al*, 2006; Klassen & Shen, 2007), although neural activity can regulate the remodeling or plasticity (Zhao & Nonet, 2000; Sachse *et al*, 2007; Tessier & Broadie, 2009; Thompson-Peer *et al*, 2012; Hart & Hobert, 2018; Cuentas-Condori *et al*, 2019). However, recent studies have shown that neuronal activity during embryogenesis regulates neuronal differentiation (Horowitz *et al*, 2019), presynaptic subcellular specificity (Wang *et al*, 2021) in *C. elegans*, indicating the important role of experience-independent activity in circuit development. In vertebrates, it is well known that neural activity regulates synaptic formation and plasticity (Hooks & Chen, 2020; Pan & Monje, 2020). During embryogenesis, neurons fire spontaneously before receiving any stimulation. Spontaneous activity is required for circuit formation in the visual system (Galli & Maffei, 1988; Shatz & Stryker, 1988; Sretavan *et al*, 1988; Herrmann & Shatz, 1995), auditory system (Moore & Kitzes, 1985; Kitzes *et al*, 1995), olfactory system (Yu *et al*, 2004), hippocampus (Ben-Ari *et al*, 1989), cerebellum (Watt *et al*, 2009), and somatosensory cortex (Anton-Bolanos *et al*, 2019). However, the underlying mechanisms are largely unknown.

Neuronal activity can regulate the trafficking of synaptic components (Tiruchinapalli *et al*, 2003; Sears & Broadie, 2017; Lorenz-Guertin & Jacob, 2018). In this study, we showed that Wnt-neuroendocrine signaling pathway is required for AIY activity and synaptic formation. Together, we could propose a model in which Wnt-neuroendocrine signaling in the gut regulates synaptic formation through neuronal activity-dependent synaptic component trafficking. This model not only provides mechanistic insights into the synaptic formation during development, but also reveals a novel gut–brain interaction.

## Materials and Methods

#### Strains and maintenance

All *C. elegans* strains were grown with *E. coli* OP50 on standard NGM plates at 21°C (Brenner, 1974). A detailed information of all the strains is listed in Table EV1. Animals were used in this study at adult Day 1 stage unless specified.

#### Plasmids and transformation

Constructs were made either with pSM (derivation of pPD49.26) (Mello & Fire, 1995; Shen & Bargmann, 2003) or with L4440 vector (derivation of pPD129.36) (Kamath & Ahringer, 2003). Detailed information is described in Table EV2.

Transgenic strains were generated by microinjections as previously described (Mello & Fire, 1995). We used P*hlh-17::mCherry* (20 ng/μl), P*unc-122::*GFP (20 ng/μl), *Prab-3::*mCherry (20 ng/μl) or P*ttx-3::sl2::*mCherry (20 ng/μl) as co-injection markers. Detailed information is described in Table EV1.

#### RT and qRT-PCR

qRT-PCR was performed as the previous study (Shen *et al*, 2012). In brief, for mRNA, total RNA was prepared by traditional Trizol extraction methods from ~ 100 worms. cDNA was subsequently generated by GoScript™ Reverse Transcription system for RT-qPCR (Bio-Rad). qRT-PCR was performed with 2xNovoStart®SYBR qPCR SuperMix Plus (Novoprotein) on a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Three technical replicates were performed in each reaction. The housekeeping gene actin (*act-1*) was used as internal control in the qRT-PCR experiments and *nlp-40* mutants were used as references for mRNA quantification, the results were from at least three biological replicates. qRT-PCR primers for *nlp-40* were designed and was listed in Table EV2.

#### Endogenous synaptic GFP reconstruction

The split GFP system was conducted as previous study (Kamiyama *et al*, 2016). To visualize the endogenous synaptic proteins, we built HA::GFP11$_{x7}$ knock-in strain PHX2157(*rab-3(syb2157)*, which was conducted by SunyBiotech (http://www.sunybiotech.com), then expressed cytoplasmic GFP1-10 in a transgene. Specifically, to label RAB-3 in AIY interneurons, a codon-optimized GFP1-10 was driven by *ttx-3 g* promoter in a transgene. The integrated array *shcIs54 [Pttx-3::sl2::GFP1-10; Pttx-3::mCherry]* was used in this study.

#### RNA interference

RNAi constructs were transformed into HT115. RNAi feeding experiments were performed based on the previous study(Fraser *et al*, 2000). For tissue-specific RNAi experiment, we used the worm strain FDU2851[*sid-1(qt9)*, *alxIs9(Pvha-6::sid-1::sl2::GFP)*, *olaIs12 (Pttx-3::mCherry::rab-3*, *Phlh-17::GFP)*] and FDU3111[*sid-1(pk3321)*, *uIs69(Punc-119::sid-1*, *Pmyo-2::mCherry); wyIs45(Pttx-3::GFP::rab-3*, *Punc-122::RFP)*] for gut and neuronal-specific RNAi, respectively. To test the efficiency of tissue-specific RNAi, we used the worm strain FDU4186[*sid-1(qt9)*, *alxIs9(Pvha-6::sid-1::sl2::GFP)*, *jsIs682 (Prab-3::GFP::rab-3)]* and FDU4288[*sid-1(pk3321)*, *auIs69 (Punc-119::sid-1; Pmyo-2::mCherry)*, *jsIs682(Prab-3::GFP::rab-3)*, *syIs319 (Pnlp-40::gfp)*], then transferred 10 synchronized Day 1 adults to the control and GFP RNAi plates, allow them to lay eggs for 2 h, then remove those adults. Finally, we quantified the GFP fluorescence intensity in the nerve ring or intestinal region of the F1s at the

adult Day 1 stage. We used the same procedure to screen the intestinal neuropeptide encoding genes (*nlp-1*, *nlp-2*, *nlp-6*, *nlp-8*, *nlp-9*, *nlp-13*, *nlp-14*, *nlp-15*, *nlp-16*, *nlp-18*, *nlp-20*, *nlp-21*, *nlp-27*, *nlp-29*, *nlp-40*, *nlp-47*, *nlp-67*, *flp-21*).

#### Calcium imaging

The animals of FDU424[*olaIs17(Pmod-1::GCaMP6*, *Pttx-3::mCherry*, *Punc-122::dsRed)*], FDU3631[*cfz-2(ok1201)*, *olaIs17(Pmod-1::GCaMP6*, *Pttx-3::mCherry*, *Punc-122::dsRed)*], FDU4566[*cfz-2(ok1201)*, *shcEx2340 (Pcfz-2::cfz-2*, *Plin-44::GFP)*, *olaIs17(Pmod-1::GCaMP6*, *Pttx-3::mCherry*, *Punc-122::dsRed)*], FDU4567[*cfz-2(ok1201)*, *shcEx2341(Pcfz-2::cfz-2*, *lin-44::GFP)*, *olaIs17(Pmod-1::GCaMP6*, *Pttx-3::mCherry*, *Punc-122:: dsRed)*], FDU3604[*nlp-40(tm4085)*, *olaIs17(Pmod-1::GCaMP6*, *Pttx-3:: mCherry*, *Punc-122::dsRed)*], FDU4562[*nlp-40(tm4085)*, *shcEx2336* (*Pnlp-40::nlp-40*, *Plin-44::GFP)*, *olaIs17(Pmod-1::GCaMP6*, *Pttx-3:: mCherry*, *Punc-122::dsRed)*], FDU4563[*nlp-40(tm4085)*, *shcEx2337* (*Pnlp-40::nlp-40*, *Plin-44::GFP)*, *olaIs17(Pmod-1::GCaMP6*, *Pttx-3:: mCherry*, *Punc-122::dsRed)*] and FDU3632[*aex-2(sa3)*, *olaIs17(Pmod-1:: GCaMP6*, *Pttx-3::mCherry*, *Punc-122::dsRed)*], FDU4558[*aex-2(sa3)*, *shcEx2332(Paex-2::aex-2*, *Plin-44::GFP)*, *olaIs17(Pmod-1::GCaMP6*, *Pttx-3::mCherry*, *Punc-122::dsRed)*], FDU4559[*aex-2(sa3)*, *shcEx2333 (Paex-2::aex-2*, *Plin-44::GFP) olaIs17(Pmod-1::GCaMP6*, *Pttx-3::mCherry*, *Punc-122::dsRed)*] were used for AIY calcium imaging. The P*ttx-3:: mCherry* were used as an internal control. Calcium imaging of adult Day 1 animals was performed on 10% agarose pads immobilized with 0.1 μM polystyrene beads and covered with glass coverslip.

Images were captured with Andor Dragonfly Spinning Disc Confocal Microscope with 60× objectives, 488 or 561 nm laser. Individual animals were imaged for 60 s at a rate of 2 Hz. Data were analyzed using custom-written scripts in R Studio, and the images rotation and brightness/contrast were processed with Adobe photoshop CC. The 60 s video was exported with Imaris 4.0.

#### Fluorescence microscope and confocal imaging

For general microscopy experiments, worms were anesthetized on 3% agarose pads with 50 mM muscimol. Images were captured with Andor Dragonfly Spinning Disc Confocal Microscope with 10× or 40× objectives, 488 nm (for GFP) or 561 nm (for mCherry) laser. The fluorescence intensity was quantified with Imaris 4.0/Image J, and the images rotation and brightness/contrast were processed with Adobe photoshop CC.

#### Quantification and statistical analysis

We quantified the fluorescence intensity using Imaris. The density obtained by cropping the AIY synaptic region (zone 2 and zone 3 in Fig 1A'), the whole intestine or nerve ring region (showed in Fig 6A dashed box) in Imaris or Image J (Fig EV4). To monitor the temporal role of indicated genes, we quantified the phenotype for L1, L4 and Day 1 adult after eggs laid for 12, 42, and 66 h, respectively. All quantified data were collected from at least three biological replicates. For data with transgenic animals, at least two independent lines were used. The statistics were made with GraphPad Prism 6.0 (GraphPad Software). Statistical analysis between two groups were based on Student's *t*-test (two-tailed), among more than two groups were based on one-way ANOVA followed by Dunnett's test, as indicated in the figure legends. All quantitative data were collected blindly.

## Data availability

Source data are available online for all figures. This study includes no data deposited in external repositories.

**Expanded View** for this article is available online.


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### Author contributions

**Yanjun Shi:** Conceptualization; resources; data curation; formal analysis; validation; investigation; visualization; methodology. **Lu Qin:** Resources; data curation; validation; investigation. **Mengting Wu:** Resources; data curation; investigation. **Junyu Zheng:** Data curation; investigation. **Tao Xie:** Data curation; investigation. **Zhiyong Shao:** Conceptualization; resources; formal analysis; supervision; funding acquisition; validation; project administration.

In addition to the CRediT author contributions listed above, the contributions in detail are:

YS and ZS conceived and designed the project. YS, LQ, MW, TX, and ZS performed experiments and analyzed data, interpreted the results. YS, LQ, MW, JZ, TX, and ZS wrote the manuscript.

### Disclosure statement and competing interests

The authors declare that they have no conflict of interest.

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