RESEARCH ARTICLE

DEVELOPMENT

Glutamate signaling at cytoneme synapses

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We investigated the roles of components of neuronal synapses for development of the *Drosophila* air sac primordium (ASP). The ASP, an epithelial tube, extends specialized signaling filopodia called cytonemes that take up signals such as Dpp (Decapentaplegic, a homolog of the vertebrate bone morphogenetic protein) from the wing imaginal disc. Dpp signaling in the ASP was compromised if disc cells lacked Synaptobrevin and Synaptotagmin-1 (which function in vesicle transport at neuronal synapses), the glutamate transporter, and a voltage-gated calcium channel, or if ASP cells lacked Synaptotagmin-4 or the glutamate receptor GluRII. Transient elevations of intracellular calcium in ASP cytonemes correlate with signaling activity. Calcium transients in ASP cells depend on GluRII, are activated by L-glutamate and by stimulation of an optogenetic ion channel expressed in the wing disc, and are inhibited by EGTA and by the GluR inhibitor NASPM (1-naphthylacetyl spermine trihydrochloride). Activation of GluRII is essential but not sufficient for signaling. Cytoneme-mediated signaling is glutamatergic.

The organization and development of metazoan tissues requires signaling proteins such as bone morphogenetic protein (BMP), fibroblast growth factor (FGF), epidermal growth factor (EGF), wingless/integrated (Wnt), and Hedgehog (Hh). The *Drosophila* air sac primordium (ASP) is a single-cell-thick epithelial tube of the larval tracheal system whose growth and morphogenesis are a response, in part, to Decapentaplegic (Dpp, a BMP) and Branchless (Bnl, a FGF homolog) (*1*, *2*). The ASP does not produce these proteins but does express the receptors for Dpp (Thickveins) and Bnl (Breathless), and it receives Dpp and Bnl produced and secreted by the wing imaginal disc. The ASP develops adjacent to the wing disc and receives Dpp and Bnl at distances of 5 to 40 μm. The proteins appear to transfer to the ASP at cell-cell contacts. Specialized filopodia called cytonemes extend from ASP cells and contact the disc cells (*2*, *3*).

Cytoneme-mediated signaling has also been implicated in *Drosophila* tissues such as the wing disc epithelium (*4*–*8*), abdominal histoblasts (*6*), testis (*9*), and the hematopoietic stem cell niche (*10*), and in vertebrate embryos and cultured cell systems (*11*). In signal-producing cells, cytoneme-mediated signaling is dependent on the signaling proteins, as well as on proteins that modify the signaling proteins and on proteins that deliver them for release to the cytonemes (*12*). In cells that are situated between cells that produce signaling proteins and the cells that extend cytonemes to receive them, components of the extracellular matrix and of the planar polarity system are required (*6*, *13*). In receiving cells, signaling protein receptors and cytoskeletal, cell adhesion, and vesicle processing proteins are required (*2*, *5*–*7*, *12*, *13*). In the ASP, mutant cells deficient for these proteins do not have cytonemes that extend to contact the disc, do not take up the signaling proteins, do not activate Dpp and Bnl signal transduction, and do not develop a normal ASP (*2*, *4*, *13*, *14*).

The basic outlines of cytoneme-mediated and neuronal signaling are similar—both processes involve cell extensions that contact target cells where signals are exchanged. Several proteins that are required for cytoneme-mediated signaling in the ASP [e.g., Capricious (a transmembrane cell adhesion protein), Neuroglian (an immunoglobulin and fibronectin superfamily transmembrane protein), and Shibire (a dynamin)] are also implicated in neuronal synapse formation (*15*). The inward-rectifying potassium channel Irk2 is necessary for normal Dpp release by wing disc cells (*16*). These features invite the question of whether there are more extensive and deeper homologies (*17*).

We focused on similarities between cytoneme contacts where signaling proteins transfer to receiving cells and neuronal synapses where neurotransmitters released by presynaptic cells are taken up by postsynaptic cells. We present genetic, histological, and functional evidence showing that components of the presynaptic and postsynaptic compartments of neuronal synapses, as well as Ca^{2+} influx, are essential for cytoneme-mediated signaling in the ASP.

Calcium transients in cells and cytonemes of the *Drosophila* ASP

The ASP of the late third instar larva has ~120 cells that form a tube with a narrow proximal stalk, a bulbous medial region, and a rounded distal tip (Fig. 1, A and B). The ASP is not radially symmetric: the cells closest to the disc epithelium (lower-layer cells) have a smaller circumference than the cells that are not juxtaposed to the disc (upper-layer cells). Cells in the lower-layer medial region show Dpp signaling [detected with a *dad*–green fluorescent protein (GFP) transgene that contains a promoter fragment from *daughters against Dpp*, a transcription target of Dpp signaling] (Fig. 1C). Upper- and lower-layer cells at the tip show Bnl signaling [detected by immunolocalization of double phosphorylated extracellular signal–regulated kinase (ERK)] (Fig. 1C). Cytonemes that extend from the lower medial region contain the Dpp receptor and take up Dpp from the disc; cytonemes that extend from the tip contain the Bnl receptor and take up Bnl from the disc (Fig. 1, A and B′) (*4*). Cytonemes that take up signaling proteins contact the disc at distances comparable to a synaptic cleft, as indicated by GRASP (GFP reconstitution across synaptic partners) fluorescence (Fig. 1D) (*2*). The GRASP system presents two complementary fragments of GFP expressed on different cells; the fragments are tethered to transmembrane domains and are extracellular. GRASP fluorescence marks sites of stable, close juxtaposition (~20 to 40 nm) that allow interaction of the GFP fragments and fluorescence. The system was developed to label synaptic contacts (*18*).

To investigate calcium signaling in the ASP, we expressed the genetically encoded calcium sensor GCaMP6 (see supplementary materials) in the ASP and detected low fluorescence in most ASP cells (Fig. 1, E to E′′′′, and movie S1). Approximately one cell per two ASPs had brighter fluorescence that did not vary during an observation period of 8 min, but approximately six cells in every ASP (n = 10 specimens) had transient flashes of brighter fluorescence that averaged ~35 s. GCaMP6 fluorescent transients were detected in the proximal stalk (which has 10 cells), in the upper-layer medial region between the stalk and tip (which has 50 cells), the upper-layer tip (which has 10 cells), the lower-layer-medial region (which has 40 cells), and the lower-layer tip (which has 9 cells) (Fig. 1F). The frequencies with which we detected bright transients ($\Delta F/F \geq 30\%$) in the lower-layer cells of the tip and medial region were approximately the same (0.15 and 0.18, respectively; n = 10) (Fig. 1G); these cells also showed Bnl (tip) and Dpp signaling (medial region). In upper-layer optical sections, transient flashes at the tip (0.2) were approximately three times more frequent on a per-cell basis than in either the upper medial region (0.06) or stalk (0.06; n = 10) (Figs. 1G and 2I). The upper medial and stalk cells show less Bnl and Dpp signaling than cells in either the tip or lower medial regions. These observations indicate that calcium oscillations were more frequent in cells that also showed Dpp and Bnl signaling. Expression in the ASP of RNA interference (RNAi) directed against calmodulin, a ubiquitous, calcium-binding protein, perturbed

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ASP morphogenesis (fig. S1A), suggesting that calcium signals may influence ASP cells that are active in signal transduction during ASP development.

We also analyzed changes to calcium concentration in ASP cytonemes by imaging GCaMP6 fluorescence. However, because the fluorescence of GCaMP6 in ASP cytonemes was too low to detect (fig. S2), we constructed a modified sensor that tethers GCaMP6 to the C-terminal cytoplasmic domain of the vesicular synaptic protein Synaptotagmin-4 (Syt4). The design was based on the possibility that this domain of Syt4 might concentrate the chimeric GCaMP6 in cytonemes. To correlate GCaMP6 fluorescence with the roles of ASP cytonemes in Dpp and FGF signaling, we expressed Syt4:GCaMP6 together with the Dpp receptor Thickveins fused to mCherry (Tkv:mCherry) or with the Bnl receptor Breathless fused to mCherry (Btl:mCherry). We imaged 24 preparations from animals that expressed Syt4:GCaMP6 and Tkv:mCherry, and we imaged 33 preparations from animals that expressed Syt4:GCaMP6 and Btl:mCherry. In these genotypes, uniform fluorescence marked both cell bodies and cytonemes, but time-lapse imaging detected bright fluorescent transients in ASP cytonemes (average duration: ~25 s) (movies S2 and S3). Every cytoneme marked by Syt4:GCaMP6 transients had an average of two to three transients during the period of observation (8 min, 5-s exposure interval). These transients report spikes of intracellular Ca^{2+} concentration in the cytonemes, most likely due to influx of extracellular Ca^{2+}.

The cytonemes with Ca^{2+} transients also had motile puncta containing either Tkv or Btl (Fig. 1H and movies S2 and S3). Puncta of Tkv and Btl are present in cytonemes that contact target disc cells; motile Tkv and Btl puncta have not been detected in cytonemes that do not contact disc cells (*2*). Although our images did not reveal how the Ca^{2+} transients relate to puncta motility or ligand uptake, the Ca^{2+} transients were observed only in cytonemes with motile Tkv- or FGF receptor–containing puncta and not in cytonemes that lacked motile puncta. This suggests that signal uptake and transport in cytonemes may be associated with local changes in intracellular Ca^{2+} concentration.

Calcium oscillations in the ASP linked to cytoneme-mediated signaling

To investigate the basis for transient changes in intracellular Ca^{2+} concentration in the ASP, we perturbed maintenance of intracellular Ca^{2+} by suppressing production of the endoplasmic reticulum adenosine triphosphatase SERCA, which allows Ca^{2+} uptake into the endoplasmic reticulum. We expressed an RNAi targeted to SERCA (*19*). ASP growth was inhibited and ASP morphogenesis was perturbed (Fig. 2, A and B), the number of ASP cytonemes decreased (Fig. 2C), and calcium transients in ASP cells were eliminated (zero transients $\Delta F/F \geq 0.3$ in eight ASPs) (Fig. 2D and movie S4). In addition, Dpp signal transduction in the ASP, which is essential for

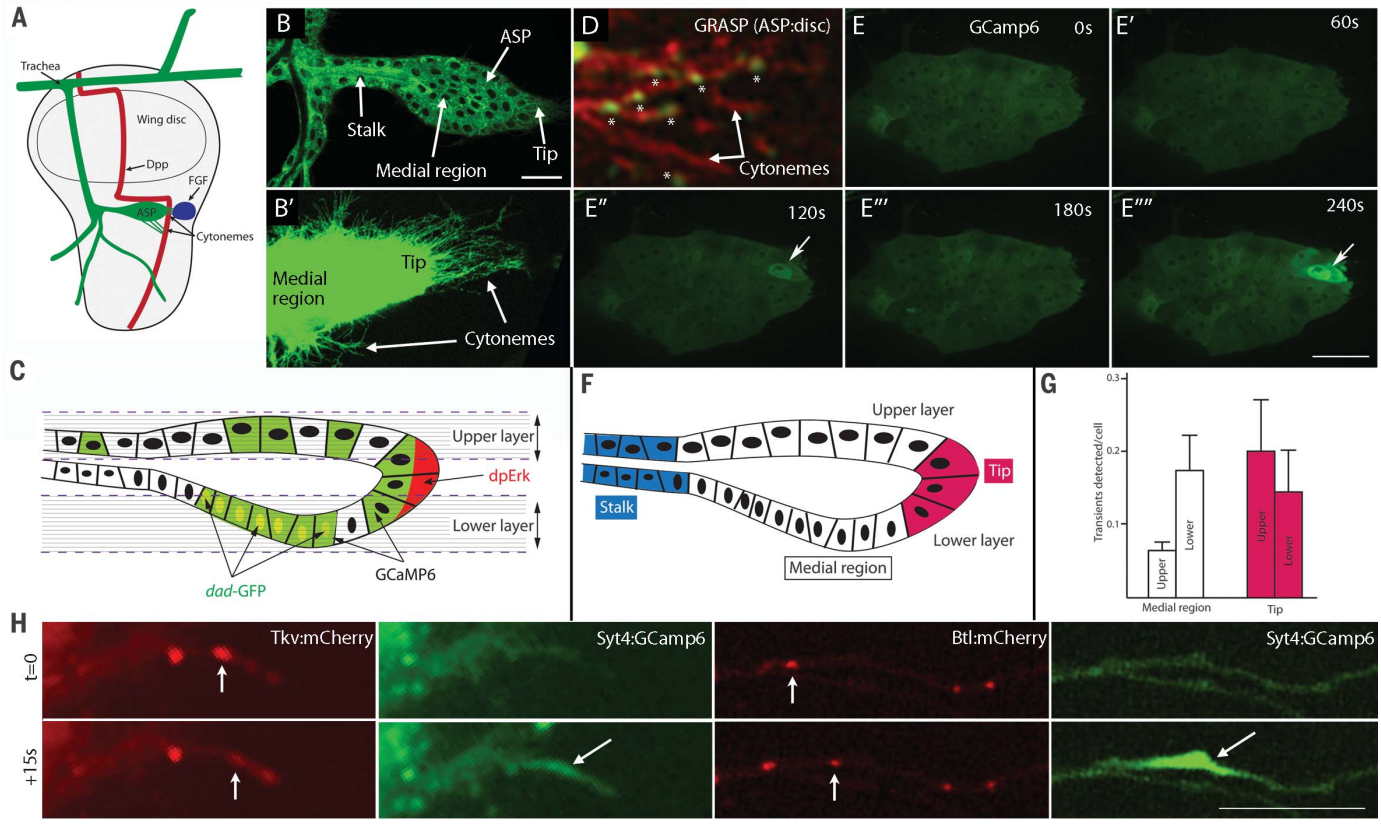

Fig. 1. Calcium transients in the ASP and ASP cytonemes. (**A**) Cartoon showing a wing imaginal disc and associated trachea, with Dpp- (red) and FGF-expressing (blue) cells indicated. (**B**) ASP expressing CD8:GFP from a late third-instar larva. (**B′**) Cytonemes labeled by CD8:GFP extending from the ASP. (**C**) Cartoon showing sagittal view of the ASP. Dashed lines represent approximate locations of the upper and lower optical sections. FGF-responsive cells are at the tip of the ASP [double phosphorylated ERK (dpErk), red], and Dpp-responsive cells are at the medial region (*dad*-GFP, green). (**D**) Contacts that ASP cytonemes (asterisks, red) make with disc cells visualized by GRASP (green). (**E** to **E⁗**) Calcium transients in an ex vivo–cultured ASP expressing GCaMP6. Arrows point to the region of calcium increase. (**F**) Schematic drawing of an ASP with stalk (blue), medial region (white), and tip (red) indicated. (**G**) Bar graph showing numbers of GCaMP6 transients per ASP cell. Error bars represent SD. (**H**) Frames from movies S2 and S3. Tkv:mCherry and Btl:mCherry receptors (indicated by arrows and imaged at an interval of 15 s) coinciding with calcium transient (green) in ASP cytonemes. Scale bars: 30 μm (B), 30 μm (E⁗), and 15 μm (H). Genotypes: (B and B′) *btl-Gal4 UAS-CD8:GFP/+*; (D) *btl-Gal4 dpp-LHG/UAS-CD8:Cherry; UAS-CD4:GFP$^{1\text{-}10}$ lexO-CD4:GFP^{11}/+*; (E to E⁗) *btl-Gal4 UAS-GCaMP6/+*; (H) *btl-Gal4/+; UAS-syt4:GCaMP6/UAS-Tkv:mCherry;* and *btl-Gal4 UAS-Btl:mCherry/+; UAS-syt4:GCaMP6/+*.

normal ASP growth and morphogenesis and is cytoneme-dependent (*2*), decreased (monitored by *dad*>GFP fluorescence) (Fig. 2, A′ and B′). Because cytonemes extend and retract rapidly (*6*) and because the contacts they make are short-lived (*6*), this steady-state measure of cytoneme number does not distinguish between effects on rates of cytoneme extension and retraction or on contact duration. Reducing SERCA did not change the numbers of dividing and apoptotic cells in the ASP (fig. S3).

To investigate whether extracellular calcium is required for the Ca^{2+} transients, we decreased extracellular calcium concentration with EGTA, a Ca^{2+} chelator. Incubation of ASP preparations with EGTA eliminated calcium transients in ASP cells (zero transients $\Delta F/F \geq 0.3$; $n = 5$) and ASP cytonemes (zero cytoneme transients $\Delta F/F \geq 0.3$; $n = 8$) (Fig. 2D and movie S4). In the absence of EGTA, time-lapse imaging detected motile Tkv:mCherry puncta that moved in both anterograde and retrograde directions at ~0.41 μm/s, reaching distances of 10 to 20 μm (Fig. 2E and movie S5). In the presence of EGTA (Fig. 2E′ and movie S6), Tkv:mCherry puncta had a more limited range of movement (all < 5 μm). Thus, motility of Tkv puncta in cytonemes may depend on uptake of extracellular Ca^{2+}.

To investigate the process that generates transient increases in intracellular Ca^{2+} concentration, we expressed RNAi constructs directed against the transient receptor potential (Trp) channels or GluR calcium-permeable receptors. We targeted 13 identified *Drosophila* Trp family genes, one of which [*inactive* (*iav*)] promotes Ca^{2+} release from the endoplasmic reticulum and is required for synapse development and neurotransmission (*20*). Expression in the ASP of RNAi directed against each of the 13 Trp family

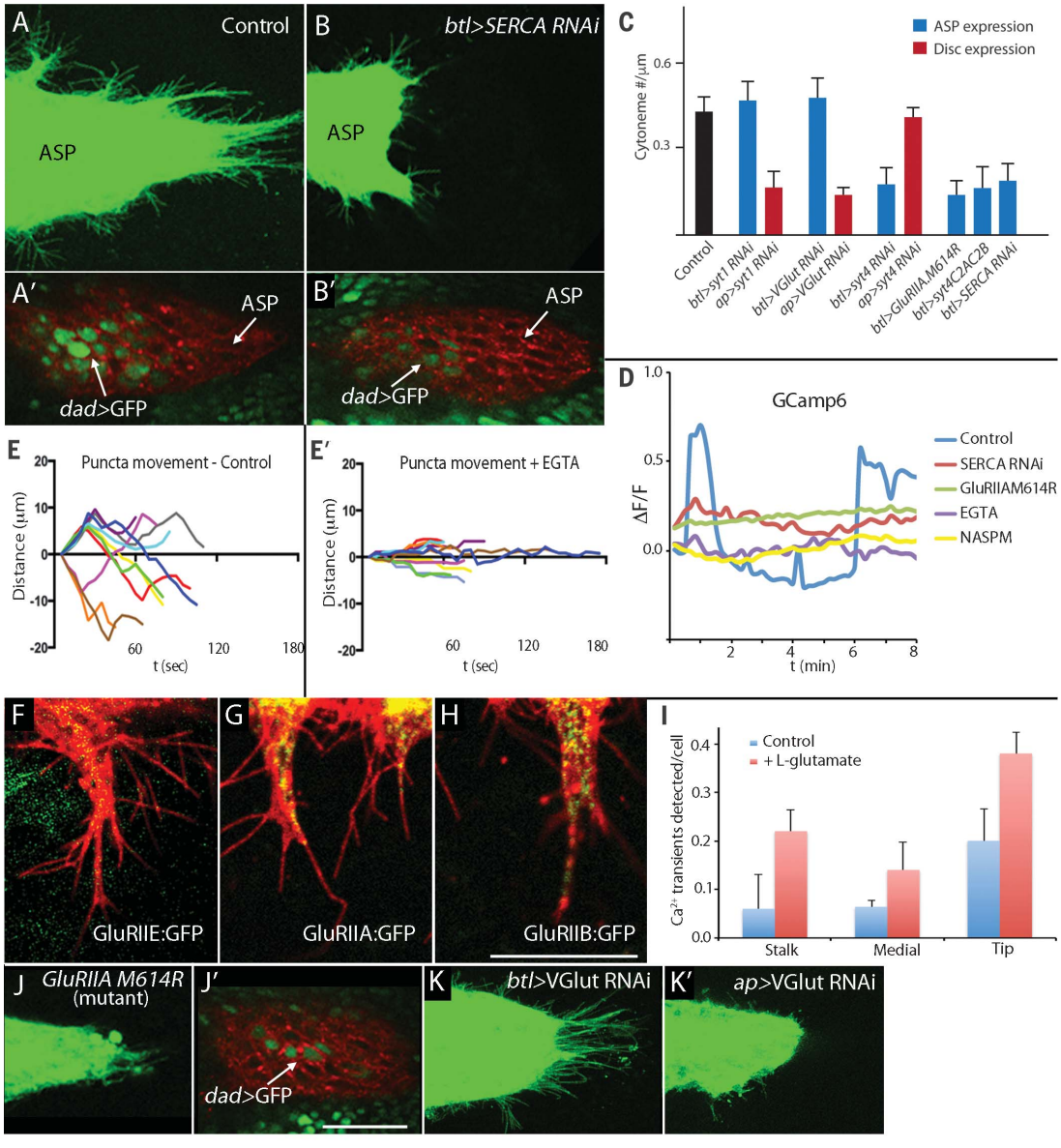

Fig. 2. Cytoneme-mediated transport requires SERCA and GluR. (**A** to **B′**) Dependence of cytoneme-mediated Dpp signaling on SERCA. Down-regulation of SERCA perturbed ASP development and decreased the number of ASP cytonemes [(A) and (B)] and expression of *dad*-GFP [(A′) and (B′)]. (**C**) Bar graph plots the relative number of cytonemes in ASPs of control, *SERCARNAi*, *GluRIIA.M614R*, *syt1RNAi*, *syt4RNAi*, *syt4 C2A C2B*, and *VGlutRNAi* larvae; *P* values for difference between ASP and disc expression: $P < 0.001$ (*SERCARNAi*), $P < 0.0001$ (*GluRIIA.M614R*), $P < 0.001$ (*syt1RNAi*), $P < 0.001$ (*syt4RNAi* and *syt4 C2A C2B*), and $P < 0.0001$ (*VGlutRNAi*). Error bars represent SD. (**D**) Traces of Ca^{2+} transients in control, SERCA RNAi, GluRIIA.M614R, EGTA-treated, and NASPM-treated ASPs. (**E** and **E′**) Kymographs depict the displacement of 10 Tkv:mCherry puncta in control (E) and in presence of 2 mM EGTA (E′). (**F** to **H**) Localization of GluRIIE:GFP (F), GluRIIA:GFP (G), and GluRIIB:GFP (H) in ASP cytonemes. (**I**) Graph representing the number of calcium transients per cell in the stalk, medial, and tip regions of the upper layer of the ASP with standard deviation indicated; *P* values for difference between control and L-glutamate conditions: stalk ($P < 0.001$), medial ($P < 0.01$), and tip ($P < 0.001$) regions. Error bars represent SD. (**J** and **J′**) Expression of *GluRIIA.M614R* decreased the number of ASP cytonemes and expression of *dad*-GFP. (**K** and **K′**) Expression of VGlutRNAi in the disc perturbed the ASP (K′) but expression in the ASP did not (K). Scale bars: 15 μm (H) and 30 μm (J′). Genotypes: (A) *btl-Gal4 UAS-CD8GFP/+*; (A′) *btl-Gal4 UAS-CD8Cherry/Gal80^{ts}; dad-GFP/+*; (B) *btl-Gal4 UAS-CD8GFP/+; UAS-SERCA RNAi/+*; (B′) *btl-Gal4 UAS-CD8Cherry/Gal80^{ts}; dad-GFP/SERCARNAi*; (J) *btl-Gal4 UAS-CD8GFP/ UAS-GluRIIA.M614R*; (J′) *btl-Gal4 UAS-CD8Cherry/UAS-GluRIIA.M614R; dad-GFP/Gal80^{ts}*; (K) *btl-Gal4 UAS-CD8GFP/+; UAS-VGlut RNAi/+*; (K′) *ap-Gal4/+; btl-LHG lexO-CD2GFP/UAS-VGlut RNAi.*

genes did not affect ASP growth or ASP cytonemes (fig. S1, B to N). RNAi-mediated depletion of the *N*-methyl-D-aspartate (NMDA)–type glutamate receptor was also without apparent effect (fig. S1O). In contrast, the number of ASP cytonemes was lower than normal and morphogenesis of the ASP was abnormal in preparations in which the non-NMDA ionotropic glutamate receptor (GluRII) was decreased (Fig. 2C).

GluRII controls calcium permeability and has been implicated in retrograde signaling at the *Drosophila* neuromuscular junction (*21*, *22*). Isoforms of GluR have three common subunits (GluRIIC, GluRIID, and GluRIIE) and a fourth subunit that is either GluRIIA or GluRIIB (*23*). We examined GFP-tagged GluR subunits to monitor GluR presence in the ASP, characterizing a GluRIIE construct expressed at endogenous levels by a fosmid transgene, and GluRIIA:GFP and GluRIIB:GFP that were ectopically overexpressed. Fluorescence of GluRIIE:GFP expressed in endogenous amounts and fluorescence of overexpressed GluRIIA:GFP and GluRIIB:GFP were detected in ASPs and in ASP cytonemes (Fig. 2, F to H). These observations suggest that GluR is present in the ASP and in ASP cytonemes.

GluRIIA.M614R is a mutant GluR subunit that lacks channel conductance and acts as a dominant-negative receptor that decreases quantal size of excitatory junctional potentials (*23*). Whereas expression of RNAi transgenes that target either GluRIIC or GluRIID had small effects on the shape of the ASP and on the number of ASP cytonemes (fig. S1, P and Q), the presence of exogenous GluRIIA.M614R in the ASP decreased the number of ASP cytonemes, eliminated calcium transients in the ASP (zero transients $\Delta F/F \geq 0.3$ in eight ASPs) (movie S4), perturbed ASP growth and morphogenesis, and decreased Dpp signaling (Fig. 2, A , A′, C, J, and J′). Expression of GluRIIA.M614R did not change the numbers of dividing and apoptotic cells in the ASP (fig. S3). In ex vivo cultures of ASP preparations, the presence of 1-naphthylacetyl spermine trihydrochloride (NASPM), a pharmacological inhibitor of GluR (see supplementary materials), also eliminated ASP calcium transients (zero transients $\Delta F/F \geq 0.3$ per ASP; $n = 5$) (Fig. 2D and movie S4). In contrast, activation of GluR by the addition of L-glutamate (1 mM) to ex vivo cultures of ASPs increased the number of ASP calcium transients (13 transients per ASP; $n = 5$). The increase in calcium transient number affected all regions of the ASP, including the stalk, upper-layer medial region, and tip, and was not restricted to the lower layer or tip, which have active calcium transients in control conditions (Fig. 2I and movie S7). Exogenously added L-glutamate increased both the amplitude by a factor of 2.3 to 4.1 and frequency of calcium transients by a factor of 1.7 to 2.7.

The effect of exogenous glutamate in the ASP suggests that the presynaptic wing disc cell might release glutamate. To test this, we decreased expression of the vesicular glutamate transporter (VGlut) in either disc cells or ASP cells (Fig. 2, K and K′). Whereas decreasing abundance of VGlut in the ASP had no apparent effect on ASP morphology or on the number of ASP cytonemes, targeting VGlut in the disc decreased the number of ASP cytonemes and resulted in a deformed, truncated ASP (Fig. 2, C, K, and K′). Together with the observations that calcium transients in the ASP increased in response to L-glutamate and decreased in the presence of the GluR inhibitor NASPM, these data suggest possible signaling by glutamate at cytoneme synapses.

Postsynaptic functionality of Syt4 and Neuroligin-2 in cytoneme-mediated signaling

Vesicle fusion and receptor internalization in neuronal presynaptic and postsynaptic compartments involve Synaptotagmin Ca^{2+}-binding proteins. In *Drosophila*, the Syt1 isoform is the sensor for presynaptic vesicle exocytosis (*24*), and the Syt4 isoform in postsynaptic compartments is

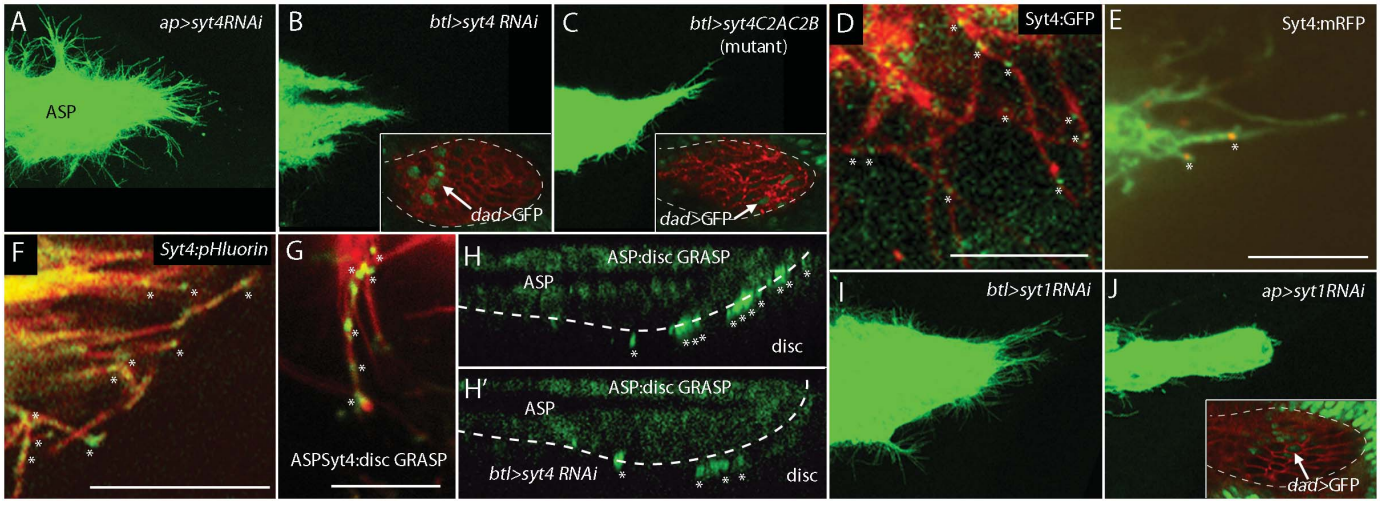

Fig. 3. Cytoneme-mediated transport requires Syt4. (**A** and **I**) Syt1 in ASP or Syt4 in disc is dispensable for the ASP. (**B**, **C**, and **J**) ASP cytonemes and expression of *dad*-GFP (insets, arrows) were decreased with expression of syt1-RNAi in the disc, and syt4-RNAi or a mutant form of Syt4 that lacked Ca^{2+}-binding sites in the ASP. *dad*-GFP analysis was performed in preparations expressing RNAi for 24 hours (by repressing $Gal80^{ts}$ conditionally). (B and J) The ASP depends on Syt1 in wing disc and Syt4 in ASP. ASPs marked by membrane-tethered GFP and expressing RNAi constructs in either the ASP (driven by *btl-Gal4*) or dorsal compartment of the wing disc (driven by *ap-Gal4*). (**D**) Syt4:GFP in ASP cytonemes. Asterisks indicate Syt4:GFP puncta. (**E**) Image showing localization of Syt4:mRFP in ASP cytonemes. Asterisks indicate Syt4:mRFP puncta. (**F**) Syt4-pHlourin fusion protein expressed in the ASP. Asterisks indicate fluorescence of Syt4-pHlourin in ASP cytonemes. (**G**) GRASP fluorescence with Syt4:GFP^{1-10} in the ASP and GFP^{11} in *dpp*-expressing wing disc cells. Asterisks indicate syt4GRASP fluorescence in ASP cytonemes. (**H** and **H′**) Sagittal images showing GRASP fluorescence at contacts (asterisks) between ASP cytonemes and Dpp-expressing cells. ASPs are outlined by dashed white lines. Scale bars: 15 μm (E); 10 μm [(D), (F), and (G)]. Genotypes: (A) *ap-Gal4/+; btl-LHG lexO-CD2:GFP/UAS-syt4RNAi*; (B) *btl-Gal4 UAS-CD8GFP/+; UAS-syt4RNAi/+*; (B, inset) *btl-Gal4 UAS-CD8Cherry/Gal80^{ts}; dad-GFP/syt4RNAi*; (C) *btl-Gal4 UAS-CD8GFP/+; UAS–syt4[C2A D4N, C2B D3,4N]/+*; (C, inset) *btl-Gal4 UAS-CD8Cherry/+; dad-GFP/UAS–syt4[C2A D4N, C2B D3,4N]*; (D) *btl-Gal4 UAS-CD8Cherry/+; syt4:GFP/+*; (E) *btl-Gal4 UAS-CD8GFP/+; UAS-syt4:mRFP/+*; (F) *btl-Gal4 UAS-CD8Cherry/+; UAS-Syt4-pHluorin/+*; (G) *btl-Gal4 dpp-LHG/UAS-CD8:Cherry; UAS-syt4:GFP$^{1-10}$/lexO-CD4:GFP^{11}*; (H) *btl-Gal4 dpp-LHG/+; UAS-CD4:GFP$^{1-10}$ lexO-CD4:GFP^{11}/+*; (H′) *btl-Gal4 dpp-LHG/+; UAS-CD4:GFP$^{1-10}$ lexO-CD4:GFP^{11}/UAS-syt4RNAi*; (I) *btl-Gal4 UAS-CD8GFP/+; UAS-syt1RNAi/+*; (J) *ap-Gal4/+; btl-LHG lexO-CD2:GFP/UAS-syt1RNAi*; (J, inset) *ap-Gal4/Gal80^{ts}; dad-GFP/UAS-syt1RNAi*.

involved in retrograde signaling (*25*). To investigate a possible role of Syt4 in cytoneme-mediated signaling, and whether that role is specific for the postsynaptic compartment, we expressed a Syt4-specific RNAi in either the ASP or wing disc. Whereas ASPs developed normally when Syt4 RNAi was expressed in the wing disc (Fig. 3A), expression of Syt4 RNAi in the ASP decreased the number of ASP cytonemes (Fig. 2C), decreased Dpp uptake (fig. S4) and Dpp signal transduction (Fig. 3B), and resulted in abnormally shaped ASPs (Fig. 3B). ASP expression of a mutant Syt4 that does not bind Ca^{2+} (*26*) decreased Dpp signal transduction and produced abnormally shaped ASPs (Fig. 3C); and in the viable $Syt4^{BA1}$ null mutant, adult dorsal air sacs were decreased in size and malformed (fig. S5). These observations indicate that Syt4 may act specifically in the ASP and contribute to Dpp signaling.

To determine whether Syt4 can be localized to cytonemes, we examined several engineered forms of Syt4. Syt4:GFP is a fusion protein generated by CRISPR-mediated insertion of GFP into the endogenous *syt4* gene; it has wild-type Syt4 function and is expressed in endogenous amounts (*27*). Cytonemes extending from ASPs that express Syt4:GFP and CD8:mCherry had multiple fluorescent GFP puncta (Fig. 3D). Overexpression of a Syt4:mRFP fusion protein (*btl-Gal4 UAS-Syt4:mRFP*) also revealed the presence of exogenous Syt4 in puncta in ASP cytonemes (Fig. 3E).

To investigate the topology of exogenously expressed Syt4 in the cytoneme membrane, we expressed Syt4-pHluorin, a Syt4 chimera tagged with a pH-sensitive variant GFP (*26*). pHluorin fluorescence is not detectable at the low pH of the interior of intracellular vesicles but increases by a factor of ~20 at neutral pH and is detectable when exposed to the extracellular environment. Expression of exogenous Syt4-pHluorin in the ASP produced spots of bright GFP fluorescence along ASP cytonemes (Fig. 3F), suggesting that if endogenous Syt4 is in cytonemes, it may be membrane-associated and exposed to the extracellular space.

The spots with Syt4-pHluorin fluorescence did not move in ex vivo cultures, in contrast to the cytoneme-associated puncta containing fluorescent Tkv or Btl (movies S2 and S3). To characterize these spots further, we constructed a Syt4:GFP^{1-10} chimera and applied the GRASP technique (*18*). GRASP can identify the proximity of ASP cytonemes to cells that express GFP^{11}, the peptide that complements GFP^{1-10} (*17*). Expression of Syt4:GFP^{1-10} in the ASP and of CD4:GFP^{11} in the *dpp* expression domain of the wing disc produced GFP fluorescence at discrete spots along ASP cytonemes (Fig. 3G). This pattern of GRASP fluorescence is similar to the pattern of Syt4:pHlourin fluorescence and is consistent with the possibility that the Syt4 extracellular domain is exposed on the cytoneme surface. It is also consistent with the possibility that ASP cytonemes make multiple contacts with disc cells. Multiple contacts are thought to exist between cytonemes

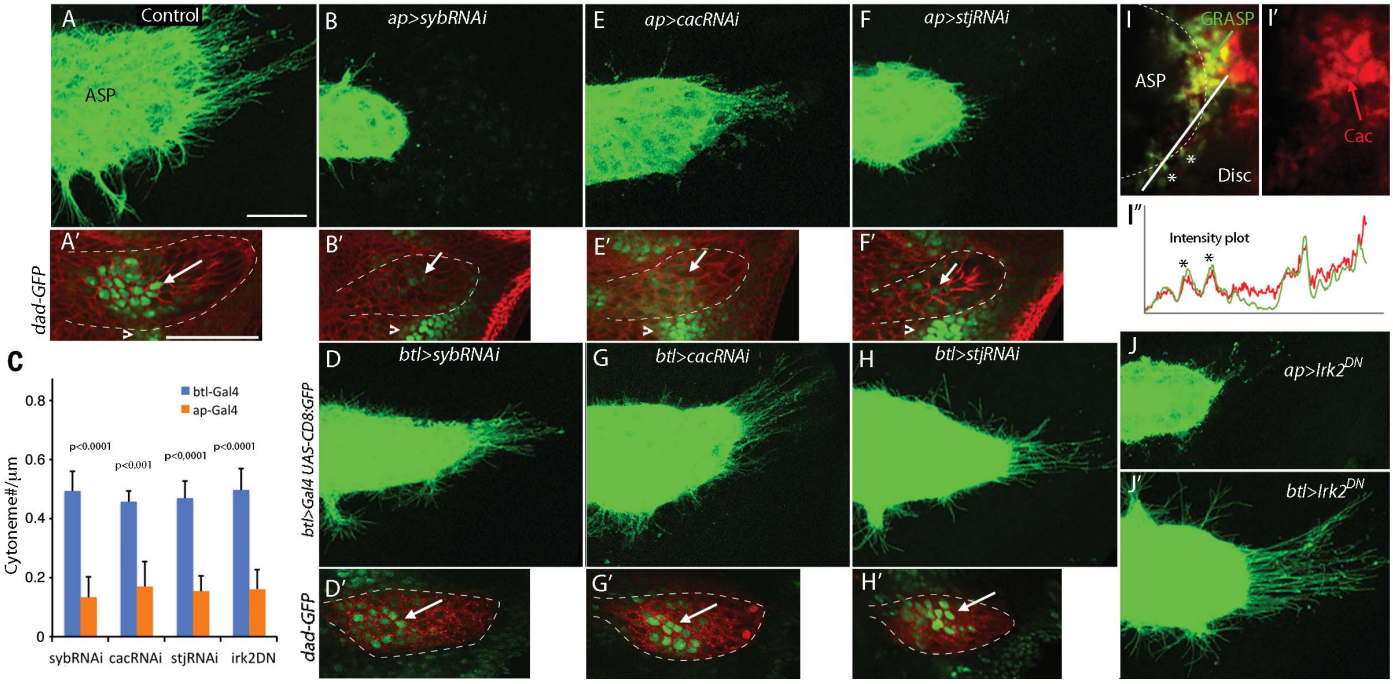

Fig. 4. The ASP depends on the VGCC and Syb SNARE in the wing disc. (**A** to **C** and **E** to **F'**) Down-regulation in the wing disc of the Cac and Stj subunits of the VGCC and of Syb decreased the number of ASP cytonemes and expression of *dad*-GFP. (C) Bar graph plots the relative number of cytonemes in ASPs under conditions of *sybRNAi, cacRNAi, stjRNAi,* and $irk2^{DN}$ expression in the wing disc (*ap-Gal4*) or ASP (*btl-Gal4*). *P* values for difference between disc and ASP expression for each genotype. Error bars represent SD. (**D**, **D'**, and **G** to **H'**) Expression of *cacRNAi, stjRNAi,* or *sybRNAi* in the ASP did not affect the ASP or cytoneme-mediated Dpp signal transduction. Arrows point to the *dad*-GFP expression in the ASP and arrowheads point to its expression in the disc. Scale bars: 25 µm (A) and 50 µm (A'). (**I** to **I''**) Cac:TdTomato and GRASP fluorescence coincide at contacts between ASP cytonemes and FGF-expressing wing disc cells. (I) Merged GFP + TdTomato fluorescence. ASP tip outlined with dashed white line, Asterisks indicate coinciding points of GRASP and TdTomato fluorescence; (I') TdTomato fluorescence; (I'') intensity plot generated with ImageJ of GFP and TdTomato fluorescence in 3-pixel-wide stripe (white line in I), normalized to equivalent minimum and maximum values. (**J** and **J'**) Expression of $irk2^{DN}$ in the disc perturbed the ASP (J') but expression in the ASP did not (J). Genotypes: (A) *ap-Gal4/+; btl-LHG lexO-CD2:GFP/+*; (A') *ap-Gal4/+; dad-GFP/+*; (B) *ap-Gal4/UAS-sybRNAi; btl-LHG lexO-CD2:GFP/+*; (B') *ap-Gal4/UAS-sybRNAi; dad-GFP/+*; (D) *btl-Gal4 UAS-CD8GFP/UAS-sybRNAi; $Gal80^{ts}$/+*; (D') *btl-Gal4 UAS-CD8Cherry/sybRNAi; dad-GFP/$Gal80^{ts}$*; (E) *ap-Gal4/UAS-cacRNAi; btl-LHG lexO-CD2:GFP/+*; (E') *ap-Gal4/UAS-cacRNAi; dad-GFP/+*; (F) *ap-Gal4/UAS-stjRNAi; btl-LHG lexO-CD2:GFP/+*; (F') *ap-Gal4/UAS-stjRNAi; dad-GFP/+*; (G) *btl-Gal4 UAS-CD8GFP/UAS-cacRNAi*; (G') *btl-Gal4 UAS-CD8Cherry/cacRNAi; dad-GFP/+*; (H) *btl-Gal4 UAS-CD8GFP/UAS-stjRNAi*; (H') *btl-Gal4 UAS-CD8Cherry/stjRNAi; dad-GFP/+*; (I to I'') *UAS-CD4:GFP^{1-10} lexO-CD4:GFP^{11}/+; btl-LHG bnl-Gal4/UAS-cac-TdTomato*. (J) *ap-Gal4/UAS-$irk2^{DN}$; btl-LHG lexO-CD2GFP/$Gal80^{ts}$*; (J') *btl-Gal4 UAS-CD8GFP/UAS-$irk2^{DN}$; $Gal80^{ts}$/+*.

that extend from cells of the wing disc that produce the Hh signaling protein and wing disc cells that receive Hh (*7*).

To determine whether endogenous Syt4 may be required for the contacts that ASP cytonemes make with disc cells, we compared the GRASP GFP fluorescence associated with ASP cytonemes in animals with normal or decreased amounts of Syt4. In controls, expression of CD8:GFP$^{1\text{-}10}$ in the ASP and of CD8:GFP^{11} in the *dpp* expression domain of the disc generated bright GFP fluorescence concentrated in the area between the lower layer of the ASP and the disc (Fig. 3H). Expression of Syt4RNAi in the ASP decreased GRASP fluorescence by ~75% ($P < 0.001$; $n = 5$) (Fig. 3H′). This result is similar to the effect on GRASP fluorescence of mutants that lack normal Capricious function in ASP cytonemes (*2*).

Neuroligins are synaptic adhesion proteins that participate in neuronal synapse formation in vertebrates and *Drosophila* (*28*). *Drosophila* has four Neuroligin orthologs (Nlg1 to Nlg4) (*28*). We depleted Nlg2 in the ASP by expression of Nlg2-RNAi and observed that the number of ASP cytonemes was decreased and the morphology of the ASP was abnormal (fig. S1S). In contrast, expression of Nlg2-RNAi in the wing disc (fig. S1T) or expression of Nlg1-RNAi in the ASP (fig. S1U) had no apparent effect. These results indicate that Nlg2 may be required for normal ASP formation.

Synaptic functions in signal protein–producing wing disc cells

To investigate whether functions that are required in the presynaptic compartment of neuronal synapses also function in cytoneme-dependent signaling, we analyzed animals defective for Syt1, Synaptobrevin (Syb/dVAMP). To investigate the role of Syt1, we expressed a Syt1-specific RNAi construct. Whereas Syt1 RNAi expression in the ASP had no apparent effect, its expression in the wing disc decreased the number of ASP cytonemes, decreased Dpp signal transduction in the ASP, and resulted in ASPs with abnormal morphology (Figs. 2C and 3, I and J). Quantitative polymerase chain reaction (qPCR) analysis (fig. S6) indicated that *syt1* is expressed in the wing disc. These findings suggest that Syt1 may contribute to the disc component of the cytoneme synapse.

Vertebrate Syb is a R-SNARE family component of neurotransmitter-containing vesicles that mediates the rapid docking response to inflow of Ca^{2+} ions in the active zone of presynaptic compartments (*29*). *Drosophila* has two homologs, one of which (n-Syb) is specific to neurons. n-Syb is functionally interchangeable with the other (Syb), which is expressed ubiquitously and has a general role in membrane trafficking (*30*). In the wing pouch primordium of the wing disc, Syb is required for Wingless signaling (*31*), and expression of sybRNAi in Hh-producing cells decreases both Hh signaling and the number of cytonemes produced by Hh-receiving cells (*8*). Wing disc expression of sybRNAi stunted ASP growth, decreased the number of ASP cytonemes, and decreased Dpp signal transduction (Fig. 4, A to C). In contrast, expression of this sybRNAi in the ASP was without effect (Fig. 4, D and D′). These results show that the defects caused by sybRNAi are tissue specific, and they also suggest

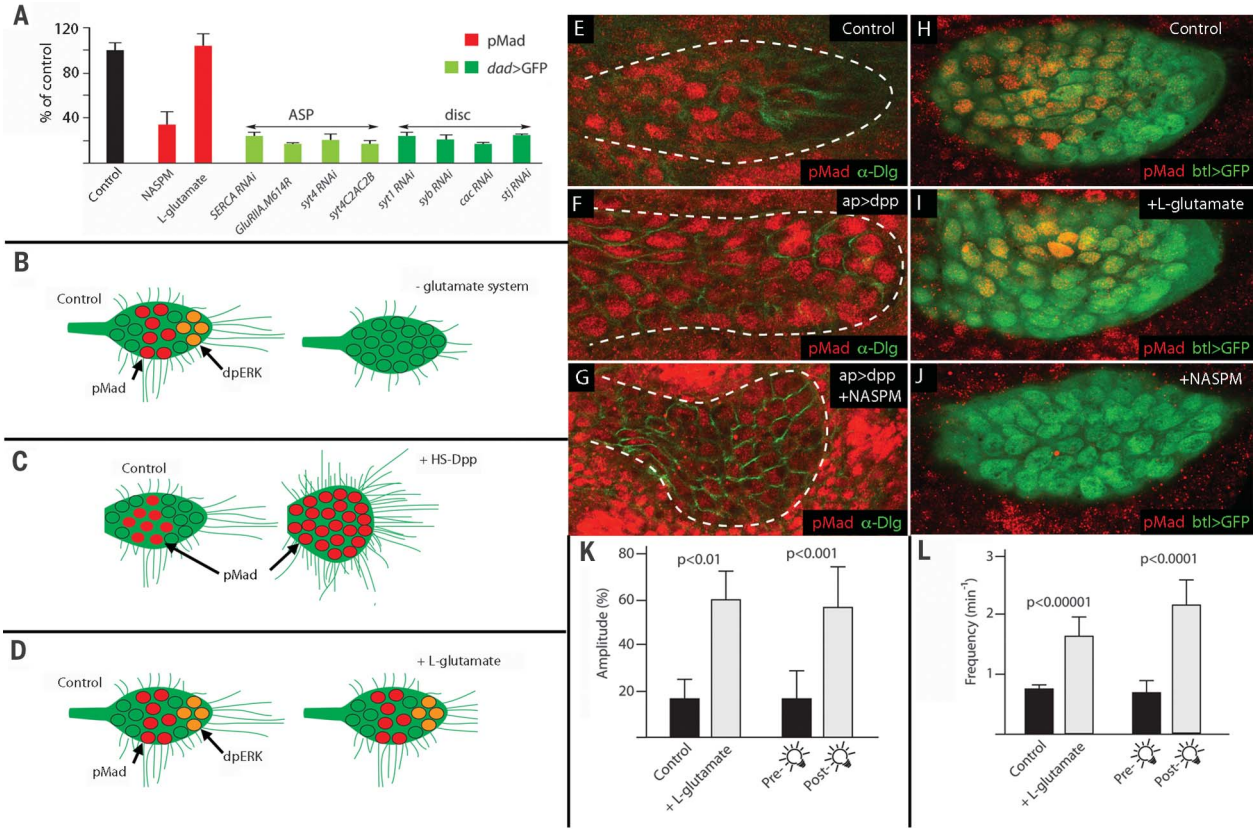

Fig. 5. Dependence of Dpp signaling on glutamate and synaptic proteins. (**A**) Bar graph showing amount of Dpp signal transduction in the ASP as indicated by immunohistological staining for pMad or expression of *dad*-GFP, shown as fold changes relative to respective control. Error bars represent SD. (**B** to **D**) Drawings depicting changes of ASP cytonemes, signaling and morphology in response to (B) glutamate system inhibition, (C) ectopic Dpp overexpression (*4*), and (D) addition of L-glutamate. (**E** to **G**) Increased Dpp signal transduction (anti-pMad staining) in the ASP in response to ectopic Dpp expression in the disc (F) was blocked by presence of NASPM (G) (see supplementary materials and methods for protocol for conditional expression of *dpp*). (**H** to **J**) Dpp signal transduction in ASP preparations incubated for 3 hours in mock (H), in 1 mM L-glutamate (I), or in 10 μM NASPM (J). (**K** and **L**) Response of ASP to L-glutamate and photostimulation of disc cells; amplitude (K) and frequency (L) of Ca^{2+} transients. Error bars represent SD. Genotypes: [(F) and (G)] *ap-Gal4/+; UAS-dpp/tub-Gal80^{ts}*; [(H) to (J)] *btl-Gal4; UAS-GFP*; [(K) and (L)] *btl-Gal4 UAS-GCaMP6/+* for L-glutamate and *ap-Gal4/+; btl-LHG lexO-GCaMP6/UAS-Chrimson* for photostimulation.

that the requirement for Syb function is specific to the wing disc side, a property that recalls the presynaptic role of n-Syb in neurons.

The voltage-gated calcium channel (VGCC) is an essential component of the neuronal presynaptic compartment; it initiates neurotransmitter exocytosis in response to membrane depolarization. The *Drosophila* genes *straightjacket* (*stj*) and *cacophony* (*cac*) encode two of the four subunits of the channel (*32*, *33*). Using qPCR analysis, we determined that *cac* and *stj* are expressed in the wing disc (fig. S6) and observed that expression in the wing disc of RNAi constructs targeting either *cac* or *stj* transcripts decreased the numbers of ASP cytonemes, changed ASP morphology, and decreased Dpp signal transduction in the ASP (Fig. 4, C and E to F′). Control ASPs were normal in animals with the same genotype but lacked either RNAi transgene (Fig. 4A). Expression of the *cac* and *stj* RNAi constructs in the ASP was without apparent effect (Fig. 4, G to H′). Thus, ASP cytonemes and Dpp signaling in the ASP require the VGCC specifically in the wing disc.

To determine whether exogenously expressed VGCC can be positioned at ASP–wing disc contacts, we expressed a functional, fluorescent-tagged Cac transgene in the *dpp*-expressing cells of wing disc together with GRASP constructs that marked the cytoneme apposition. In developing neurons, this tagged Cac protein localized to the presynaptic active zone (*34*). In the wing disc, exogenous Cac was present at sites of contact between ASP cytonemes and wing disc cells (Fig. 4, I to I″). These results, and the fact that suppression of *stj* transcripts by RNAi caused phenotypes that were similar to the stj^2 mutant (Fig. 4F and fig. S1V), are consistent with the possibility that the VGCC functions in the wing disc cells for Dpp signaling from the wing disc to the ASP. Given the role of the VGCC at the neuronal synapse, we speculate that cytoneme signaling might also be sensitive to membrane depolarization.

We investigated the inward-rectifying potassium channel Irk2 for a role in signaling by the wing disc. Irk2 regulates Dpp release from wing disc cells and wing disc development and participates in the generation of calcium transients in the wing disc (*16*). We detected calcium transients in the wing disc (movie S9), and expression of a dominant-negative mutant Irk2 in the wing disc decreased the number of ASP cytonemes and changed the shape of the ASP (Fig. 4J) in ways that are consistent with inadequate Dpp signaling. In contrast, expression of $Irk2^{DN}$ in the ASP was without effect (Fig. 4J′).

An essential role for glutamate in cytoneme-mediated signaling

Dpp signal transduction in the ASP was sensitive to suppression of the VGCC and Syb in the Dpp-producing disc cells and to suppression of SERCA, GluRII, or Syt4 in the ASP (Fig. 5A). In the late third-instar larval ASP, cells with the highest level of Bnl signaling are at the tip and cells with the highest level of Dpp signaling are in the medial region (Figs. 1C and 5, B, E, and H). Ubiquitous ectopic expression of Dpp induced Dpp signaling in cells throughout the ASP (as indicated by anti-pMad immunohistochemistry), cytonemes extended outwards without apparent directional bias and in greater numbers, and the ASP had a more bulbous form (Fig. 5, C and F) (*4*). Because our genetic tests showed that the components of glutamatergic neuronal synapses contribute to Dpp signaling in the ASP, we tested for responses to glutamate and to NASPM during ex vivo culture. Whereas expression of Dpp throughout the region of the disc near the ASP induced uniform activation of Dpp signaling in the ASP (Fig. 5F), the presence of L-glutamate, which induced repetitive Ca^{2+} transients throughout the ASP (Fig. 5, K and L, and movie S7), did not change the amount or pattern of Dpp signaling in the ASP (Fig. 5, A, D, H, and I). In contrast, the presence of NASPM decreased Dpp signaling (Fig. 5, A, B, G, and J). NASPM did not decrease the number of ASP cytonemes (fig. S7). Thus, glutamate signaling appears to promote, but is not sufficient for, cytoneme-mediated Dpp signaling, and the glutamate system contributes to cytoneme function.

Transmission at the cytoneme synapse

The apposition between the cytoneme tip and target cell shares basic structural and biochemical features with the neuronal synapse. These features include contact at the tip of a cellular extension, constitution with common components, and a gap of 20 to 40 nm. One of the common components is the VGCC, which is expressed in wing disc cells that ASP cytonemes target and is in the presynaptic compartment of neuronal synapses. To test whether cytoneme-mediated signaling between the disc and ASP could be initiated by depolarization, we monitored calcium transients in ASP preparations dissected from animals that expressed a channelrhodopsin that functions as a light-gated ion channel (*35*). Wing disc–specific expression of the channelrhodopsin and photostimulation induced calcium transients in the ASP (Fig. 5, K and L, and movie S8). The increased amplitude and frequency of the optogenetically induced transients were similar to those observed with stimulation by exogenous L-glutamate (Fig. 5, K and L, and movie S7). Thus, calcium transients in the ASP can respond to altered ionic currents.

Discussion

Signaling between cells of the wing disc and ASP is cytoneme mediated and involves the intercellular transfer of signaling proteins at sites of contact between cytonemes and disc cells (*2*). Cytoneme contacts are characterized by GRASP fluorescence (*2*, *7*), a technique that marks sites of approximately 20- to 40-nm cell-cell apposition and that was developed to identify neuronal synapses (*18*). Cytoneme contacts and cytoneme-mediated signaling depend on the adhesion proteins Capricious and Neuroglian, which also have essential transsynaptic roles in neuronal synapses (*15*, *36*). This work provides evidence for additional components and features that are common to both cytoneme contacts and neuronal synapses, including voltage-activated glutamate transmission.

Because most of the genes we characterized are essential and null mutants are lethal early in development, the genetic conditions we report were partial loss-of-function—either in a hypomorphic mutant or generated by tissue-specific expression of partially defective proteins or gene-specific RNAi constructs. Expression conditions were adjusted to have no discernable effects on cell viability or cell division (fig. S3). Partial loss-of-function conditions in the wing disc that targeted *syt1*, *syb*, *cac*, *stj*, *Irk2*, *VGlut*, or *Atpα*, all essential components of presynaptic neuronal compartments, decreased the presence of cytonemes and signaling in the ASP. In contrast, targeting these genes in the ASP had no apparent phenotype. Partial loss-of-function conditions in the ASP that targeted *GluRII* or *syt4*, both essential components of postsynaptic neuronal compartments, decreased signaling in the ASP; but targeting these genes in the wing disc had no effect on the ASP. This specificity, which implicates glutamatergic functions of neuronal presynaptic compartments only in the signal-transmitting cells of the wing disc and glutamatergic functions of neuronal postsynaptic compartments only in the signal-receiving cells of the ASP, provides genetic evidence that the observed phenotypes are correctly attributed to the gene targets.

Histological characterizations showed that GluRII is present in cytonemes that take up signaling proteins from disc cells (Fig. 2, F to H), that exogenous VGCC expressed in the disc cells can be detected at cytoneme contacts, and that Syt4 expressed exogenously can be exposed on the plasma membrane of cytoneme contacts (Fig. 3, E to G) (*26*). These findings show that the GluRII, Syt4, and the VGCC components of the glutamatergic neuronal synapse are segregated in similar ways at cytoneme contacts. Further similarities include the fact that postsynaptic GluRII regulates Dpp signaling at the *Drosophila* neuromuscular junction (*37*) and that optogenetic stimulation of a channelrhodopsin expressed specifically in the wing disc induced Ca^{2+} transients in the ASP. These similarities are consistent with the possibility that cytoneme contacts are functional glutamatergic synapses.

Comparative analysis of the GluR gene family suggests that GluR signaling is present in animals that lack nervous systems, such as *Trichoplax* (*38*). Plants also regulate Ca^{2+} influx with GluR-like channels that are required for growth and cell-cell communication (*39*, *40*). This work shows that glutamate-dependent signaling is also present in non-neuronal epithelial cells of the *Drosophila* wing disc and ASP.

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S7
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Movies S1 to S9

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Glutamate signaling at cytoneme synapses

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Cytoneme signaling in fly development

During development of certain tissues in the fruit fly, signaling from one tissue to another appears to occur through specialized filopodia called cytonemes. The cytonemes contain receptors and reach close toward the signal-producing cells. Huang *et al.* report that components of neuronal signaling at synapses also function in proper formation of cytoneme appositions and signaling. Various manipulations of calcium signaling, expression of dominant-negative glutamate receptor proteins, or depletion of vesicle transport proteins or components of voltage-gated calcium channels influenced the presence of cytonemes and signaling.

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