Wnts Promote Synaptic Assembly Through T-Cell Specific Transcription Factors in Caenorhabditis elegans

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Synapses are specialized neuronal connections essential for neuronal function. Defects in synaptic assembly or maintenance usually lead to various neurological disorders. Synaptic assembly is regulated by secreted molecules such as Wnts. Wnts are a large family of conserved glycosylated signaling molecules involved in many aspects of neural development and maintenance. However, the molecular mechanisms by which Wnts regulate synaptic assembly remain elusive due to the large number of ligands/ receptors, the diversity of signaling cascades and the complexity of the nervous system. In this study, through genetic manipulation, we uncover that C. elegans Wnt-2 (CWN-2) is required for synaptic development. The CWN-2 signal is required during both embryonic and postembryonic development, in the nervous system and intestine, for promoting synaptic assembly. Furthermore, we provide genetic evidence for CWN-2 promoting synaptogenesis through the Frizzled receptor (FZD) CFZ-2, the Dishevelled (DVL) DSH-2, the β-catenin SYS-1 and the only T-cell specific transcription factor POP-1/TCF. Importantly, it is the first time to report the requirement of a TCF for presynaptic assembly. These findings expand our understanding of the synaptogenic mechanisms and may provide therapeutic insights into Wnt-related neurological disorders.

Keywords: synaptic assembly, CWN-2/Wnt, canonical Wnt pathway, CFZ-2/Frizzled, DSH-2/Dishevelled, SYS-1/β-catenin, POP-1/TCF/LEF, intestine-neuron cross talk

INTRODUCTION

Normal neural function requires precise synaptic connections, and defects in the connection often result in neurological disorders (Caracci et al., 2016; Remedio et al., 2016; Song et al., 2016, 2017; Roeper, 2017; Zhai et al., 2017; Moretto et al., 2018). Chemical synapses are junctional connections composed of presynaptic terminals, postsynaptic targets and the synaptic cleft (Pappas and Purpura, 1972; Cowan et al., 2001). Synaptic formation and maintenance are finely regulated by signaling molecules such as Wnts (Wu et al., 2010; Budnik and Salinas, 2011; Henriquez and Salinas, 2012; Park and Shen, 2012; Dickins and Salinas, 2013).

Abbreviations: ADAMT, A disintegrin and metalloproteinase with thrombospondin motifs; AIY, Amphid interneuron; CAMKII, Type II calcium/calmodulin-dependent protein kinase; DVL, Dishevelled; ECM, Extracellular matrix; FZD, Frizzled receptor; GFP, Green fluorescent protein; IgSF, Immunoglobulin superfamily; JNK, c-Jun N-terminal kinase; LEF, Lymphoid enhancing factor; MAP, Microtubule-associated protein; NMJ, Neuromuscular junction; PCP, Planer cell polarity; PK, Prickle; PKC, Protein kinase C; RNAi, RNA interference; TCF, T-cell specific transcription factor.
Wnts are a large family of conserved glycosylated secreted signaling molecules, with 19 members in mammals, seven in fly, and five in nematode (Gordon and Nusse, 2006; Willert and Nusse, 2012; Barik et al., 2014). Upon binding to their receptors, Wnts trigger conserved signaling cascades including the canonical β-catenin/TCF pathway and noncanonical planar cell polarity (PCP) and Ca\(^{2+}\) pathways (Supplementary Figure S1; Mlodzik, 1999; Patapoutian and Reichardt, 2000; Ciani and Salinas, 2005; Montcouquiol et al., 2006). In Drosophila, Wnts also act through the Frizzled (FZD) nuclear import signaling pathway (Mathew et al., 2005).

Wnts play complex roles at multiple levels in synaptic development due to the large number of ligands/receptors and the diversity of signal cascades. Wnts activate different signaling pathways (Supplementary Figure S1; Lucas and Salinas, 1997; Hall et al., 2000; Cerpa et al., 2008; Davis et al., 2014). Upon binding to their receptors, Wnts trigger conserved signaling cascades including the canonical Wnt pathway, was associated with memory consolidation in mice (Fortress et al., 2013), the requirement of TCF/LEF molecules for 1 (LEF1), the downstream components in the canonical Wnt pathway, was associated with memory consolidation in mice (Fortress et al., 2013), the requirement of TCF/LEF molecules for synaptic assembly or maintenance has not been reported in any system.

C. elegans has proven to be an excellent model for addressing molecular mechanisms underlying synaptogenesis in vivo at the single cellular level in live animals (Jin, 2005). Wnt signal pathways are conserved in the nematode C. elegans (Sawa and Korswagen, 2013), and regulate neuromuscular junction (NMJ) synaptic assembly and plasticity (Klassen and Shen, 2007; Jing et al., 2009; Jensen et al., 2012a; Park and Shen, 2012). Although it is well known that Wnts are required to regulate synaptic assembly, many questions remain. For example, systematic studies of Wnts in synaptic assembly are missing. Additionally, although the expression of T-cell specific transcription factor 1 (TCF1) and Lymphoid Enhancing Factor 1 (LEFI), the downstream components in the canonical Wnt pathway, was associated with memory consolidation in mice (Fortress et al., 2013), the requirement of TCF/LEF molecules for synaptic assembly or maintenance has not been reported in any system.

C. elegans has proven to be an excellent model for addressing molecular mechanisms underlying synaptogenesis in vivo at the single cellular level in live animals (Jin, 2005). Wnt signal pathways are conserved in the nematode C. elegans (Sawa and Korswagen, 2013), and regulate neuromuscular junction (NMJ) synaptic assembly and plasticity (Klassen and Shen, 2007; Jensen et al., 2012b; Mizumoto and Shen, 2013; Pandey et al., 2017). However, it remains unknown if Wnts are required for non-NMJ presynaptic formation in the nematode nerve ring, which is analogous to the vertebrate brain. To address this question, we systematically examined the requirement of all five Wnts, four Frizzled receptors (FZDs), three Dishevelled (DVLs), four β-catenin and only one POP-1/TCF for the presynaptic assembly in the Amphid interneurons (AIY). We found that genes encoding components in the canonical Wnt pathway, including cwn-2/Wnt, cfz-2/Fzd, dsh-2/Dvl, sys-1/β-catenin and the pop-1/TCF, are required for promoting AIY synaptic assembly during both embryonic and postembryonic stages both in the nervous system and in the intestine.

**MATERIALS AND METHODS**

**Strains and Genetics**

All worms were fed with *E. coli* OP50 on standard NGM plates as described (Brenner, 1974). Strains used in this study were maintained at 21°C and detailed information is listed in Supplementary Table S1. The mutants and transgenic alleles were used in this study: wyls5 (Pttx-3::GFP::rab-3, Punc-122::RFP) X, cwn-1(ok546) II, cwn-2(ok895) IV, lin-44(n1792) I, eg-20(n585) IV, mig-1(e1787) I, lin-16(e620) X, lin-17(n671) I, c-fz-2(ok1201) V, dsh-1(ok1445) II, bar-1(mu63) X, cdc-42(ok825) II, vang-1(ok1142) X, pop-1(9/u9) I, olais10(Ptxx-3::mCherry::rab-3, Pttx-3::GFP::syd-1, Punc-122::RFP), shecEx293 (Pcwn-2::GFP, Punc-122::RFP), shecEx312(Pcwn-2::GFP, Punc-122::RFP), shecEx112(Pcwn-2::cwn-2, Punc-122::GFP), shecEx113(Pcwn-2::cwn-2, Punc-122::GFP), shcEx305(Pttx-3::cwn-2, Punc-122::GFP), shcEx373(Prab-3::cwn-2, Punc-122::GFP), shcEx374(Prab-3::cwn-2, Punc-122::GFP), shecEx375(Prab-3::cwn-2, Punc-122::GFP), shecEx267(Pmyo-2::cwn-2, Punc-122::GFP), shecEx279(Pmyo-2::cwn-2, Pmyo-3::cwn-2, Punc-122::GFP), shecEx400(Pmyo-3::cwn-2, Punc-122::GFP), shecEx448(Pttx-3::cwn-2, Punc-122::GFP), shecEx449(Pttx-3::cwn-2, Punc-122::GFP), shecEx450(Pges-1::cwn-2, Punc-122::GFP), shecEx451(Pges-1::cwn-2, Punc-122::GFP), shecEx452(Pges-1::cwn-2, Punc-122::GFP), shecEx454(Pfz::cfz-2, Punc-122::GFP), shecEx485(Pfz::cfz-2, Punc-122::GFP), shecEx444(Pttx-3::cfz-2, Punc-122::GFP), shecEx445(Pttx-3::cfz-2, Punc-122::GFP), shecEx446(Pttx-3::cfz-2, Punc-122::GFP), shecEx453(Prab-3::cfz-2, Punc-122::GFP), shecEx454(Prab-3::cfz-2, Punc-122::GFP), shecEx455(Prab-3::cfz-2, Punc-122::GFP), shecEx479(Pges-1::cfz-2, Punc-122::GFP), shecEx480(Pges-1::cfz-2, Punc-122::GFP), shecEx481(Pges-1::cfz-2, Punc-122::GFP), shecEx482(Pmyo-3::cfz-2, Punc-122::GFP), shecEx483(Pmyo-3::cfz-2, Punc-122::GFP), shecEx311(Pfz::cfz-2, Punc-122::GFP), shecEx661(Pttx-3::mCherry, Pfz::cfz-2, Punc-122::GFP), shecEx662(Pttx-3::mCherry, Pwcn-2::GFP, shcEx665(Pttx-3::mCherry), shcEx666(Pttx-3::mCherry), shcEx667(cfz-2 genomic::GFP, phlh-17::mCherry), shcEx668(cwn-2 genomic::GFP, phlh-17::mCherry).

**Cloning and Transgenes**

We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites derived of pPD49.26 (A. Fire) with extra cloning sites. We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites. We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites derived of pPD49.26 (A. Fire) with extra cloning sites. We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites derived of pPD49.26 (A. Fire) with extra cloning sites. We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites derived of pPD49.26 (A. Fire) with extra cloning sites. We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites derived of pPD49.26 (A. Fire) with extra cloning sites. We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites derived of pPD49.26 (A. Fire) with extra cloning sites. We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites derived of pPD49.26 (A. Fire) with extra cloning sites. We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites derived of pPD49.26 (A. Fire) with extra cloning sites. We created the expression clones using the pSM vector, a derivative of pPD49.26 (A. Fire) with extra cloning sites derived of pPD49.26 (A. Fire) with extra cloning sites.
RESULTS

CWN-2/Wnt Is Required for AIY Presynaptic Clustering

*C. elegans* AIYs are a pair of bilateral symmetric interneurons located in the nerve ring (*Figures 1A,B*, White et al., 1986). AIY neurites can be divided into three Zones based on the anatomic location: the ventral part proximal to the soma, called Zone 1; the distal axon in nerve ring, called Zone 3; and the middle elbow region called Zone 2 (*Figure 1B*; White et al., 1986; Colón-Ramos et al., 2007). AIY forms large presynaptic clusters in Zone 2, which can be labeled with the synaptic vesicle marker GFP::RAB-3, and the synaptic clustering phenotype is highly reproducible across individual animals (*87%, Figures 1D,G*; Colón-Ramos et al., 2007).

To test whether Wnts are required for synaptic clustering, we examined the AIY synaptic vesicle marker GFP::RAB-3 clustering phenotype in all four viable Wnt loss of function mutants: *cwn-1*(ok546), *cwn-2*(ok895), *egl-20*(n585), *lin-44*(n1792); their genetic lesions are shown in *Figure 1C*, Supplementary Figures S2A–C), and in the essential Wnt *mom-2* knockdown animals. Among those mutant alleles we examined, *cwn-1*(ok546), *cwn-2*(ok895) and *lin-44*(n1792) are most likely to be null alleles since the first two are big deletions and the third is an early stop (*Hereman et al., 1995; Zinovyeva and Forrester, 2005*). The *egl-20*(n585) is probably a strong loss of function mutation since the altered a highly conserved cysteine at position 99 to a Serine (*Maloof et al., 1999*). We only found that *cwn-2* is required for the synaptic clustering as revealed by the synaptic vesicle GFP::RAB-3 marker (*Figures 1E,G,H* and Supplementary Figures S2F–J). In *cwn-2*(ok895) mutants, the coherent Zone 2 GFP::RAB-3 clustering is fragmented in 74.7% animals (*p < 0.0001, Figures 1E,G*). Additionally, we quantified the relative Green fluorescent protein (GFP) intensity and found that the intensity of GFP::RAB-3 is reduced by 60.2% (*p < 0.001, Figures 1E,H*). To confirm the requirement of *cwn-2* for AIY synaptic clustering, we knocked down *cwn-2* by RNAi and found robust AIY Zone 2 synaptic fragmentation in *cwn-2* RNAi treated animals as well (*P < 0.001, Figure 1G*). The requirement of *cwn-2* for GFP::RAB-3 clustering is further confirmed by the fact that the AIY synaptic defect in *cwn-2*(ok895) mutants can be rescued by expressing the wild type *cwn-2* transgene in *cwn-2*(ok895) mutants (*P < 0.0001 for Zone 2 fragmentation, *P < 0.05* for GFP intensity, *Figures 1F–H*). To examine if the AIY Zone 3 region is affected by *cwn-2*(ok895), we quantified the GFP intensity and found that the GFP intensity in the AIY Zone 3 region is normal in *cwn-2*(ok895) mutants (Supplementary Figure S3).

Synaptic vesicle and synaptic active zone proteins are assembled independently (*Zhen and Jin, 2004*). To address if *cwn-2* is also required for AIY synaptic active zone protein assembly, we examined the synaptic active zone marker GFP::SYD-1 (*Hallam et al., 2002*). We found that GFP::SYD-1 colocalizes with the synaptic vesicle marker mCherry::RAB-3, as reported previously (*Figures 2A–D*; Stavoe and Colon-Ramos, 2012; Shao et al., 2013). Similar to the RAB-3 marker, the intensity of SYD-1::GFP is dramatically reduced and the Zone
2 GFP is fragmented in cwn-2(ok895) mutants (P < 0.0001 for Zone 2 fragmentation, P < 0.01 for fluorescent intensity, Figures 2A–D,E,F). Together, these data suggest that cwn-2 is required for both AY synaptic vesicle and active zone protein assembly in the Zone 2 region. Since SYD-1 marker and RAB-3 marker are colocalized and the presynaptic defect is the same for both markers in cwn-2(ok895), we only use GFP::RAB-3 for our further analysis.

CWN-2 Promotes Synaptogenesis During Both Embryonic and Postembryonic Development

The previous described synaptic phenotype in cwn-2(ok895) could result from the defect of synaptic assembly during embryogenesis or synaptic maintenance during postembryonic stages. To differentiate those two, we examined the AY synaptic marker GFP::RAB-3 at four larval stages (L1–L4) and the adult stages. To determine where cwn-2 acts, we first determine the expression pattern by the transcription reporter Pcwn-2::GFP. Consistent with previous findings, Pcwn-2::GFP is expressed beginning in early embryonic stages, mainly in the digestive and nervous systems, with weak expression in the body wall muscles at adult stage (Supplementary Figure S4 and data not shown; Kennerdell et al., 2009; Song et al., 2010). The GFP reporter is only seen in the intestine before 2-fold stage (Supplementary Figures S4A–C). In late embryonic stage, Pcwn-2::GFP is expressed both in the intestine and the pharynx (Supplementary Figures S4D,E). After hatching, Pcwn-2::GFP is mainly seen in the pharynx, some head neurons, the body wall muscle and the intestine (Supplementary Figures S4F,G). However, Pcwn-2::GFP is not seen in the AY (Supplementary Figures S4H–J).

FIGURE 1 | cwn-2/Wnt is required for presynaptic vesicle clustering. (A) Cartoon diagram of a nematode C. elegans. (B) The head region shown in the dashed box in (A). Bilateral asymmetric Amphid interneurons (AY) are indicated in gray, whose neurites innervate in the nerve ring. AY presynapses form a distinct and highly reproducible pattern: the ventral region proximal to soma with no synapse (Zone 1), the elbow turn region with a large synaptic cluster (Zone 2) and the distal region with a few scattered synapses (Zone 3; Colón-Ramos et al., 2007; Altun et al., 2002–2018). (C) cwn-2(ok895) is a 905 bp deletion allele. The boxes and lines represent exons and introns of cwn-2 gene. Black and gray indicate coding sequence and UTRs. The line beneath indicates the deletion region. (D–F) Confocal images of AY synaptic marker GFP::RAB-3 in wild type (D), cwn-2(ok895) mutants (E) and cwn-2(ok895) mutants rescued by a transgene (tg[Pcwn-2::cwn-2]) (F). The Zone 2 of AY forms a large presynaptic cluster in wild type animals, while the cluster is broken into multiple pieces, which is named fragmentation, in cwn-2(ok895) mutants. This defect is rescued by transforming a copy of wild type cwn-2

To confirm the requirement of cwn-2 during the postembryonic development further, we treated wild type animals with cwn-2 RNAi beginning at different stages (L1, L2, L4 and day 1 adult) and examined the AY presynaptic phenotype 3 days later. We found that postembryonic knockdown of cwn-2 from either L1 or L2, but not from L4 or adult day 1 stage, also led to a synaptic fragmentation in the AY Zone 2 (P < 0.0001 at L1, P < 0.01 at L2, Figure 3H).

CWN-2 Acts Both in the Nervous System and in the Intestine to Regulate AY Synaptic Clustering

To determine where cwn-2 acts, we first determine the expression pattern by the transcription reporter Pcwn-2::GFP. Consistent with previous findings, Pcwn-2::GFP is expressed beginning in early embryonic stages, mainly in the digestive and nervous systems, with weak expression in the body wall muscles at adult stage (Supplementary Figure S4 and data not shown; Kennerdell et al., 2009; Song et al., 2010). The GFP reporter is only seen in the intestine before 2-fold stage (Supplementary Figures S4A–C). In late embryonic stage, Pcwn-2::GFP is expressed both in the intestine and the pharynx (Supplementary Figures S4D,E). After hatching, Pcwn-2::GFP is mainly seen in the pharynx, some head neurons, the body wall muscle and the intestine (Supplementary Figures S4F,G). However, Pcwn-2::GFP is not seen in the AY (Supplementary Figures S4H–J).
Given that cwn-2 is mainly expressed in the nervous system, intestine, pharynx and body wall muscle, we next expressed cwn-2 in those tissues with tissue-specific promoters Prab-3 (neurons), Pges-1 (intestine), Pmyo-2 (pharynx) or Pmyo-3 (muscles) in cwn-2(ok895) mutants. AIY Zone 2 fragmentation is rescued when cwn-2 is expressed in the nervous system with the pan-neuronal rab-3 promoter or the AIY-specific ttx-3 promoter, or in the intestine with the ges-1 promoter, but not in the pharynx or body wall muscle (P < 0.0001 for Prab-3::cwn-2, Pttx-3::cwn-2 and Pges-1::cwn-2. Figures 4A–I). However, only intestinal expression of cwn-2 rescues the GFP::RAB-3 intensity (Figure 4J). The data suggest that the presynaptic morphology is regulated by either the local neuronal or the distant intestinal CWN-2, while the presynaptic GFP::RAB-3 intensity is only regulated by the intestinal CWN-2.

**CFZ-2/FZD Is Required for AIY Synaptic Clustering**

Wnts bind to FZD receptors and activate downstream cascade signaling pathways. C. elegans has four genes encoding FZDs: mig-1, lin-17, cfz-2 and mom-5. To address the requirement...
of FZDs for AIY synaptic clustering, we examined the AIY synaptic marker GFP::RAB-3 in the loss-of-function mutants mig-1(e1787), lin-17(n671) and cfz-2(ok1201). The mutant alleles for mig-1(e1787), lin-17(n671) and cfz-2(ok1201) are likely to be null since mig-1(e1787) and lin-17(n671) are nonsense mutations, and cfz-2(ok1201) delete 194 predicted amino acids and result in a frameshift (Figure 5A, Supplementary Figures S5A–B). The requirement of mom-5 for AIY synaptic assembly was assayed by RNAi, due to its essential role during development. Significant synaptic fragmentation in AIY are only observed in cwn-2(ok895) mutants (p < 0.0001, Supplementary Figures S5C–G). To examine if the AIY Zone 2 synaptic clustering defect in cfz-2(ok1201) mutants is due to the AIY morphologic defect, we looked the AIY cytoplasmic mCherry and found that the AIY morphology is grossly normal in the cfz-2(ok1201) mutants (Supplementary Figure S7).

If CFZ-2 acts as the CWN-2 receptor, the cfz-2; cwn-2 double mutants would phenocopy either cwn-2(ok895) or cfz-2(ok1201) single mutants. To test this hypothesis, we made cwn-2(ok895); cfz-2(ok1201) double mutants and found that the AIY synaptic fragmentation in the double mutants is similar to that in cwn-2(ok895) single mutants (P = 0.29, Figures 5C–E). We address if they are required for AIY synaptic clustering, we examined the synaptic marker GFP::RAB-3 in dsh-1(ok1445), a putative hypomorphic allele (Supplementary Figure S8A; Klassen and Shen, 2007), and dsh-2 and mig-5 knockdown animals.

**DSH-2/DVL and SYST-1/β-Catenin Are Required for AIY Synaptic Clustering**

The binding of Wnt to FZD receptors activates DVL. Three C. elegans DVLs are encoded by: dsh-1, dsh-2 and mig-5. To address if the AIY synaptic marker GFP::RAB-3 in dsh-1(ok1445), a putative hypomorphic allele (Supplementary Figure S8A; Klassen and Shen, 2007), and dsh-2 and mig-5 knockdown animals.
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While we did not observe an AIY synaptic fragmentation in dsh-1(ok1445) or mig-5 knockdown animals, the AIY Zone 2 GFP::RAB-3 morphology in dsh-2 knockdown animals is similar to that in cwn-2(oks895) or cfz-2(ok1201) mutants (p < 0.0001, Figures 6A,B,D, Supplementary Figures S8C,E,H, and data not shown).

To test whether dsh-2 acts in the same pathway as cwn-2 and cfz-2, we treated cwn-2(oks895) or cfz-2(ok1201) mutants with dsh-2 RNAi. We first determined that the dsh-2 RNAi efficiency is robust, as assayed by the quantifying synaptic fragmentation of the dsh-2 RNAi-treated wild type animals (Figure 6D). Interestingly, dsh-2 RNAi knockdown did not enhance the AIY Zone 2 fragmentation of either cwn-2(oks895) or cfz-2(ok1201) mutants (P = 0.06 for cwn-2, P = 0.15 for cfz-2, Figure 6E), indicating that dsh-2 acts in the cwn-2/cfz-2 pathway to promote the synaptic clustering.

In the canonical Wnt pathway, the β-catenin stabilized by DVLs enters nuclei to activate their downstream targets through binding to TCF/LEF. To address if any β-catenin is required for AIY synaptic clustering, we examined the presynaptic marker GFP::RAB-3 in β-catenin knockout or knockdown animals. Four C. elegans β-catenin are encoded by bar-1, hmp-2, wrm-1, sys-1. While the hypomorphic bar-1(mu63) allele (Natarajan et al., 2004, Supplementary Figure S8B), or knockdown of wrm-1 or hmp-2 by RNAi does not affect the GFP::RAB-3 clustering in AIY, knockdown of sys-1 by RNAi results in a significant GFP::RAB-3 fragmentation in AIY Zone 2 (p < 0.0001, Figures 6C,D, Supplementary Figure S8D,F,G), suggesting that sys-1 is required for the AIY Zone 2 presynaptic assembly.

To test whether sys-1 acts in the same pathway as cwn-2 and cfz-2, we treated cwn-2(oks895) or cfz-2(ok1201) mutants with sys-1 RNAi. We first determined that the sys-1 RNAi was efficient as the GFP::RAB-3 fragmentation in the AIY Zone 2 was highly penetrant (Figure 6D). We observed that sys-1 RNAi did not enhance the AIY synaptic fragmentation of either cwn-2(oks895) or cfz-2(ok1201) mutants (P = 0.43 for cwn-2, P = 0.99 for cfz-2

**FIGURE 4** cwn-2 acts in nervous system and intestine to regulate AIY synaptic clustering. (A-H) Confocal micrographs of the AIY presynaptic pattern of GFP::RAB-3. Presynaptic marker GFP::RAB-3 forms a large continuous cluster at AIY Zone 2 in wild type animals (A), and the cluster is fragmented in cwn-2(ok895) mutants (B). This Zone 2 fragmentation in cwn-2(oks895) is rescued by driving expression of cwn-2 with its own promoter (C), pan-neuronal rab-3 promoter (D), AIY-specific ttx-3 promoter (E) or intestinal-specific ges-1 promoter (F), but not with pharyngeal-specific myo-2 promoter (G) or body wall muscle-specific myo-3 promoter (H). Dashed boxes highlight AIY zone 2 and asterisks indicate the position of AIY soma. The scale bar represents 10 µm. (I,J) Quantification of the GFP::RAB-3 fragmentation (I) or fluorescence intensity (J) in AIY Zone 2. The Zone 2 fragmentation phenotype is rescued by expressing cwn-2 either in the nerve system (with pan-neuronal rab-3 promoter or with AIY-specific ttx-3 promoter) or in the intestine (with ges-1 promoter), while the GFP::RAB-3 intensity can only be rescued by expressing cwn-2 in the intestine, not in the nerve system. Data for each genotype are averaged from at least three biological replicates. Transgenic data are averaged from at least two independent lines. ns: not significance, ****p < 0.001, analyzed by one-way ANOVA Dunnett’s test. Error bars represent 95% confidence interval.
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FIGURE 5 | cfz-2/Fzd receptor acts with cwn-2 to promote AIY synaptic clustering. (A) A diagram of cfz-2, ok1201 is a 1174 bp deletion allele. The boxes and lines represent exons and introns. Black and gray boxes indicate coding sequence and UTRs, respectively. The line beneath indicates the deletion region. (B–J) Confocal micrographs of the AIY presynaptic pattern of GFP::RAB-3. The GFP::RAB-3 forms a large cluster at Zone 2 in wild type animals (A), which is fragmented in cfz-2(ok1201) (B), cwn-2(ok895) (C) and cwn-2(ok895); cfz-2(ok1201) double mutants (D). The Zone 2 fragmentation in cfz-2(ok1201) is rescued by expressing cfz-2 with its own promoter (F), pan-neuronal rab-3 promoter (G), AIY-specific ttx-3 promoter (H), intestinal-specific ges-1 (I) or pharyngeal-specific myo-2 promoter (J). Dashed boxes highlight AIY zone 2 and asterisks indicate the position of AIY soma. Scale bar is 10 μm. (K,L) Quantification of AIY Zone 2 fragmentation. The penetrance of the fragmentation in mig-1(e1787), lin-17(n671), cfz-2(ok1201), cwn-2(ok895), cfz-2(ok1201); cwn-2(ok895), mom-5 RNA interference (RNAi) animals (K) and tissue specific rescued strains (L). Data indicate that cfz-2 acts in the cwn-2 pathway both in the AIY and in the intestine to promote synaptic clustering. ns: not significance, ***p < 0.001, ****p < 0.0001, analyzed by one-way ANOVA Dunnett’s test (K,L) and two-tailed Student’s t-test (K). Error bars represent 95% confidence interval (K,L) and SEM (K) respectively.

Figure 6E). These results indicate that sys-1 acts in the cwn-2/cfz-2 pathway to regulate AIY synaptic clustering.

POP-1/TCF Is Required for AIY Presynaptic Assembly

The β-catenin interacts with the TCF transcription factors to activate the expression of downstream targets. C. elegans has only one TCF homolog encoded by pop-1 (Lin et al., 1998). To address the requirement of pop-1 for AIY synaptic assembly, we first examined the synaptic phenotype in the pop-1(hu9) mutants, which harbors a mutation at the β-catenin interaction site (Figure 7A; Korswagen et al., 2002). We found that the AIY Zone 2 fragmentation in pop-1(hu9) mutants was similar to that in cwn-2(ok895) or cfz-2(ok1201) mutants or knockdown of dsh-2 or sys-1 animals (Figures 7B,C,E). To further confirm the requirement of pop-1 for AIY presynaptic assembly, we knocked down pop-1 by RNAi. We found that pop-1 knockdown also resulted in a robust AIY Zone 2 fragmentation (Figures 7D,F,G).

To determine if pop-1 acts in the same pathway as cwn-2, first we examined the AIY synaptic phenotype at L1, L2, L3, L4 and
adult stages in pop-1(hu9) mutants. Similar to cwn-2(ok895) mutants, the AIY Zone 2 presynaptic fragmentation appears starting from L1 and continues into adult stage ($P < 0.0001$ for L1–L3 and adult stages, $P < 0.001$ for L4 stage, Figure 7E). Next, we treated wild type animals with pop-1 RNAi starting at L1, L2, L4 and day 1 adult stages and examined the GFP::RAB-3 in AIY 3 days later. Similar to the results from cwn-2 RNAi, we observed the synaptic fragmentation in animals treated with pop-1 RNAi starting at L1 or L2, but not after L4 ($P < 0.001$ for L1, $P < 0.01$ for L2, $P = 0.21$ for L4, and 0.14 for adult stages, Figure 7F), suggesting that pop-1 is required in larval stages for the presynaptic assembly.

To confirm that pop-1 acts in the cwn-2/cfz-2 signal pathway, we knocked down pop-1 in cwn-2(ok895), cfz-2(ok1201), or cwn-2(ok895); cfz-2(ok1201) double mutants. We first determined the pop-1 RNAi efficiency by the penetrance of the AIY Zone 2 presynaptic fragmentation ($P < 0.001$), and embryonic lethality of F1 (data not shown). Supporting our hypothesis, pop-1 RNAi did not aggravate the expressivity or penetrance of the AIY Zone 2 presynaptic fragmentation of cwn-2(ok895), cfz-2(ok1201), or cwn-2(ok895); cfz-2(ok1201) double mutants ($P = 0.14, 0.13, 0.42$ for cwn-2(ok895), cfz-2(ok1201), and cwn-2(ok895); cfz-2(ok1201) double mutants, respectively, Figure 7G). These results suggest that pop-1 acts in the same pathway as cwn-2/Wnt and cfz-2/Frizzled.

Wnts can also act through non-canonical PCP or Ca2+ signaling pathways, which are mediated by Vangl or CDC42 (Supplementary Figure S1). We examined the effect of deletion
alleles vang-1(ok1142) and cdc-42(ok825) (Supplementary Figures S2D,E) on the AIY synaptic marker GFP::RAB-3. Neither of them shows Zone 2 synaptic fragmentation (Supplementary Figures S2K–M), suggesting that vang-1 or cdc-42 is not required for synaptic clustering in AIY Zone 2.

Collectively, our data suggest that CWN-2 functions through the canonical Wnt signal pathway, which requires CFZ-2/FZD, DSH-2/DVL, SYS-1/β-catenin and the only POP-1/TCF to promote AIY presynaptic assembly. This CWN-2/Wnt signaling acts both cell-autonomously in the AIY and
non-cell-autonomously in the intestine, both during embryonic and postembryonic development to promote the AIY presynaptic assembly (Figure 7H).

DISCUSSION

Synapses are key structures for neuronal function, and synaptic assembly is precisely regulated. In this study, we reported a molecular mechanism by which CWN-2/Wnt regulates the presynaptic assembly in interneuron AIY in *C. elegans*. Our results demonstrate that CWN-2 regulates the presynaptic assembly during embryonic and postembryonic development through the canonical Wnt signaling pathway, requiring CFZ-2, DSH-2, and SYS-1, and the TCF transcription factor POP-1.

Our genetic data strongly support that specific components in the canonical Wnt signaling promotes *C. elegans* nerve ring interneuron presynaptic assembly. However, the limitation of this work is that we cannot conclusively exclude the requirement of some components in the pathway for two reasons. First, we tested for the requirement of essential genes through RNAi, which could not deplete their expression; second, we scored the synaptic defect mainly based on the fragmentation at Zone 2 region, which will miss those genes that only affect the GFP::RAB-3 intensity or size.

**CWN-2/Wnt Promotes Synaptic Assembly**

In this study, we found that *C. elegans* CWN-2 promotes presynaptic assembly as supported by several lines of evidence. First, loss-of-function mutation *cwn-2(ok895)* results in reduction of both synaptic vesicle and active zone markers at AIY presynaptic sites; second, the synaptic defect in *cwn-2(ok895)* is rescued by transforming a wild type copy of *cwn-2*; third, knockdown of *cwn-2* with RNAi decreases the AIY synaptic vesicle GFP::RAB-3 clustering.

*C. elegans* have five Wnts: CWN-1, CWN-2, EGL-20, LIN-44 and MOM-2 (Shackleford et al., 1993; Herman and Horvitz, 1994; Thorpe et al., 1997; Maloof et al., 1999). At presynaptic sites of NMJ, LIN-44 and EGL-20 inhibit synaptic assembly (Klassen and Shen, 2007; Mizumoto and Shen, 2013). The findings that CWN-2 promotes the interneuron AIY presynaptic assembly expand our understanding of the roles of Wnts in *C. elegans* presynaptic assembly. CWN-2 is the closest to Wnt5 in *Drosophila* and mammals (Prud’homme et al., 2002). Similar to the role of CWN-2 in AIY presynaptic assembly, *Drosophila* Wnt5 promotes synaptic formation (Liebl et al., 2008). However, mammalian Wnt5a has a more complex role in hippocampal synaptic development (Davis et al., 2008; Farias et al., 2009; Cuitino et al., 2010; Varela-Nallar et al., 2010; Thakar et al., 2017). Wnt5a was found to promote both glutamatergic spine morphogenesis and GABA receptor trafficking in rat cultured hippocampal neurons (Cuitino et al., 2010; Varela-Nallar et al., 2010), but to inhibit glutamatergic synaptic development in mouse hippocampal neurons (Davis et al., 2008; Thakar et al., 2017). In addition to Wnt5a, other Wnts have been found to either promote or inhibit synaptogenesis in different organisms, suggesting evolutionally conserved roles of Wnts in synaptic development (Budnik and Salinas, 2011; Park and Shen, 2012; Barik et al., 2014). The complex roles of Wnts are partly due to the diversity of Wnts and receptors, various signaling cascades and the complexity and dynamics of synapses in the nervous system. Combined with previous studies (Klassen and Shen, 2007; Mizumoto and Shen, 2013), our findings suggest that like in mammals, *C. elegans* Wnts have both positive and negative roles in regulating presynaptic assembly.

**The POP-1/TCF Mediated Canonical Wnt Pathway Is Required for Presynaptic Assembly**

Wnts function through either canonical or noncanonical pathways (Ciani and Salinas, 2005). The canonical pathway is mediated by FZD receptors, DVLs, β-catenin and TCF transcription factors (Wisniewska, 2013). Our study found that CWN-2 promotes presynaptic assembly through the canonical Wnt signaling pathway supported by the following evidence. First, mutation in *cfz-2/Fzd*, or knockdown of *dsh-2/Dvl* or *sys-1/β-catenin* by RNAi mimics the AIY Zone 2 presynaptic fragmentation in either *cwn-2* or *cfz-2* mutants. Second, loss-of-function mutation or knockdown of *pop-1/Tcf* resembles the AIY presynaptic fragmentation observed in either *cwn-2* or *cfz-2* mutants. Third, combination of mutations, or mutations and knockdown of two or three genes described above shows similar degree of the AIY presynaptic fragmentation to that in any single mutants. Collectively, these data suggest that CWN-2 regulates AIY presynaptic assembly through the canonical signal pathway.

In *C. elegans*, at the presynaptic sites, LIN-44 and EGL-20 inhibit synaptic assembly independent of the TCF/POP-1 (Klassen and Shen, 2007; Mizumoto and Shen, 2013). Although mutations of POP-1 enhanced the DD neuron presynaptic assembly defect in FSN-1 mutants, POP-1 single mutants showed normal presynaptic phenotype (Tulgren et al., 2014). In vertebrates or *Drosophila*, the TCF/LEF family of transcription factors can be activated by Wnts (Eastman and Grosschedl, 1999; Korswagen and Clevers, 1999), and is associated with memory consolidation (Fortress et al., 2013), but no evidence indicates its role in synaptic assembly thus far. Our findings showed for the first time that the β-catenin SYS-1 and the TCF transcription factor POP-1 are required for presynaptic assembly in the interneuron AIY.

**Embryonic and Postembryonic Requirement for the Cell-Autonomous and Non-Cell-Autonomous CWN-2 Signal to Promote Synaptic Clustering**

CWN-2 is expressed both during embryonic and postembryonic developmental stages, and our data suggests that CWN-2 has a role in synaptic assembly during both stages. First, we found that in *cwn-2(ok895)* or *pop-1(mu9)* mutants, the AIY Zone 2 presynaptic fragmentation appeared in newly hatched L1, suggesting that *cwn-2* and *pop-1* are required during embryonic development. Second, postnatal knockdown of *cwn-2*, *dsh-2*, or *pop-1* with RNAi results in the AIY presynaptic assembly defect,
indicating that the Wnt signal is required during larval stages for AIY presynaptic assembly.

AIY presynaptic assembly is largely established during embryonic stages and is maintained throughout the life of the animal (Colón-Ramos et al., 2007; Shao et al., 2013). However, during postembryonic development, as the animal grows, the nervous system architecture, including synaptic structure, scales up (Bénard and Hobert, 2009). We found that while the AIY synaptic distribution is maintained during postnatal development and adult stages, the size or intensity of synaptic marker increases as animals grow (Figure 3). At the NMJs, extracellular matrix (ECM) components such as type IV collagen EMB-9 and ECM remodeling ADAMT proteases such as GON-1 are required for maintaining the synaptic structures during the postnatal stages (Kurshan et al., 2014; Qin et al., 2014). The immunoglobulin superfamily (IgSF) protein ZIG-10 was recently identified for maintaining synaptic densities during development and adulthood (Cherra and Jin, 2016).

Wnts can act either locally or at long distances. Our studies showed that cwn-2 expressed either in the nervous system (or AIY) or in the intestine rescues the AIY Zone 2 fragmentation in the cwn-2 loss-of-function mutants, suggesting that both neuronal and intestinal CWN-2/Wnt regulates the AIY presynaptic assembly. However, the presynaptic GFP::RAB-3 intensity can only be rescued by expressing cwn-2 in the intestine, not in the nerve system, suggesting that the fragmentation effect and the reduction of the GFP::RAB-3 intensity are regulated independently. Alternatively, the fragmentation could be a more severe reduction of the GFP::RAB-3 intensity. Further experiments need to be done to differentiate those possibilities. The data also indicate that CWN-2 from the intestine is probably more important than that from nerve system. Similar to CWN-2, the Frizzled receptor CFZ-2 acts both cell-autonomously and non-cell-autonomously for the fragmentation phenotype. We speculate that the intestinal CWN-2/Wnt signaling is indirect and probably acts through secreted signaling molecules. Consistent with this hypothesis, molecules involved in exocytosis or secretion in the intestine are found to regulate neuronal function (Doi and Iwasaki, 2002; Mahoney et al., 2008). Further studies are needed to determine how the CWN-2/Wnt signaling in the intestine regulates synaptogenesis in the AIY neurons.

Wnts are evolutionarily conserved signaling molecules playing critical roles in neural development, including synaptogenesis (Koles and Budnik, 2012; Park and Shen, 2012). Wnt signaling dysfunction is often associated with neurodevelopmental and neurodegenerative disorders such as autism, schizophrenia, bipolar disorder and Alzheimer’s disease (Gould and Manji, 2002; Inestrosa et al., 2012; Kwan et al., 2016). The most common feature for those disorders is the defects of synaptic function. Wnt signaling blockade leads to synaptic disassembly in mature hippocampal neurons and probably some neurodegenerative disorders (Purro et al., 2014). Our finding that the presynaptic development requires the canonical Wnt signal and TCF transcriptional factors might provide cues to develop therapeutic strategies for related neurological disorders.

**AUTHOR CONTRIBUTIONS**

YS and ZS conceived, designed the project, YS, QL and ZS performed experiments and analyzed data, interpreted the results. YS and ZS wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00194/full#supplementary-material

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