Development

Carrier of Wingless (Cow) Regulation of Drosophila Neuromuscular Junction Development

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Abstract

The first Wnt signaling ligand discovered, Drosophila Wingless [Wg (Wnt1 in mammals)], plays critical roles in neuromuscular junction (NMJ) development, regulating synaptic architecture, and function. Heparan sulfate proteoglycans (HSPGs), consisting of a core protein with heparan sulfate (HS) glycosaminoglycan (GAG) chains, bind to Wg ligands to control both extracellular distribution and intercellular signaling function. Drosophila HSPGs previously shown to regulate Wg trans-synaptic signaling at the NMJ include the glypican Dally-like protein (Dlp) and perlecan Terribly Reduced Optic Lobes (Trol). Here, we investigate synaptogenic functions of the most recently described Drosophila HSPG, secreted Carrier of Wingless (Cow), which directly binds Wg in the extracellular space. At the glutamatergic NMJ, we find that Cow secreted from the presynaptic motor neuron acts to limit synaptic architecture and neurotransmission strength. In cow null mutants, we find increased synaptic bouton number and elevated excitatory current amplitudes, phenocopying presynaptic Wg overexpression. We show cow null mutants exhibit an increased number of glutamatergic synapses and increased synaptic vesicle fusion frequency based both on GCaMP imaging and electrophysiology recording. We find that membrane-tethered Wg prevents cow null defects in NMJ development, indicating that Cow mediates secreted Wg signaling. It was shown previously that the secreted Wg deacylase Notum restricts Wg signaling at the NMJ, and we show here that Cow and Notum work through the same pathway to limit synaptic development. We conclude Cow acts cooperatively with Notum to coordinate neuromuscular synapse structural and functional differentiation via negative regulation of Wg trans-synaptic signaling within the extracellular synaptomatrix.

Key words: Drosophila; HSPG; neuromuscular junction; synaptomatrix

Significance Statement

Wnt intercellular signaling is disrupted in numerous devastating neurologic disorders, including Alzheimer’s disease. Therefore, an understanding of Wnt signaling regulation is important for the design and implementation of targeted treatments. As a disease model, the Drosophila glutamatergic neuromuscular junction (NMJ) system is large, accessible, and genetically malleable, and thus well suited for discovering the molecular and cellular mechanisms of Wnt signaling regulation. Extracellular heparan sulfate proteoglycans (HSPGs) are important players as regulators of Wnt intercellular signaling. Here, we show secreted HSPG Carrier of Wingless (Cow), which directly binds to the founding Wnt-1 ligand, regulates NMJ structure and function. The mammalian homolog of Cow, Testican-2, is highly expressed in the brain. Studying this HSPG in Drosophila should inform mechanisms of Wnt regulation in human brain.

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Introduction

The developing nervous system requires the coordinated action of many signaling molecules to ensure proper synapse formation and function. One key class of signals is the Wnt ligands. The first discovered Wnt, Drosophila Wingless (Wg), is secreted from presynaptic neurons (Packard et al., 2002) and glia (Kerr et al., 2014) at the developing glatmateric neuromuscular junction (NMJ) to bind to the Frizzled-2 (Fz2) receptor (Bhanot et al., 1996) in both anterograde and autocrine signaling. In the postsynaptic muscle, Wg binding to Fz2 activates the noncanonical Frizzled Nuclear Import (FNI) pathway, which leads to Fz2 endocytosis and cleavage of the Fz2 C terminus (Fz2-C; Mathew et al., 2005). The Fz2-C fragment is trafficked to the nucleus to control translation of synaptic mRNAs and glutamate receptors (GluRs; Speese et al., 2012). In presynaptic neurons, Wg binding to Fz2 activates a divergent canonical pathway inhibiting glycosyn-thase kinase 3β (GSK3β) homolog Shaggy (Sgg) to control microtubule cytoskeletal dynamics via the microtubule-associated protein 1B (MAP1B) homolog Futsch (Miech et al., 2008), resulting in synaptic bouton growth (Franco et al., 2004; Ataman et al., 2008). The Wg signaling ligand must be tightly regulated in the synaptic extracellular space (synaptomatrix) to ensure proper NMJ development.

One critical category of proteins regulating Wg ligand in the synaptomatrix is heparan sulfate proteoglycans (HSPGs; Kamimura and Maeda, 2017). HSPGs consist of a core protein to which heparan sulfate (HS) glycosylphosphatidylinositol (GAG) chains are covalently attached. HS GAG chains are composed of repeating disaccharide subunits expressing variable sulfation patterns (the “sulfation code”; Masu, 2016). These GAG chains bind secreted extracellular ligands to regulate intercellular signaling. There are three HSPG families: transmembrane; glycerophosphatidylinositol (GPI) anchored; and secreted. The Drosophila genome encodes only five HSPGs, with the following three known to affect NMJ development: transmembrane syndecan (Johnson et al., 2006); GPI-anchored Dally-like protein (Dlp; Johnson et al., 2006; Dani et al., 2012); and secreted perlecain (Kamimura et al., 2013). A second secreted HSPG recently characterized in Drosophila was named Carrier of Wingless (Cow; Chang and Sun, 2014). In the developing wing disk, Cow directly binds secreted Wg and promotes its extracellular transport in an HS-dependent manner. Cow shows a biphasic effect on Wg target genes. Removing Cow results in a Wg overexpression (OE) phenotype for short-range targets, and a loss-of-function phenotype for long-range targets (Chang and Sun, 2014).

The mammalian homolog of Cow, Testican-2, is highly expressed within the developing mouse brain (Vannahme et al., 1999), and inhibits neurite extension in cultured ne-urons (Schnepp et al., 2005), although the mechanism of action is not known. We therefore set out to characterize Cow functions at the developing Drosophila NMJ. We use the larval NMJ model because it is large, accessible and particularly well characterized for HSPG-dependent Wg trans-synaptic signaling (Sears and Broadie, 2018). Each NMJ terminal consists of a relatively stereotypical innervation pattern, with consistent axonal branching and synaptic bouton formation (Menon et al., 2013). Boutons are the functional unit of the NMJ, containing presynaptic components required for neurotransmission including glutamate-containing synaptic vesicle (SV) pools and specialized active zone (AZ) sites for SV fusion. AZs contain Bruchpilot (Brp) scaffolds, which both cluster Ca2+ channels (Kittel et al., 2006) and tether SVs (Hallermann et al., 2010). AZs are directly apposed to GluR clusters in the postsynaptic muscle membrane (Schuster et al., 1991). This spatially precise juxtaposition is critical for high-speed and efficient synaptic communication between neuron and muscle.

In this study, we sought to test Cow functions at the NMJ, with the hypothesis that Cow should facilitate extracellular Wg transport across the synapse. Structurally, cow null mutants display overelaborated NMJs with more boutons and more synapses, phenocopying Wg overexpression. This phenotype is replicated with targeted neuronal Cow knockdown, but not muscle Cow knockdown, which is consistent with Cow secretion from the presynaptic terminal. Functionally, cow null mutants display increased synaptic transmission strength. Both electrophysiology recording and postsynaptically targeted GCaMP imaging show increased SV fusion, indicating elevated presynaptic function. Replacing native Wg with a membrane-tethered Wg blocks secretion (Alexandre et al., 2014). Tethered Wg has little effect on NMJ development, but when combined with the cow null suppresses the synaptic bouton increase, indicating that Cow mediates only secreted Wg signaling. It was recently shown that Notum, a secreted Wg deacetylase, also restricts Wg signaling at the NMJ (Kopke et al., 2017). We show here that combining null cow and notum heterozygous mutants causes a synergistic increase in NMJ development, indicating nonallelic noncomplementation. Moreover, combining null cow and notum homozygous mutants did not cause an increase in NMJ development compared with the single nulls, indicating an interaction within the same pathway. We conclude that Cow functions via negative regulation of Wg trans-synaptic signaling.

Materials and Methods

Drosophila genetics

All Drosophila stocks were reared on standard cornmeal/agar/molasses food at 25°C in a 12 h light/dark cycling incubator. Mixed sexes were used for all experiments except the SynapGCaMP imaging (females only). The genetic background control was w1118 D. The cow5A mutant, UAS-cow-miRNA-1 (referred to as UAS-cow-RNAi) and UAS-SP-eGFP-cow (referred to as UAS-Cow::eGFP) lines (Chang and Sun, 2014) were obtained from Yi Henry Sun (Institute of Molecular Biology, Academia Sinica, eNeuro.0285-19.2020 March/April 2020}
The cowGD
dP #03259 (y[1] w[+]; Mi[y
[+mDint2]=MIC])Cow[Ml03259]/TM3, Sb[1] Ser[1] and
cowGD
P #12802 (y[1] w[+]; Mi[y[+mDint2]=MIC])Cow
Ml02802) mutants, and the cow Df #6193 (w[1118]; Df
(3R)Exel6193, P[w[+mc]=XP-U]Exel6193/TM6B, Tb[1]) and
cow Df #619 (w[1118]; Df(3R)BSC619/TM6C, cu[1]
Sb[1]) deficiencies were all obtained from the Bloomington
Drosophila Stock Center (stock #40757, #58669, 7672, and
25694, respectively; Indiana University, Bloomington, IN).
Cow-Gal4 was obtained from the Vienna Tile (VT) collection of
the Vienna Drosophila Resource Center (VT046086; Vienna,
Austria). Neuronal vesicular glutamate transporter (vglut)-Gal4 and muscle-specific 24B-Gal4 driver lines were
obtained from the Bloomington Drosophila Stock Center.
The MHC-CDS-GCaMP6f-Sh Ca
P reporter (SynapGCaMP6f;
Newman et al., 2017) was obtained from Ehud Isacoff
(University of California, Berkeley, CA). Control
antibody (Chang and Sun, 2014). New rabbit anti-Cow
Cow antibodies were also made by ABclonal against amino
acids 36–236. Three antiserums were recovered and
affinity purified (29, 30, 31). Cow antibody 31 was pre-
absorbed against cow nulls (cowGD
P) for imaging studies. Cow antibody 31 was used for Figures 1, 2
and 4).

Western blotting
Staged Drosophila eggs (18–24 h postfertilization for
maximum expression; www.fruitfly.org) were dechorio-
ated using bleach for 30 s and washed with dH2O three
times, and embryos were genotyped using a GFP marker
with an epifluorescent microscope. Twenty-five embryos
were placed into an Eppendorf tube with 24 μl RIPA buffer
(150 mM sodium chloride, 1% Triton X-100, 0.5% sodium
deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris)
and protease inhibitor cocktail (catalog #P8340, Sigma-
Aldrich), then immediately snap frozen in a dry ice etha-
nol bath. Samples were sonicated (settings: 90% duty,
output 2; Sonifier 250, Branson) for 20 s, vortexed (speed 4;
Standard Mini Vortexer, VMR Scientific Products) for 5 s,
and then centrifuged at 14,000 rpm for 10 min. The super-
natant was then transferred to new tubes with 1×
Invitrogen NuPage LDS buffer (catalog #NP007, Thermo
Fisher Scientific) and 5% 2-mercaptoethanol (catalog
#M7154, Sigma-Aldrich), then vortexed as above. Samples
were incubated at room temperature for 20 min, heated at
100°C for 10 min, then centrifuged as above. Equal
volumes of lysates were loaded into precast NuPage 4–12%
Invitrogen Bis-Tris gels (catalog #NP0336, Thermo Fisher
Scientific) with Life Technologies NuPage running buffer
(catalog #NP0005, Thermo Fisher Scientific) and Invitrogen
NuPage antioxidant (catalog #NP0005, Thermo Fisher
Scientific). Electrophoresis was performed at 150 V for
2 h. Protein was then transferred overnight at 4°C with
constant 30 mA current to nitrocellulose mem-
branes (catalog #Protran NBA085C001EA, PerkinElmer)
in the Life Technologies NuPage transfer buffer (catalog
#NP0006-1, Thermo Fisher Scientific) supplemented with
20% methanol (catalog # AH230-4, Honeywell). Following
transfer, membranes were rinsed with dH2O, air dried at
room temperature for 1 h, and then blocked with 2% nonfat
powdered milk in TBS-T (0.1% Tween-20, 150 mM sodium
chloride, 5 mM potassium chloride, and 25 mM Tris, pH 7.6)
at room temperature for 1 h with rotation. Primary antibodi-
es were incubated overnight in 2% milk in TBST.
Membranes were then washed in TBST (five times for 6
min), followed by incubation in secondary antibody at room
temperature in 2% milk in TBST for 1 h with rotation, and
washed again as before. Imaging was performed on a LI-
COR Odyssey Imager with analysis on Image Studio Lite
(LI-COR). Total protein was assessed via the REVERT total
protein stain (catalog #926–11011, LI-COR). Primary antibo-
dies: rabbit anti-Cow (Ab 31, this study) and goat anti-
GFP (catalog #ab6662, Abcam), both at 1:1000. The fol-
lowing secondary antibodies were used: IRDye 680 donkey
anti-rabbit (catalog #926–68 073, LI-COR) and IRDye 800
donkey anti-goat (catalog #926–32 214, LI-COR), both at
1:10,000.

Cow antibodies
We used a well characterized, published anti-Cow
antibody (Chang and Sun, 2014). New rabbit anti-Cow
Confocal imaging

Wandering third instars were dissected in physiological solution containing the following (in mM): 128 NaCl, 2 KCl, 0.2 CaCl₂, 4 MgCl₂, 70 sucrose, 5 HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) at pH 7.2. The samples were fixed with 4% paraformaldehyde (catalog #15 714, EMS) diluted in PBS (catalog #46–013-CM, Corning). For intracellular labeling, samples were permeabilized with 0.2% Triton X-100 (catalog #BP151-100, Thermo Fisher Scientific) three times for 10 min each. Embryos were bleached for dechorionation, fixed with heptane and paraformaldehyde, and devitillinized with methanol. The following primary antibodies were used: mouse anti-Discs Large (DLG; 1:250; catalog #4F3), mouse anti-Wg (1:1; catalog #4D4), and mouse anti-Brp (1:200, catalog #nc82), all from the Developmental Studies Hybridoma Bank; Alexa Fluor 488-conjugated goat anti-horseradish peroxidase (HRP; 1:250; catalog #123–545-021), Cy3-conjugated goat anti-HRP (1:250; catalog #123–165–021), and Alexa Fluor 647-conjugated goat anti-HRP (catalog #123–605–021; 1:250), all from Jackson ImmunoResearch; rabbit anti-GluRIIC (1:5000; Marrus et al., 2004); and rabbit anti-GFP (1:1500; catalog #ab290, abcam). Preparations were incubated with primary antibodies overnight at 4°C and secondary antibodies at room temperature for 2 h, washed three times for 10 min each, and then mounted in Fluoromount-G (catalog #17984–25, EMS) onto 25 × 75 × 1 mm slides (Fisher Scientific, 12–544-2) with a 22 × 22-1 coverslip (catalog #12-542-B, Thermo Fisher Scientific) and sealed with clear nail polish (Sally Hansen). Imaging was performed on a Zeiss LSM 510 META laser-scanning confocal microscope, with images projected in Zen (Zeiss) and analyzed using ImageJ (NIH). NMJ intensity measurements were
made with HRP signal-delineated z-stack areas of maximum projection using ImageJ threshold and wand-tracing tools.

**Two-electrode voltage-clamp electrophysiology**

Wandering third instars were dissected longitudinally along the dorsal midline, internal organs were removed, and body walls were glued down (Vetbond, 3M). Peripheral motor nerves were cut at the base of the ventral nerve cord (VNC). Dissections and two-electrode voltage-clamp (TEVC) recordings were both conducted at 18°C in physiological saline as follows (in mM): 128 NaCl, 2 KCl, 4 MgCl2, 1.5 CaCl2, 70 sucrose, and 5 HEPES, pH 7.2. Preparations were imaged using a Zeiss Axioskop microscope with a Zeiss 40× water-immersion objective. Muscle 6 in abdominal segments 3–4 was impaled with two intracellular electrodes (1 mm outer diameter borosilicate capillaries; catalog #1B100F-4, World Precision Instruments) of ~15 MΩ resistance filled with 3 M KCl. The muscles were clamped at ~60 mV using an Axoclamp-2B amplifier (Axon Instruments). Spontaneous miniature excitatory junction current (mEJC) recordings were made in continuous 2 min sessions and low-pass filtered. For EJC
records, the motor nerve was stimulated with a fire-polished suction electrode using 0.5 ms suprathreshold voltage stimuli at 0.2 Hz from a Grass S88 stimulator. Nerve stimulation-evoked EJC recordings were filtered at 2 kHz. To quantify EJC amplitude, 10 consecutive traces were averaged, and the average peak value was recorded. Clampex 9.0 was used for data acquisition, and Clampfit 9 was used for data analysis (Axon Instruments).

**SynapGCaMP imaging**

For SynapGCaMP quantal imaging experiments, wandering third instars were dissected and type 1b NMJs were imaged in physiological saline as follows (in mM): 70 NaCl, 5 KCl, 1.5 CaCl₂, 25 MgCl₂, 10 NaHCO₃, 5 trehalose, 115 sucrose, and 5 HEPES, pH 7.2. Fluorescence images were acquired with a Vivo Spinning Disk Confocal microscope (3I Intelligent Imaging Innovations), with a 63×1.0 numerical aperture (NA) water-immersion objective (Zeiss), LaserStack 488 nm (50 mW) laser, Yokogawa CSU-X1 A1 spinning disk, and EMCCD camera (Photometrics Evolve). Image capture and analysis were performed as reported previously (Newman et al., 2017). Briefly, spontaneous events were imaged at 20 Hz (50 ms exposures, in streaming capture mode) for 30 s. Movies 1, 2 were then filtered, registered, and bleach corrected prior to ΔF conversion. Using the ΔF data, an XYT local maxima algorithm was applied to the thresholded ΔF data to identify where and when quantal release events occur (Newman et al., 2017). Quantal coordinates were used to calculate ΔF/F amplitudes and frequencies (normalized to the baseline SynapGCaMP6f 2D area).

**Structured illumination microscopy**

Dissected wandering third instar preparations were imaged using a Nikon N-SIM in 3D SIM (structured illumination microscopy) mode, configured with a 100× EX V-R diffraction grating, automated TIE inverted fluorescence microscope stand, 100× SR Apo 1.49 NA objective, Andor DU-897 EM-CCD, and 488/561 nm lasers. Image acquisition was managed through NIS-Elements (Nikon Instruments), and stacks were acquired with a 0.12 μm step size. Stack reconstruction of the raw data were used prior to rendering and analysis. To acquire larger fields of view and capture whole NMJs, SIM images were stitched together using the automated tiling method within NIS-Elements software.

**Laser-scanning confocal imaging analysis**

We used Imaris Version 9.3.0 to quantify LSM (laser-scanning confocal imaging) images using the “surfaces” function to identify the number and volume of Brp punctae, as follows:

1. Open image file and click “add new surfaces” to start the wizard.
2. Algorithm settings click “segment only a region of interest” (ROI).
3. Select ROI in X, Y, and Z.
4. Select “source channel” and click “thresholding conditions.”
5. Adjust threshold until all spots are selected.

**SIM image analysis**

We used Imaris Version 9.3.0 to quantify SIM images using the “spots” function to identify the number of Brp punctae and GluR clusters, as follows:

1. Open image file and click “add new spots” to start the wizard.
2. Algorithm settings click “segment only a region of interest” with “different spot sizes (region growing).”
3. Select ROI in X, Y, and Z.
4. Select “source channel” and click “background subtraction.”
5. Classify spots with a “quality” filter type and adjust by eye.

**Movie 1.** SynapGCaMP imaging of spontaneous quantal events in the control NMJ. Example of muscle 4 type 1b NMJ imaged in the control (vglut-Gal4/+; SynapGCaMP6f/+). [View online]

**Movie 2.** SynapGCaMP imaging of spontaneous quantal events in cow KD NMJ. Example of muscle 4 type 1b NMJ imaged following motor neuron-targeted cow RNAi (vglut-Gal4>UAS-cow-RNAi; SynapGCaMP6f/+). [View online]
Statistical analyses

All statistical measurements were performed within GraphPad Prism (version 7.04 for Windows). The D’Agostino–Pearson K-squared normality test was performed on all datasets to check for normality. For comparisons of two genotypes, a t test (normally distributed) or Mann–Whitney test (not normally distributed) was performed. For all other comparisons of more than two genotypes, an ordinary one-way ANOVA (normally distributed) or Kruskal–Wallis test (not normally distributed) was performed. All graphs were made in Prism, and the data are represented in scatter plots with the mean ± SEM.

Results

Carrier of wingless (cow) genetic locus, mutants and expression profiles

The cow gene encodes three transcripts (cow-RC, cow-RD, cow-RE), with cow-RD containing a long 3’-UTR (Fig. 1A). We acquired a reported cow null mutant (cow^{5A}; Chang and Sun, 2014), two mutations from the Gene Disruption Project (cow^{GDP} 03259 and 12802; Bellen et al., 2004; Nagarkar-Jaiswal et al., 2015), and two cow deficiencies from the Bloomington Drosophila Stock Center (Df[619] and Df[6193]). The cow^{5A} mutant has a 9119 bp deletion starting in the 3’-UTR that does not remove cow coding sequence, but is published as a well characterized protein null (Chang and Sun, 2014). The cow^{GDP} lines are minos-mediated integration cassette (Mi{MIC}) insertions; 03259 in cow intron 1, and 12802 in cow intron 4. Df[619] completely removes cow and 31 other genes, while cow Df[6193] removes cow and 41 other genes. PCR tests were performed using primers in the cow^{5A} deletion region (Fig. 1A). As expected, there are no PCR products from cow^{5A} or either cow Df (Fig. 1B). Next, RT-PCR tests were performed using primers spanning an exon–exon junction to ensure mRNA amplification (Fig. 1A). The RNA extraction was confirmed using primers for a control gene (dfmr1; Fig. 1C). The cow transcript in the genetic background control w^{111B} is present at similar levels in the cow^{5A} line (Fig. 1D). There is no detectable cow transcript in either of the cow Dfs, or in one of the cow^{GDP} lines (03259), and only a very faint product in the other cow^{GDP} line (12802; Fig. 1D). Thus, cow^{GDP} 03259 is an RNA null allele.

The published cow^{5A} mutation has been reported to have transcript levels similar to those of wild type, but to have no detectable Cow protein expression (Chang and Sun, 2014). We therefore next examined protein levels via Western blotting using the published, well characterized Cow antibody (Chang and Sun, 2014), as well as three new antibodies made for this study (see Materials and Methods). Cow protein has a predicted molecular weight of ~75 kDa (without HS chains) and ~100 kDa (with HS chains). The two Cow protein bands are clearly present in the w^{111B} controls and absent in both cow deficiency lines (Fig. 1E). Cow protein is also undetectable in the cow^{GDP} lines, even at heightened levels of protein loading (Fig. 1E). In stark contrast to previously published work (Chang and Sun, 2014), both Cow protein bands are present at normal levels in cow^{5A} mutants (Fig. 1E, arrows). In our studies, cow^{5A} mutants typically die as early-stage larvae, and the few escapers can be raised to the third instar only with constant care. In contrast, both cow^{GDP} protein nulls are fully adult viable, both as homozygotes and as heterozygotes over Df[619]. Thus, our evidence indicates that cow^{5A} does not affect Cow expression, but has a second site larval lethal mutation. Further, the Cow protein is not required for full adult viability. For the remainder of experiments, cow^{GDP} 03259 and cow Df[619] were used, as both show complete removal of Cow RNA and protein.

To assess Cow protein expression in controls and null mutants, we performed anti-Cow labeling and Cow-Gal4 to drive UAS-Cow::eGFP (Fig. 2). In control embryos, Cow is widely expressed, including localization in the VNC (Fig. 2A). In cow null mutants (cow^{GDP}/cow^{GDP}), antibody labeling is undetectable (Fig. 2A, right). Since Cow has a signal peptide, and has been previously established to be secreted (Chang and Sun, 2014), we tested Cow expression at the NMJ using antibody labeling with nonpermeabilizing conditions. In the w^{111B} control wandering third instar NMJ, Cow appears secreted from a dynamic subset of type 1b synaptic boutons (Fig. 2B, arrows). Cow is also present in a punctate pattern along the peripheral nerve bundle (arrowhead). In cow nulls, neuronal and synaptic antibody labeling is lost (Fig. 2B, right). Within NMJ synaptic boutons colabeled for both Cow and Wg antibody, the two secreted proteins have overlapping expression patterns, colocalizing in the extracellular synaptotomatrix surrounding the same boutons (Fig. 2C). Using Cow-Gal4 to drive a UAS-Cow::eGFP, GFP is present throughout the wandering third instar wing imaginal disk, including punctae surrounding the wing pouch (Fig. 2D, left). Cow::eGFP is also present at the NMJ in punctae within and surrounding the synaptic boutons within a single confocal slice (Fig. 2D, right). Overall, Cow is expressed in both neuronal and non-neuronal tissue in embryos, larvae, and imaginal discs, and colocalizes with Wg at the NMJ.

Presynaptic cow restricts NMJ growth and synaptic bouton formation

Wg trans-synaptic signaling regulates NMJ growth and synaptic bouton formation (Packard et al., 2002), thus we hypothesized that if Cow regulates Wg at the NMJ, Cow loss should affect the NMJ architecture. Each NMJ terminal consists of a relatively stereotypical muscle innervation pattern, with a consistent number of axon branches and large synaptic boutons (Menon et al., 2013). Wg signaling bidirectionally regulates synaptic development, with Wg knockdown decreasing NMJ synaptic bouton number and Wg OE increasing boutons (Packard et al.,
2002; Kopke et al., 2017), including an increase in satellite boutons [small boutons connected to the mature (parent) bouton or adjacent axon; Torroja et al., 1999; Gatto and Broaddie, 2008]. To test Cow requirements in synaptic architectural development, we labeled the wandering third instar NMJ. Anti-HRP was used to label the NMJ terminal by binding to extracellular fucosylated N-glycans associated with the presynaptic neural membrane (Jan and Jan, 1982; Parkinson et al., 2013). Anti-DLG was used to label the postsynaptic scaffold in the subsynaptic reticulum (SSR; Lahey et al., 1994; Parnas et al., 2001). We used cow\textsuperscript{GDP/DF} (referred to as cow null) to eliminate cow globally, and characterized cow RNAi lines (Chang and Sun, 2014) for both motor neuron (vglut-Gal4) and muscle (24B-Gal4) cell-targeted knock-down studies. Sample images and the summary of results are shown in Figure 3.

Cow restrains NMJ development, specifically restricting synaptic bouton formation. When Cow is knocked out completely, there is a clear increase in boutons in both 2002; Parkinson et al., 2013). The percentage of satellite boutons (24B-Gal4/+), 3.16 ± 1.16% vs 24B\textsuperscript{>cow}, 5.48 ± 1.58; p = 0.2486). We next assayed synaptic functional differentiation to test whether these structural changes have functional consequences.

Cow restricts presynaptic vesicle fusion and neurotransmission strength

We used the following two methods to assay NMJ synaptic functional differentiation and neurotransmission strength: (1) TEVC electrophysiology (Dani et al., 2012; Parkinson et al., 2013; Kopke et al., 2017); and (2) imaging genetically encoded calcium reporter SynapGCaMP6f (Newman et al., 2017). For assaying evoked transmission, muscle 6 was clamped (–60 mV), while the motor nerve was stimulated with a suction electrode (1.5 mM \(\text{Ca}^{2+}\)). EJC traces were recorded (0.2 Hz, 10 consecutive stimuli) to measure the average amplitude. For assaying mEJC events, spontaneous synaptic vesicle fusions were recorded, measuring frequency and amplitude. The mEJC frequency indicates presynaptic vesicular release (number of active synapses, fusion probability), and mEJC amplitude indicates number of activated postsynaptic receptors. For quantal imaging, the SynapGCaMP reporter (MHC-CD8-GCaMP6f-Sh) contains a myosin heavy chain (MHC) promoter for muscle targeting, CD8 transmembrane domain for membrane targeting, and Shaker (Sh) \(\text{K}^+\) channel C-terminal tail for postsynaptic targeting (Newman et al., 2017). By imaging transmission, we are able to specifically determine the changes in quantal activity at the convergent motor neuron inputs separately. Live-imaging recordings were made of the SynapGCaMP reporter at muscle 4, with spontaneous event frequency divided by the NMJ synaptic area, and event amplitude measured as the change in the fluorescence signal over the baseline NMJ fluorescence (\(\Delta F/F_0\)). Representative recordings and summarized data are shown in Figure 5.

With nerve stimulation, evoked transmission is clearly and consistently increased in cow nulls compared with \(w^{1118}\) controls (Fig. 5A). Quantified measurements show EJC amplitude significantly elevated (\(w^{1118}\), 175.4 ± 9.93 nA vs \(w^{\text{cow}}\), 214.6 ± 12.24; p = 0.023; \(w^{1118}\), 175.4 ± 9.93 vs \(w^{\text{cow}}\), 254.2 ± 14.99; p = 0.012; Fig. 5B). Although the \(w^{\text{cow}}\)/DF mutants show a slight increase in mEJC frequency, no change was observed in the \(w^{\text{cow}}\) nulls. We found no change in amplitude (Fig. 5C). In quantified measurements, mEJC frequency is slightly increased in homozygous mutants and more increased in the \(w^{\text{cow}}\)/DF (\(w^{1118}\), 1.396 ± 0.19 Hz vs \(w^{\text{cow}}\)/DF, 1.764 ± 0.23; p = 0.58; \(w^{1118}\), 1.396 ± 0.19 vs \(w^{\text{cow}}\)/DF, 2.41 ± 0.49; p = 0.05; Fig. 5D, left). There is no significant change in mEJC amplitude (\(w^{1118}\), 0.75 ± 0.03 nA vs \(w^{\text{cow}}\)/DF, 0.87 ± 0.06; p = 0.189; \(w^{1118}\), 0.75 ± 0.03 nA vs \(w^{\text{cow}}\)/DF, 0.72 ± 0.05; p = 0.886; Fig. 5D, right)). Neuronally targeted cow-RNAi causes an increase in mEJC frequency (vglut-Gal4/+,
Figure 3. Presynaptically secreted Cow limits NMJ synaptic bouton number. **A**, Confocal images of the muscle 4 NMJ colabeled with anti-HRP (green) to mark the presynaptic membrane and anti-DLG (red) to mark the postsynaptic domain in the genetic background control (w^1118, left) and the cow null mutant (cow^GDP/Df, right). Synaptic bouton number is shown in a scatter plot, with mean ± SEM. **B**, Representative confocal NMJ images of motor neuron-targeted Gal4 driver control (vglut-Gal4/+, left), UAS-RNAi transgene control (UAS-cow-RNAi/+, middle) and cow RNAi knockdown (vglut>cow-RNAi, right). Satellite boutons (asterisks) are shown in the inset. Right, Synaptic bouton number is shown in a scatter plot, with mean ± SEM. **C**, Representative confocal NMJ images of muscle-targeted Gal4 driver control (24B-Gal4/+, left), UAS-RNAi transgene control (UAS-cow-RNAI/+, middle) and cow RNAi knockdown (24B>cow-RNAi, right). Synaptic bouton number is quantified to the right. The p values are shown for each statistical comparison.
| Feature Structure | Comparison                  | Data structure (D’Agostino normality test) | Type of test                     | Sample size (# of NMJs, # of animals) | Mean Statistic | p Value | Outlier test |
|------------------|-----------------------------|-------------------------------------------|----------------------------------|---------------------------------------|----------------|---------|--------------|
| Bouton number    | $w^{1118}$ vs cow$^{GDF/D}$ | Normal                                    | Unpaired two-tailed t test       | $w^{1118}$ (15,8); cow null (16,8)    | t = 8.296 df = 29 | p < 0.0001 |              |
| Bouton number    | $vglut$-GAL4 vs UAS-cow-RNAi | Not normal                                | Kruskal-Wallis with Dunn’s multiple-comparisons test | $vglut$-GAL4 (16,8); UAS-Cow-RNAi (16,8) | 26.69 vs 28.8 | Mean rank diff = -2.938 | p = 0.9999 |
| Bouton number    | 24B-GAL4 vs UAS-cow-RNAi    | Not normal                                | Kruskal-Wallis with Dunn’s multiple-comparisons test | 24B-GAL4 (16,8); UAS-cow-RNAi (16,8)  | 30.63 vs 31.5 | Mean rank diff = -6.188 | p = 0.6307 |
| Bouton number    | $vglut$ vs Cow             | Normal                                    | Unpaired two-tailed t test       | $vglut$ (16,8); VGlu (16,8)           | t = 1.122 df = 30 | p = 0.2706 |              |
| Bouton number    | 24B$^+/+$ vs 24B$^−$/Cow   | Normal                                    | Unpaired two-tailed t test       | 24B$^+/+$ (16,8); 24B$^−$/Cow (16,8) | 30.38 vs 29.81 | Mean rank diff = 0.2317 df = 30 | p = 0.8183 |
| Bouton number    | FRT-Wg vs FRT-Wg; Cow      | Not normal                                | Kruskal-Wallis with Dunn’s multiple-comparisons test | FRT-Wg (24,12); FRT-Wg; Cow (24,12)    | 26.71 vs 31.71 | Mean rank diff = -22.29 | p = 0.0030 |
| Bouton number    | $w^{1118}$ vs cow$^{GDF/+}$ | Not normal                                | Kruskal-Wallis with Dunn’s multiple-comparisons test | $w^{1118}$ (15,8); cow$^{GDF/+}$ (15,8) | 28.33 vs 35.73 | Mean rank diff = -15.93 | p = 0.0929 |
| Bouton number    | $w^{1118}$ vs Notum$^{KO/+}$ | Normal                                    | Ordinary one-way ANOVA with Tukey’s multiple-comparisons test | $w^{1118}$ (18,10); cow$^{GDF/+}$ (19,10) | 22.94 vs 33.74 | q = 9.731 df = 76 p < 0.0001 |              |

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Table 1: Statistical tests used to analyze data
Table 1: Continued

| Feature | Comparison | Data structure | Type of test | Sample size | Mean | Statistic | p Value | Outlier test |
|---------|------------|----------------|--------------|-------------|------|-----------|---------|--------------|
| % Satellite Boutons | \( w^{118} \) vs \( \text{cow}^{GFP} / \text{cow}^{GFP} / \) | Normal | Unpaired two-tailed \( t \) test | \( w^{118} \) (15,8); cow null (15,8) | 3.301 vs 3.336% | \( t = 0.03021 \) df = 28 | \( p = 0.9761 \) | ROUT, \( q = 1\% \), removed 1 \( \text{cow}^{GFP} / \) \( \text{DF} \) value |
| % Satellite Boutons | vglut-GAL4/+ vs | Normal | Ordinary one-way ANOVA with Tukey’s multiple-comparisons test | vglut-GAL4/+ (15,8); UAS-Cow-RNAi/+ (15,8) | 2.895 vs 2.908% | \( q = 0.016 \) df = 42 | \( p > 0.9999 \) | ROUT, \( q = 1\% \), removed 2 vglut-->Cow-RNAI values |
| % Satellite Boutons | UAS-Cow-RNAi/+ vs vglut-GAL4/+ | Normal | Kruskal–Wallis with Dunn’s multiple-comparisons test | UAS-Cow-RNAi/+ (15,8); vglut-GAL4/+ (15,8); 24B-GAL4/+ (16,8) | 0.88 vs 2.381% | Mean rank diff = −8.656 | \( p = 0.1328 \) | |
| % Satellite Boutons | vglut-GAL4/+ vs vglut->Cow-RNAI | Normal | Mann–Whitney test | vglut->Cow (16,8); vglut-GAL4/+ (16,8) | 2.326 vs 7.121% | \( U = 38 \) | \( p = 0.0003 \) | |
| % Satellite Boutons | 24B/+ vs 24B->Cow | Normal | Unpaired two-tailed \( t \) test | 24B/+ (16,8); 24B>Cow (16,8) | 3.164 vs 5.476% | \( t = 1.177 \) df = 30 | \( p = 0.2486 \) | |
| % Satellite Boutons | FRT-Wg vs FRT-Wg; CowGFP | Not normal | Kruskal–Wallis with Dunn’s multiple-comparisons test | FRT-Wg (16,8); FRT-Wg; CowGFP (16,8) | 2.038 vs 1.002% | Mean rank diff = 5.167 | \( p > 0.9999 \) | |
| % Satellite Boutons | FRT-Wg vs NRT-Wg | Not normal | | FRT-Wg (16,8); NRT-Wg (16,8) | 2.038 vs 8.304% | Mean rank diff = −26.08 | \( p = 0.0021 \) | |
| % Satellite Boutons | FRT-Wg vs NRT-Wg; CowGFP | Not normal | | FRT-Wg (16,8); NRT-Wg; CowGFP (16,8) | 2.038 vs 3.595% | Mean rank diff = −5.452 | \( p > 0.9999 \) | |
| % Satellite Boutons | FRT-Wg; CowGFP vs NRT-Wg | Not normal | | FRT-Wg (16,8); NRT-Wg; CowGFP (16,8) | 1.002 vs 8.304% | Mean rank diff = −31.25 | \( p = 0.0001 \) | |
| % Satellite Boutons | NRT-Wg vs NRT-Wg; CowGFP | Not normal | | NRT-Wg (16,8); NRT-Wg; CowGFP (16,8) | 1.002 vs 3.595% | Mean rank diff = −10.62 | \( p > 0.9999 \) | |
| % Satellite Boutons | w\(^{118}\) vs Notum\(^{KO}\) | Not normal | Kruskal–Wallis with Dunn’s multiple-comparisons test | w\(^{118}\) (15,8); cow\(^{GFP} / \) Notum\(^{KO} / \) (15,8) | 1.89 vs 3.079% | Mean rank diff = −7.867 | \( p > 0.9999 \) | |
| w\(^{118}\) vs Notum\(^{KO}\) | w\(^{118}\) (15,8); Notum\(^{KO} / \) (16,8) | | | 1.89 vs 3.379% | Mean rank diff = −10.95 | \( p = 0.4586 \) | |
| w\(^{118}\) vs cow\(^{GFP} / \) Notum\(^{KO}\) | w\(^{118}\) (15,8); cow\(^{GFP} / \) Notum\(^{KO} / \) (16,8) | | | 1.89 vs 3.337% | Mean rank diff = −13.2 | \( p = 0.1961 \) | |
| cow\(^{GFP} / \) Notum\(^{KO}\) vs cow\(^{GFP} / \) Notum\(^{KO}\) | cow\(^{GFP} / \) (15,8); cow\(^{GFP} / \) Notum\(^{KO} / \) (16,8) | | | 3.079 vs 3.379% | Mean rank diff = −3.079 | \( p > 0.9999 \) | |
| cow\(^{GFP} / \) Notum\(^{KO}\) vs cow\(^{GFP} / \) Notum\(^{KO}\) | cow\(^{GFP} / \) (15,8); cow\(^{GFP} / \) Notum\(^{KO} / \) (16,8) | | | 3.079 vs 3.337% | Mean rank diff = −5.329 | \( p > 0.9999 \) | |
Table 1: Continued

| Feature | Comparison | Data structure (D’Agostino normality test) | Type of test | Sample size (# of NMJs, # of animals) | Mean | Statistic | p Value | Outlier test |
|---------|------------|------------------------------------------|--------------|--------------------------------------|------|----------|---------|-------------|
| w1118 vs cowGDP/NotumKO | NotumKO+/− vs cowGDP/NotumKO | Not normal | Kruskal-Wallis with Dunn’s multiple-comparisons test | NotumKO+/− (16,8); cowGDP/NotumKO (16,8) | 3.379 vs 3.337% | Mean rank diff = −2.25 | p > 0.9999 |  |
| w1118 vs NotumKO/NotumKO | cowGDP/NotumKO vs cowGDP/NotumKO | Normal | Mann-Whitney test | vglut/ (16,8); vglut/Cow (16,8) | 1 vs 3.035 | U = 0 | p < 0.0001 |  |
| Cow intensity | vglut+ vs vglut− | Normal | Mann-Whitney test | vglut+ (16,8); vglut− (16,8) | 1 vs 3.907 | U = 0 | p < 0.0001 |  |
| Wg intensity | vglut+ vs vglut− | Normal | Mann-Whitney test | vglut+ (16,8); vglut− (16,8) | 1 vs 0.6731 | U = 46 | p = 0.0014 |  |
| Brp punctae number | w1118 vs cowGDP/ | Normal | Unpaired two-tailed t test | cowGDP+/− (15,8); cowGDP− (15,8) | 1 vs 0.885 | q = 1.328 df = 56 | p = 0.7840 |  |
| Brp punctae Volume | w1118 vs cowGDP/ | Normal | Unpaired two-tailed t test | cowGDP+/− (15,8); cowGDP− (15,8) | 1 vs 1.094 df = 56 | q = 1.094 df = 56 | p = 0.8660 |  |
| Brp punctae number | w1118 vs cowGDP/ | Normal | Unpaired two-tailed t test | cowGDP+/− (15,8); cowGDP− (15,8) | 1 vs 0.9014 | q = 1.139 df = 56 | p = 0.8515 |  |
| GluR cluster number | w1118 vs cowGDP/ | Normal | Unpaired two-tailed t test | cowGDP+/− (15,8); cowGDP− (15,8) | 1 vs 0.885 | q = 2.422 df = 56 | p = 0.3268 |  |
| Function amplitude | w1118 vs cowGDP/ | Normal | Ordinary one-way ANOVA with Tukey’s multiple-comparisons test | cowGDP+/− (20,18); cowGDP− (20,18) | 171.6 vs 212.1 nA | q = 3.868 df = 53 | p = 0.0227 | ROUT, Q = 1%, removed 1 cowGDP value |
| w1118 vs cowGDP/DF | cowGDP+ vs cowGDP− | Normal | Unpaired two-tailed t test | cowGDP+/− (10,9); cowGDP− (10,9) | 171.6 vs 254.2 nA | q = 4.197 df = 53 | p = 0.0123 | ROUT, Q = 1%, removed 1 cowGDP value |

Data structure (Continued)

| Value | Outlier test |
|-------|-------------|
| N/A   |  |
| 1%    |  |

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1.5 ± 0.33 Hz vs vglut-Cow-RNAi, 2.45 ± 0.3; p = 0.045), but not amplitude (vglut-Gal4/+), 0.8 ± 0.03 nA vs vglut-Cow-RNAi, 0.85 ± 0.42; p = 0.4325). SynapGCaMP imaging also shows increased fusion frequency in type Ib boutons (Fig. 5E). In quantal imaging measurements, spontaneous fusion frequency increases (vglut-Gal4/+, 1.62 ± 0.47 Hz/\mu m^2 vs vglut-Cow-RNAi, 2.98 ± 0.36; p = 0.051; Fig. 5F, left). Interestingly, event magnitude also significantly increases (vglut-Gal4/+, 0.79 ± 0.04 \Delta F/F_0 vs vglut-Cow-RNAi, 1.06 ± 0.09; p = 0.012; Fig. 5F, right). These results demonstrate that Cow limits evoked neurotransmission strength and suggest that neuronally secreted Cow regulates synaptic vesicle fusion at the presynaptic active zone.

**Cow restricts presynaptic active zone and glutamatergic synapse formation**

We next used imaging to assay presynaptic and postsynaptic molecular components of the synapse to test the hypothesis of increased NMJ synapse number in cow mutants. The presynaptic AZ is the specialized site of SV

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**Table 1: Continued**

| Feature | Comparison | Data structure | Type of test | Sample size | Mean | Statistic | p Value | Outlier test |
|---------|------------|----------------|--------------|-------------|------|-----------|---------|--------------|
| EJC amplitude | w^{1118} vs cow^{GFP}/+ | Normal | Ordinary one-way ANOVA with Tukey’s multiple-comparisons test | w^{1118} (10,6); cow^{GFP}/+ (11,6) | 217.2 vs 234.9 nA | q = 0.9383 df = 40 | p = 0.9101 | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
| | w^{1118} | | \(w^{1118}\) | 217.2 vs 214.1 nA | q = 0.1649 df = 40 | p = 0.9994 | | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
| mEJC Frequency | w^{1118} vs cow^{GFP}/Df | Normal | Ordinary one-way ANOVA with Tukey’s multiple-comparisons test | w^{1118} (22,17); cow^{GFP}/Df (13,11) | 1.396 vs 1.765 Hz | q = 3.406 g = 53 | p = 0.0503 | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
| | cow^{GFP}/ vs cow^{GFP}/Df | Normal | Ordinary one-way ANOVA with Tukey’s multiple-comparisons test | cow^{GFP}/ (21,15); cow^{GFP}/Df (13,11) | 1.764 vs 2.41 Hz | q = 2.15 df = 53 | p = 0.2897 | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
| mEJC amplitude | w^{1118} vs cow^{GFP}/ | Normal | Ordinary one-way ANOVA with Tukey’s multiple-comparisons test | w^{1118} (21,16); cow^{GFP}/ (13,11) | 0.7518 vs 0.8682 nA | q = 2.506 df = 52 | p = 0.1889 | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
| | cow^{GFP}/ vs cow^{GFP}/Df | Normal | Ordinary one-way ANOVA with Tukey’s multiple-comparisons test | cow^{GFP}/ (21,15); cow^{GFP}/Df (13,11) | 0.7165 vs 0.8682 nA | q = 4.64 df = 52 | p = 0.8656 | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
| mEJC Frequency | vglut-GAL4/+ vs vglut>Cow-RNAi | Normal | Unpaired two-tailed t test | vglut-GAL4/+ (10,7); vglut-Cow-RNAi (11,7) | 1.497 vs 2.449 Hz | t = 2.142 df = 19 | p = 0.0454 | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
| | vglut>GAL4/+ vs vglut>Cow-RNAi | Normal | Unpaired two-tailed t test | vglut-GAL4/+ (11,7); vglut-Cow-RNAi (11,7) | 0.8015 vs 1.617 Hz | q = 0.0513 | p = 0.4325 | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
| mEJC Frequency | vglut/>+ vs vglut>Cow-RNAi | Normal | Unpaired two-tailed t test | vglut/+ (7,4); vglut>Cow-RNAi (8,3) | 3.157 vs 2.977 Hz/\mu m^2 | U = 7 | p = 0.0513 | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
| | vglut/+ | Normal | Unpaired two-tailed t test | vglut/+ (8,4); vglut>Cow-RNAi (5,3) | 0.7912 vs 1.058 \Delta F/F_0 | t = 3.013 df = 11 | p = 0.0118 | ROUT, Q = 1%, removed 1 cow^{GFP}/+ value |
fusion that mediates the release of the glutamate neurotransmitter. Brp tethers both the voltage-gated Ca\(^{2+}\) channels and SVs to the AZ, and is the best AZ marker (Hallermann et al., 2010). Each AZ directly apposes a postsynaptic GluR cluster to mediate fast neurotransmission (Schuster et al., 1991). We used colabeling with both anti-Brp (Wagh et al., 2006) and anti-GluRIIC (aka GluRIII; Marrus et al., 2004) to compare cow null mutants to w\(_{1118}\) genetic background controls. The second panel shows HRP alone with arrows indicating distinct spots of HRP accumulation, and the third panel shows HRP expression heatmap. C, Quantification of confocal fluorescence intensity for Cow (left) and Wg (right) in the two conditions shown in a scatter plot, with mean ± SEM. D, Quantification of synaptic bouton number (left) and the percentage of satellite boutons (right) in transgenic controls versus cow neuronal overexpression shown in a scatter plot, with mean ± SEM. p Values are shown for each statistical comparison.

**Figure 4.** Presynaptic Cow elevation decreases Wg and increases satellite boutons. A, Confocal images of NMJ boutons triple labeled with anti-HRP (blue), anti-Cow (green), and anti-Wg (red), and merged (far right) comparing transgenic controls (vglut-Gal4/+ ) to motor neuron Cow overexpression condition (vglut>cow). Labeling was done in the absence of detergent to reveal only secreted Cow and Wg. B, Representative muscle 4 NMJ images colabeled for presynaptic HRP (red) and postsynaptic DLG (green) comparing controls (vglut-Gal4/+ ) to neuronal Cow overexpression (vglut>cow). Asterisks indicate satellite boutons. The second panel shows HRP alone with arrows indicating distinct spots of HRP accumulation, and the third panel shows HRP expression heatmap. C, Quantification of confocal fluorescence intensity for Cow (left) and Wg (right) in the two conditions shown in a scatter plot, with mean ± SEM. D, Quantification of synaptic bouton number (left) and the percentage of satellite boutons (right) in transgenic controls versus cow neuronal overexpression shown in a scatter plot, with mean ± SEM. p Values are shown for each statistical comparison.

Brp AZ punctae are precisely juxtaposed to GluR clusters in a functional synapse (Menon et al., 2013). For better resolution to image postsynaptic GluR clusters and quantify the synaptic apposition, SIM was used (Gustafsson, 2000). To compare with previous LSM, Brp AZs were first measured to find a consistent increase in the cow null mutants, but with larger punctae numbers,
presumably due to increased resolution ($w^{118}, 298.6 \pm 17.2$ vs $cow^{GDP}, 387.9 \pm 17.86; p = 0.0019$; Fig. 6C). There is also a similar increase in GluR clusters ($w^{118}, 382 \pm 23.21$ vs $cow^{GDP}, 542.8 \pm 29.41; p = 0.0004$; Fig. 6D). Brp punctae and GluR clusters almost always partner, with rare exceptions seen at a similar frequency in controls and mutants (Fig. 6D). There are more GluR clusters than Brp punctae in both genotypes. The GluR/Brp ratio was measured to test for defects in synaptic apposition. If there is a larger ratio in the mutants compared with controls, this would indicate more GluR clusters without a Brp AZ. Conversely, a smaller ratio would indicate more GluR clusters paired with a presynaptic partner. Quantified measurements show no difference in the GluR/Brp ratio between controls and the cow null mutants ($w^{118}, 1.29 \pm 0.04$ vs $cow^{GDP}, 1.36 \pm 0.05; p = 0.272$). Together, these results demonstrate that Cow limits NMJ synapse formation, which is consistent with strengthened neurotransmission.

**Membrane-tethering Wg prevents cow null defects in NMJ development**

Our starting hypothesis was that Cow regulates Wg by binding the ligand in the extracellular space and carrying
Figure 6. Cow limits presynaptic active zones and glutamatergic synapse number. A, Representative muscle 4 NMJ images from confocal LSM of genetic background controls (w1118, left) and cow null mutants (cowGDP, right) colabeled for presynaptic membrane marker (HRP, red) and the active zone scaffold Brp (green). Brp alone is shown in right panels and the quantified Brp punctae number is shown to the right. B, High-magnification synaptic bouton images with Brp punctate identified using Imaris software (asterisks, left) and volume indicated in a heatmap (scale, 0.01–3.4 μm³; right). Quantified Brp punctae volume shown to the right. C, Representative NMJ images from a SIM of controls (w1118) and cow nulls (cowGDP) colabeled for both presynaptic active zones (Brp, red) and postsynaptic glutamate receptors (GluRIIC, green). The quantified Brp punctae number is shown to the right. D, High-magnification SIM images of juxtaposed Brp punctae and GluR clusters at synapses. Arrowheads indicate Brp or GluR domains without a partner, which are observed at equal frequency in both genotypes. Quantified GluR cluster number is shown to the right. Data shown in scatter plots, with mean ± SEM. p Values are shown for each statistical comparison.
it across the synaptic cleft (from neuron to muscle). This hypothesis is based on published work demonstrating that Cow is secreted, directly binds secreted Wg and acts to mediate intercellular transport (Chang and Sun, 2014). To test this hypothesis, we obtained transgenic lines with the wg gene cut from its native locus via FRT sites and then replaced either without (FRT-wg; transgenic control) or with (NRT-wg) a membrane tether. Importantly, HA-tagged NRT-wg is not secreted from Wg-expressing cells and fails to maintain the expression of long-range Wg targets (Alexandre et al., 2014). We tested whether tethering Wg to the membrane affects NMJ development. Comparing FRT-wg to NRT-wg, there is increased expression of the Wg ligand around presynaptic boutons (data not shown). To determine whether tethered Wg can bind Fz2 receptors, the NMJ bouton number was measured to assess presynaptic Wg signaling. Next, NRT-wg was combined with the cow null mutant (NRT-wg; cowGDP) to test the hypothesis that Cow normally acts to regulate secreted Wg function. If Wg needs to be secreted and transported dependent on Cow function, then NRT-wg and NRT-wg; cowGDP would be predicted to have the same phenotype. Representative images and summarized data are shown in Figure 7.

In comparing the control FRT-wg and tethered NRT-wg, there is no change in mature NMJ bouton number, but there is a clear increase in the number of immature satellite boutons when Wg is tethered (Fig. 7A). In quantified measurements, NRT-wg has the same number of NMJ synaptic boutons as the control (FRT-wg, 26.71 ± 1.04 vs NRT-wg, 27.04 ± 1.72; p = 0.999; Fig. 7A,B), but a fourfold increase in the percentage of satellite boutons (FRT-wg, 2.04 ± 0.77% vs NRT-wg, 8.3 ± 1.62; p = 0.0019; Fig. 7C). When membrane-tethered Wg is placed in the cow null background (NRT-wg; cowGDP), both the mature synaptic bouton number and the percentage of satellite boutons are similar to the FRT-wg control levels (Fig. 7A). In quantified measurements, the mature bouton number is no longer different between the two genotypes (FRT-wg, 26.71 ± 1.04 vs NRT-wg; cowGDP, 26.78 ± 0.97; p = 0.999; Fig. 7B; Table 1, all other comparisons), and the satellite boutons are also restored to near-normal levels (FRT-wg, 2.04 ± 0.77% vs NRT-wg; cowGDP, 3.60 ± 1.1; p = 0.999; Fig. 7C). Together, these results suggest that Cow facilitates Wg-dependent satellite bouton formation, and that Wg has to be secreted for Cow to act on it. However, in contrast to the original hypothesis, Cow acts as a negative regulator of secreted Wg signaling at the NMJ, suggesting that it should interact with other Wg-negative regulators in the extracellular synaptomatrix.

**Cow and Notum function together to restrict NMJ growth and bouton formation**

The secreted deacylase Notum has also been recently shown to regulate NMJ synaptic bouton formation via the negative regulation of Wg trans-synaptic signaling (Kopke et al., 2017). Notum restricts Wnt signaling by cleaving the Wg palmitoyl group that binds to Fz2 receptors (Kakugawa et al., 2015). In notum null mutants, NMJ Wg signaling is elevated both presynaptically and postsynaptically, resulting in increased synaptic bouton number, synapse number, and neurotransmission strength (Kopke et al., 2017). To test the hypothesis that the increased NMJ development in cow null mutants is similarly caused by an increase in Wg trans-synaptic signaling, we performed the genetic test of combining cow and notum null heterozygotes to assay effects on NMJ synaptic bouton development. The failure of mutant alleles at two different loci to complement one another is one method to test for an interaction of the gene products in a common signaling mechanism (nonallelic noncomplementation; Yook et al., 2001; Hawley and Gilliland, 2006). In this case, the interaction tests the hypothesis that Cow and Notum have closely associated functions in the regulation of Wg synaptic signaling via direct interaction with the Wg ligand in the extracellular synaptomatrix. We compared bouton formation in genetic background control (w1118); cow null (cowGDP), and notum null (notumKO) homozygotes and heterozygotes; cow/notum trans-heterozygotes; and cow/notum double null mutant (cowGDP/notumKO) in the extracellular synaptomatrix. Representative images and summarized data are shown in Figure 8.

The trans-heterozygote has a clearly expanded NMJ with more synaptic boutons compared with controls, as well as other wg mutant phenotypes such as the appearance of ghost boutons (Fig. 8A, inset). Ghost boutons are immature boutons that contain the HRP marker, but do not yet contain the postsynaptic DLG protein (Ataman et al., 2006). The cow (cowGDP/+), and cow/notum (cowGDP/+notumKO) heterozygotes alone are no different from w1118 controls and lack synaptic features of impaired Wg signaling (Fig. 8A, Table 1). In quantified measurements, trans-heterozygotes have strongly increased bouton numbers (w1118, 28.33 ± 1.46 vs cowGDP/notumKO, 46.13 ± 1.08; p < 0.0001; Fig. 8A, right; Table 1, all other comparisons). Extracellular Wg labeling without cellular permeabilization in all these genotypes indicates no difference in the Wg fluorescence intensity (Fig. 8B). In quantified measurements, there is no detectable change in Wg ligand levels between controls and cow/notum trans-heterozygotes (normalized w1118, 1.0 ± 0.09 vs cowGDP/+ notumKO); 0.9 ± 0.09; p = 0.852; Fig. 8B, right; Table 1, all other comparisons). The double null mutants have significantly increased bouton numbers compared with controls but no increase compared with each null alone (w1118, 22.94 ± 1.60 vs cowGDP+, notumKO/cowGDP, 29.13 ± 0.97; p = 0.0005; Fig. 8C, right; Table 1, all other comparisons). Interestingly, trans-heterozygotes show no change in nerve stimulation-evoked EJC recordings (Table 1). These results indicate that Cow and Notum act in the same pathway to restrict Wg signaling in structural development, and that the level of extracellular Wg ligand alone is not predictive of signaling activity.

**Discussion**

The function of signaling ligands in the extracellular space is tightly regulated to ensure coordinated intercellular development, often via glycan-dependent mechanisms (Dani and Broadie, 2012; Parkinson et al., 2013; Shilts and Broadie, 2017). The most recently discovered
Drosophila HSPG, secreted Cow, was characterized with this role (Chang and Sun, 2014). In the developing wing disk, the Wnt Wg is produced in a stripe of cells at the dorsal/ventral margin boundary, and acts as an intercellular morphogen through Fz2 receptor signaling (Bhanot et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1997). The glypican HSPGs Dally and Dlp, bound to outer plasma membrane leaflets via GPI anchors, bind Wg to regulate both ligand distribution and intercellular signaling (Tsuda et al., 1999; Baeg et al., 2001; Dani et al., 2012; Dear et al., 2017). It has been proposed that Dally/Dlp HSPGs are involved in the movement of extracellular Wg to form a morphogen gradient (Han et al., 2005). However, in dally dl dip double mutant clones, extracellular Wg is detected far away from Wg-secreting cells, suggesting that another extracellular factor can transport Wg. Cow was shown to fill this role by binding extracellular Wg to increase stability and rate of movement from producing to receiving cells (Chang and Sun, 2014). Supporting this model, cow mutants manifest Wg ligand gain-of-function/overexpression phenotypes for short-range targets, and loss-of-function phenotypes for long-range targets.

At the NMJ, such a long-range Wg morphogen transport function is not seemingly required, except perhaps as a clearance mechanism, but Wg extracellular regulation and short-range Wg transport to cross the synaptic cleft is critical for NMJ development (Packard et al., 2002; Friedman et al., 2013; Dear et al., 2016; Parkinson et al., 2016). At the forming of NMJ, Wg from neurons and glia signals both presynaptically (neuronal) and postsynaptically (muscle) via Fz2 receptors (Packard et al., 2002; Kerr et al., 2014). In the motor neuron, Wg signaling inhibits the GSK3β homolog Sgg to regulate the MAP1B homolog Futsch to modulate microtubule dynamics controlling NMJ bouton formation (Miech et al., 2008). However, Futsch distribution and microtubule dynamics do not change with elevated Wg signaling (Kopke et al., 2017), so this pathway alone does not explain the increased bouton formation with increased Wg signaling. In the postsynaptic muscle, Wg signaling drives Fz2 endocytosis and C-terminus cleavage, with transport to the nucleus regulating mRNAs involved in synaptogenesis, including postsynaptic GluR distribution (Speese et al., 2012). In wg mutants, GluRs are more diffuse; with clusters irregular in size/shape, increased receptor numbers and a larger postsynaptic volume (Packard et al., 2002; Speese et al., 2012; Kerr et al., 2014). Thus, Wg trans-synaptic signaling controls both NMJ structure and function.

Based on the findings from Chang and Sun (2014), we hypothesized that Cow binds Wg to facilitate the transport across the synapse to Fz2 receptors on the muscle. If this is correct, we would expect a presynaptic Wg OE

**Figure 7.** Membrane-tethered Wg prevents cow null defects in bouton formation. **A**, Representative confocal images of muscle 4 NMJs colabeled with presynaptic HRP marker (green) and postsynaptic DLG marker (red) in Wg control (FRT-wg), cow null (FRT-wg; cow<sup>GDP</sup>), tethered Wg (NRT-wg), and tethered Wg in cow null background (NRT-wg; cow<sup>GDP</sup>). **B, C**, Quantification of total NMJ synaptic bouton number (B) and the percentage of satellite boutons (C) in the four genotypes shown in a scatter plot, with mean ± SEM. *p* Values are shown for each statistical comparison.
phenotype in the absence of Cow (Wg buildup at the source), and a postsynaptic Wg decrease/loss phenotype (failure of Wg transport). Presynaptically, we find increased synaptic bouton number in cow null mutants phenocopying the Wg OE condition (Kopke et al., 2017), consistent with this hypothesis. These results indicate that Cow normally inhibits NMJ bouton formation, consistent with the effects of inhibiting presynaptic Wg.

Figure 8. Cow and Notum act in the same Wg pathway to limit NMJ bouton number. 

A, Confocal images of the muscle 4 NMJ colabeled with presynaptic HRP marker (green) and postsynaptic DLG marker (red) in the genetic background control (w[1118]), cow null heterozygote (cow[GD;]+), notum null heterozygote (notum[KO;]+), and cow/notum transheterozygote (cow[GD;]/notum[KO;]). Quantified bouton number is shown to the right. 

B, High-magnification NMJ confocal images of anti-Wg labeling at synaptic boutons of the same indicated genotypes. The presynaptic HRP marker boundary is outlined in white. Quantified Wg fluorescence intensity is shown to the right, normalized to the background control (w[1118]). 

C, Confocal images of the muscle 4 NMJ colabeled with presynaptic HRP marker (green) and postsynaptic DLG marker (red) in the genetic background control (w[1118]), cow null (cow[GD;]cow[GD;]), notum null (notum[KO;]notum[KO;]), and cow/notum double null (cow[GD;]cow[GD;]notum[KO;]notum[KO;]). Quantified bouton number is shown to the right. Data shown in scatter plots, with mean ± SEM. *p Values are shown for each statistical comparison.
signaling (Packard et al., 2002). Postsynaptically, we find an increased number of GluR clusters due to elevated synapse formation in cow null mutants, but no evidence of diffuse GluR clusters of irregular size/shape and larger volume, as has been reported in wg mutants (Packard et al., 2002; Speese et al., 2012; Kerr et al., 2014). Therefore, we do not find strong support for the second prediction of the hypothesis. GluR changes within single postsynaptic domains are challenging to see even with enhanced resolution microscopy (e.g., the SIM used here; Gustafsson, 2000), but future studies could focus more on GluRIIA cluster size/shape/intensity in cow mutants. If GluR defects are detected in cow nulls, it would be interesting to test the FNI pathway (Mathew et al., 2005).

Wg signaling regulates multiple steps of NMJ development including branching, satellite bouton budding, and synaptic bouton maturation (Koles and Budnik, 2012). None of the cow manipulations cause changes in branching, indicating that Cow does not regulate this Wg signaling, likely working in concert with other Wg regulators. Wg loss (wgDf) decreases bouton formation (Packard et al., 2002), while neural Wg OE increases branching, satellite, and total bouton numbers (Packard et al., 2002; Miech et al., 2008; Kopke et al., 2017). Satellite boutons represent an immature stage of development, with small boutons connected to the mature (parent) bouton or adjacent axon (Torroja et al., 1999; Gatto and Brodie, 2008). Neuronal Cow OE does not change mature bouton number, but increases satellite bouton budding. Neuronal Cow RNAi also increases satellite boutons. Thus, changing neural Cow levels in either direction elevates satellite bouton numbers, suggesting different consequences on budding versus developmental arrest. It also appears that the cellular source of secreted Cow, or the balance between budding sources, may be important for proper Wg regulation. Importantly, glia-secreted Wg regulates distinct aspects of synaptic development (Kerr et al., 2014), with loss of glial-derived Wg accounting for some, but not all, of Wg mutant phenotypes. Similarly, cell-targeted cow manipulations cause different NMJ phenotypes. There is no evidence for normal Cow function in postsynaptic muscle, but it remains possible that Cow secreted from glia could regulate Wg trans-synaptic signaling.

Increasing Wg signaling elevates evoked transmission strength and functional synapse number (Kopke et al., 2017), which is phenocopied in cow null mutants. Block of postsynaptic Wg signaling causes increased SV fusion frequency and amplitude of miniature excitatory junctional potentials (Speese et al., 2012). With neuronal cow RNAi, there is a similar increase in event frequency and amplitude. These results suggest a decrease in postsynaptic Wg signaling when cow is lost, supporting the Wg transport hypothesis. Blocking Wg secreted from neurons or glia increases muscle GluR cluster size, albeit with differential effects on neurotransmission efficacy (Kerr et al., 2014). Reducing neuronal Wg has no effect on mEJC frequency, but reducing glial-derived Wg increases SV fusion frequency (Kerr et al., 2014). Both nerve-evoked and spontaneous neurotransmission are increased in cow null mutants, together with increased Brp active zones and postsynaptic GluR clusters forming supernumerary synapses. SynapGCaMP is an exciting new tool to test function at individual synapses (Newman et al., 2017). With targeted neuronal cow RNAi, there is an increase in both the number of SV fusion events and the postsynaptic Ca2+ signal amplitude, which is consistent with both presynaptic and postsynaptic regulation of Wg signaling (Packard et al., 2002; Speese et al., 2012; Kerr et al., 2014). These functional phenotypes, combined with coordinated changes in presynaptic and postsynaptic formation, suggest Cow regulates trans-synaptic Wg transport.

There were differences between spontaneous synaptic vesicle fusion findings between TEVC electrophysiological recordings and SynapGCaMP reporter (MHC-CDB-GCaMP6f-Sb) Ca2+ imaging (Newman et al., 2017). Motor neurons that presynaptically targeted cow RNAi showed stronger impacts on SV fusion frequency with imaging in contrast to recordings, comparable to effects in cowGDP null mutants. Moreover, SynapGCaMP imaging revealed significantly larger SV fusion event magnitudes in contrast to the lack of change found with TEVC recording. While the basis of these differences in unknown, we speculate that it is due to the differential nature or sensitivity of these two methods. The Ca2+ imaging is based on measuring the change in the fluorescence signal over the baseline NMJ fluorescence (AF/F0; Newman et al., 2017), and it may be that glutamate receptor Ca2+ permeability or intracellular Ca2+ signaling dynamics is changed in a way not directly related to detectable membrane current changes in the cow mutants. TEVC recordings capture whole NMJ activity, whereas with imaging we only captured type 1b bouton activity normalized to area. In future studies, SynapGCaMP imaging can be used to map spatial changes in synapse function by assaying quantal activity separately in convergent type 1s and 1b motor neuron inputs and within discrete synaptic boutons (Newman et al., 2017). Moreover, differences between cowGDP and cowGDP/Df conditions could be influenced by second site-enhancing mutations on the Df chromosome. Overall, it should be noted that the changes in spontaneous SV fusion frequency and amplitude in cow mutants are subtle and variable, and need to be further studied in the future.

Wg is lipid modified via palmitoylation to become strongly membrane associated (Zhai et al., 2004). The hydrophobic moiety is located at the interface of Wg and Fz2 binding, shielded from the aqueous environment by multiple extracellular transporters until signaling interaction with the receptor (Takada et al., 2017). There have been many modes of extracellular Wg transport demonstrated, primarily from work in the wing disk, including microvesicles, lipoproteins, exosomes, and cytoneme membrane extensions (Greco et al., 2001; Panáková et al., 2005; Gross et al., 2012; Huang and Komberg, 2015). These multiple mechanisms of transport are much less studied at the synapse; however, exosome-like vesicles containing the Wg-binding protein Evenness Interrupted (Evi) have been demonstrated at the Drosophila NMJ (Korkut et al., 2009). Cow could be considered an alternative extracellular Wg transport method (Chang and Sun,
acting to shield Wg while facilitating transport through the extracellular synaptomatrix (Dani and Broadie, 2012; Dear et al., 2016). In addition, HSPGs have been shown to regulate ligands by stabilizing, degrading, or sequestering the ligand, or as bifunctional coreceptors, or as facilitators of transcytosis (Lin, 2004; Dani et al., 2012; Dear et al., 2017). Results presented here are consistent with the hypothesis that Cow is mediating Wg transport across the NMJ synapse (Chang and Sun, 2014), but also that Cow has an additional role in the negative regulation of Wg synaptic signaling.

The need for secreted Wg has been recently challenged, with Wg tethering to the membrane (NRT-wg) showing Wg secretion to be largely dispensable for development (Alexandre et al., 2014). In contrast, other recent studies suggest that Wg release and spreading is necessary (Beaven and Denholm, 2018; Pani and Goldstein, 2018; Stewart et al., 2019). We find tethering Wg at the NMJ synapse increases extracellular Wg ligand levels, with no change in mature bouton numbers. This Wg accumulation shows that NRT-wg is more stable at the synaptic signaling interface, consistent with other studies (Morata and Struhl, 2014; Chaudhary et al., 2019). However, although Wg levels increase, Wg signaling is less effective. With NRT-wg, only the budding of new satellite bouton is increased, with no increase in mature bouton formation. Reducing Wg function causes Fz2 upregulation (Cadigan et al., 1998; Chaudhary et al., 2019), so we hypothesize that Wg signaling could be maintained by increased presynaptic Fz2 receptors. When Wg is tethered, Cow cannot mediate intercellular transport, so the hypothesis predicts a similar phenotype with Cow (NRT-wg) or without Cow (NRT-wg; cow<sup>GDP</sup>). Indeed, Cow removal in the NRT-wg condition does not impact synaptic bouton number, although it does block the increase in satellite boutons, consistent with a Cow role in greater Wg stability (Chang and Sun, 2014). These results show that Wg secretion is required for the elevated NMJ development characterizing cow mutant animals.

To further test how Cow is working through the Wg pathway to negatively regulate NMJ development, we turned to genetic interaction tests with the Wg-negative regulator Notum (Gerlitz and Basler, 2002; Giráldez et al., 2002; Kakugawa et al., 2015). At the NMJ, Wg trans-synaptic signaling is elevated in the absence of Notum, and null notum mutants display larger NMJs with more synaptic boutons, increased synapse number and elevated neurotransmission (Kopke et al., 2017). All these defects are phenocopied by neuronal Wg OE, showing that the positive synaptogenic phenotypes arise from lack of Wg signaling inhibition. Consistently, genetically correcting Wg levels at the synapse in notum nulls alleviates synaptogenic phenotypes (Kopke et al., 2017). We show here that cow null mutants have the same phenotypes of expanded NMJs, supernumerary synaptic boutons, greater synapse number/function, and strengthened transmission, suggesting that Cow acts like Notum in regulating Wg signaling. We performed a genetic test to ask whether Cow and Notum work in this same pathway. While cow and notum null heterozygotes do not exhibit NMJ defects, cow/notum trans-heterozygotes display grossly expanded NMJs with excess boutons. This combined haplo-insufficiency (type 3 SSNC) of nonallelic noncomplementation suggests that Cow and Notum share related roles (Yook et al., 2001; Hawley and Gilliland, 2006). When we tested full double mutants, there is no additive effect, showing that Cow and Notum restrict Wg signaling in the same pathway. However, this pathway convergence appears restricted only to the control of structural synaptogenesis but not of functional neurotransmission, although the control neurotransmission amplitude was elevated in these studies.

Cow now joins the list of synaptic HSPGs with key roles in NMJ development. HSPGs have been implicated in vertebrate NMJ synapse formation for >3 decades (Kamimura and Maeda, 2017; Condomitti and de Wit, 2018). The Agrin HSPG is secreted from presynaptic terminals to maintain postsynaptic acetylcholine receptor clustering (Godfrey et al., 1984; Hubbard and Gnanasambandan, 2013). Another secreted HSPG, perlecan, regulates acetylcholinesterase localization (Peng et al., 1999; Arikawa-Hirasawa et al., 2002). Drosophila NMJ analyses have begun to more systematically elucidate HSPG roles in NMJ formation and function (Ren et al., 2009; Kamimura and Maeda, 2017). In particular, the glypican HSPG Dlp regulates Wg signaling to modulate both NMJ structure and function, including the regulation of active zone formation and SV release (Johnson et al., 2006; Dani et al., 2012; Friedman et al., 2013; Dear et al., 2017).

Wg binds the core Dlp, with HS chains enhancing this binding, to retain Wg on the cell surface, where it can both compete with Fz2 receptors and facilitate Wg–Fz2 binding (Yan et al., 2009). This biphasic activity depends on the ratio of Wg, Fz2, and Dlp HSPG as expounded in the “exchange factor model” (Yan et al., 2009; Dear et al., 2016). Cow may impact this exchange factor mechanism as a fourth player, acting with Dlp to modulate Wg transport and Wg–Fz2 binding at the synaptic interface. It will be important to test Dlp levels and distribution in cow nulls to see how Cow fits into this model.

In addition to Cow, perlecan (Trol) is another secreted HSPG reported to regulate bidirectional Wg signaling at the Drosophila NMJ (Kamimura et al., 2013). Trol has been localized near the muscle membrane, where it promotes postsynaptic Wg accumulation. In the absence of Trol, Wg builds up presynaptically, causing excess satellite bouton formation (Kamimura et al., 2013). It is interesting to note that cow mutants enhance Wg signaling without increasing satellite boutons. In trol mutants, ghost boutons increase due to decreased postsynaptic Wg signaling (Kamimura et al., 2013). Note that cow mutants do not exhibit ghost boutons, which fails to support decreased postsynaptic Wg signaling. Other postsynaptic defects in trol mutants (e.g., reduced SSR, increased postsynaptic pockets; Kamimura et al., 2013) are NMJ ultrastructural features that could be a future focus using electron microscopy studies. Similar to cow mutants, extracellular Wg levels are decreased in the absence of Trol, speculated due to increased Wg proteolysis, since HS protects HS-binding proteins from degradation (Saksela et al., 1988). In cow mutants, it is not yet known whether
Wg is decreased due to elevated signaling (ligand/receptor endocytosis) or to increased degradation due to Cow no longer protecting/stabilizing the ligand. Given that synaptic Fz2 is internalized with Wg binding (Mathew et al., 2005), future experiments could test internalized Fz2 levels in cow mutants as a proxy of Wg signaling.

In summary, we have confirmed here new tools to study Cow HSPG function, and have discovered that Cow from presynaptic motor neurons restricts NMJ bouton formation, glutamatergic synapse number, and NMJ functional differentiation. Cow acts within the same Wg trans-synaptic signaling pathway as Notum by regulating the Wg ligand in the extracellular synaptomatrix. Secreted Cow modulates extracellular Wg ligand levels, with additional functions controlling Wg signaling efficacy, which may be independent of or dependent on Wg transport. It will be interesting to determine whether Cow core protein and/or its HS chains are important for the synaptic structural and functional phenotypes. Wg must be secreted for Cow to act on it, as shown by the membrane-tethered interaction studies, showing that secreted Cow must work on the freely diffusible Wg ligand. Perhaps most informative for our future studies will be dissection of the interactions, coordination or redundancy of the multiple synaptic HSPGs at the NMJ, to further the understanding of exacellular Wg trans-synaptic signaling regulation during synaptic development. 

*Drosophila* is a particularly well suited model to study HSPGs because of the relatively reduced complexity in this system (17 HSPGs in mammals vs 5 HSPGs in *Drosophila*; Sarrazin et al., 2011). We look forward to expanding future studies to examine multiple synaptic HSPGs in parallel, with the goal of elucidating the surprisingly complex control of trans-synaptic signaling occurring within the extracellular synaptomatrix.

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