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Journal
Developmental neurobiology, 68(3)

ISSN
1932-8451

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Publication Date
2008-02-15

Peer reviewed

Ultrastructural Analysis of Chemical Synapses and Gap Junctions Between *Drosophila* Brain Neurons in Culture

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Received 18 June 2007; revised 15 August 2007; accepted 21 August 2007

ABSTRACT: **Dissociated cultures from many species have been important tools for exploring factors that regulate structure and function of central neuronal synapses. We have previously shown that cells harvested from brains of late stage *Drosophila* pupae can regenerate their processes *in vitro*. Electrophysiological recordings demonstrate the formation of functional synaptic connections as early as 3 days *in vitro* (DIV), but no information about synapse structure is available. Here, we report that antibodies against pre-synaptic proteins Synapsin and Bruchpilot result in punctate staining of regenerating neurites. Puncta density increases as neuritic plexuses develop over the first 4 DIV. Electron microscopy reveals that closely apposed neurites can form chemical synapses with both pre- and postsynaptic specializations characteristic of many inter-neuronal synapses in the adult brain. Chemical synapses in culture are restricted to neuritic processes and some neurite pairs form reciprocal synapses. GABAergic synapses have a significantly higher percentage of clear core versus granular vesicles than non-GABA synapses. Gap junction profiles, some adjacent to chemical synapses, suggest that neurons in culture can form purely electrical as well as mixed synapses, as they do in the brain. However, unlike adult brain, gap junctions in culture form between neuronal somata as well as neurites, suggesting soma ensheathing glia, largely absent in culture, regulate gap junction location *in vivo*. Thus pupal brain cultures, which support formation of interneuronal synapses with structural features similar to synapses in adult brain, are a useful model system for identifying intrinsic and extrinsic regulators of central synapse structure as well as function.** © 2007 Wiley Periodicals, Inc. Develop Neurobiol 68: 281–294, 2008

Keywords: **electron microscopy; synapsin; electrical synapse; GABAergic; cholinergic**

INTRODUCTION

There are well-established methods for preparation of dissociated cell cultures from brains of embryonic/postnatal rodents, including mutant and transgenic mice, which support development of neurons that are capable of forming functional synaptic connections (Banker et al., 1991). These cultures have been useful in defining genes and environmental factors involved in regulation of synapse formation and function (Craig et al., 2006). Conditions for culturing neurons from a wide variety of insects, including cockroach,

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Contract grant sponsor: Korea Science & Engineering Foundation (KOSEF).

Contract grant sponsor: NIH; contract grant numbers: NS27501, NS33213.

Contract grant sponsor: Howard Hughes Medical Institute (HHMI Professors Program).

Published online 28 November 2007 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/dneu.20575

locust, honey bee, houseflies, crickets, grasshopper, moths, and fruit flies have also been established (Beadle, 2006). Although neurons in cultures from many insect species extend overlapping neuritic processes and express functional ligand-gated receptors, the only insect cultures that have been reported to support the formation of functional synaptic connections *in vitro* are from *Drosophila* (Rohrbough et al., 2003). Both cholinergic and GABAergic synaptic currents have been recorded in embryonic neurons from mid-gastrula stage *Drosophila* embryos grown in a defined medium (Lee and O'Dowd, 1999; Lee et al., 2003). Genetic and environmental manipulations in these cultures have demonstrated that cAMP-signaling regulates synaptic transmission and plasticity at cholinergic synapses (Lee and O'Dowd, 2000). A recent report has also defined conditions that support development of functional cholinergic synapses between cultured embryonic *Drosophila* neurons grown in a serum-supplemented Schneider's medium (Kuppers-Munther et al., 2004).

To identify factors that regulate synaptic form and function in *Drosophila* neurons known to be involved in mediating adult behaviors, we previously developed a technique for preparation of primary neuronal cultures from brains of late stage pupae (Su and O'Dowd, 2003). In these cultures, neurons from the central brain region that includes association areas involved in learning and memory formation, regenerate neuronal processes lost during culture preparation and form functional synaptic connections with each other. Fast inhibitory synaptic transmission is mediated by picrotoxin sensitive GABA receptors (Su and O'Dowd, 2003), similar to the pharmacology of the receptors mediating fast inhibition in antennal lobe neurons in the adult brain (Wilson and Laurent, 2005). Fast excitatory synaptic transmission in the cultured neurons is predominantly cholinergic and mediated by α-bungarotoxin sensitive nAChRs (Su and O'Dowd, 2003). The pharmacological profile of the miniature excitatory postsynaptic currents (mEPSCs) in cultured Kenyon cells is similar to mEPSCs recorded from Kenyon cells in the adult brain (Su and O'Dowd, 2003; Gu and O'Dowd, 2006).

To use the *Drosophila* pupal cultures to investigate how genetic and environmental factors influence synaptic morphology and how these might contribute to altered neural function, it is necessary to be able to visualize the structural features of inter-neuronal synapses. Here, we show by immunocytochemistry the presence of presynaptic specializations on regenerating neurites. At the electron microscopic level we report chemical synapses with distinct pre- and postsynaptic specializations, as well as gap junction profiles on the cultured neurons. Thus, *Drosophila* pupal brain cultures represent a useful model system in which to identify intrinsic and extrinsic factors that influence formation and function of central synapses, complementary to and extending similar studies *in vivo*.

METHODS

Fly Strains

The majority of cultures, and all cultures used in the immuno-electron microscopic studies were prepared from Canton-S wild-type pupae. Similar structural features were found in culture prepared from OK107-GAL4; UAS-GFP (Su and O'Dowd, 2003), and Cha-GAL; UAS-GFP, a 7.4 kb Cha-Gal4 driver line (19B) recombined with UAS-GFP (S65T) (courtesy of P. Salvaterra) pupae.

Primary Pupal Cultures

Heads were removed from animals 55–78 h after puparium formation, the brains were removed and the optic lobes discarded. Central brain regions were dissociated and cultured as previously described (Jiang et al., 2005). A video of the culture procedure and a detailed protocol can be viewed online (O'Dowd et al., 2007). Cultures were maintained in a 23°C humidified 5% CO_2 incubator for up to 7 days.

Fluorescent Immunocytochemical Staining

Cultures were fixed in 4% paraformaldehyde in PBS for 40 min at 4°C. Cultures were washed thrice with PBS and then 30 min in 0.05% $NaBH_4$ (Sigma, Saint Louis, MO). Following 3 washes with PBS/4% BSA, the cultures were incubated in 5% goat normal serum, 4% BSA, and 0.5% triton for 1 h. Incubations with primary antibodies were carried out overnight at 4°C, Synapsin (Syn) (3C11; mouse MAB, 1:500; Developmental Studies Hybridoma Bank, DSHB; University of Iowa), or Nc82 (mouse MAB, 1:1000; DSHB). The secondary antibody was a goat anti-mouse conjugated to Alexa Fluor 546 (1:2000; Invitrogen; Carlsbad, CA), applied for 2 h at RT. Cultures were co-stained with fluorescein conjugated anti-HRP antibodies (1:500; MP Biochemicals, LLC; Solon, OH) to visualize all neurons. After incubation in the secondary antibody, cultures were washed with PBS and mounted in Fluoromount-G (Southern Biotech, Birmingham, AL).

Electron Microscopy

The procedure used for electron microscopy (EM) of cultured *Drosophila* neurons was based on that developed for ultrastructural studies of cultured mammalian neurons (Schikorski and Stevens, 1999). Cultures were fixed in 2%

glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.0 at 4°C for 1 h, washed in phosphate buffer, and post-fixed in 1% OsO_4 on ice for 1 h. Cells were contrasted in 1% uranyl acetate at RT for 1 h, dehydrated in ethanol, and embedded in Epon 812 (50°C × 24 h then 60°C for 48 h). The block was immersed in hydrofluoric acid (49%) for 10–15 min to remove the coverslip. Ultrathin (~60 nm) serial sections of cultured neurons were cut on a Reichert Ultracut Ultramicrotome with a diamond knife and mounted on Formvar-coated single slot grids. Sections were stained again in 2% uranyl acetate for 2 min, followed by Reynold's lead citrate for 2 min. Sections were examined on a Philips CM10 transmission electron microscope. Electron micrographs were taken with a Gatan Utrascan US1000 digital camera.

Post-Embedding Immunogold Electron Microscopy

Cultures were immersed in 2% paraformaraldehyde and 1% glutaraldehyde in 0.1*M* phosphate buffer at 4°C for 1 h, and then processed as described earlier with the omission of the first uranyl acetate staining. After thin sectioning, grids were incubated in 10% normal goat serum in Tris-buffered saline (TBS), pH 7.4 for 30 min, followed by an overnight incubation at 4°C in a polyclonal anti-GABA antibody (Sigma, St. Louis) diluted at 1:200 in Buffer A (TBS+1% normal goat serum, 0.1% Tween 20, 1% BSA in TBS). Sections were rinsed 3 times with Buffer A and incubated with a 10-nm gold particle conjugated goat anti-rabbit IgG (1:10, EMS, Hatfield, PA), 1 h at room temperature. Unbound secondary antibody was removed with 3 × 5 min wash in 0.1*M* phosphate buffer followed by 3 × 5 min wash in distilled water. Sections were counterstained with 2% uranyl acetate for 2 min, followed by Reynold's lead citrate for 2 min.

Analysis of Images

Digital electron micrographs were analyzed using Adobe Photoshop and NIH Image. Cross sectioned synapses selected for analysis met the following three criteria: (1) a widened synaptic cleft, (2) presence of a cluster of synaptic vesicles associated with the presynaptic membrane, and (3) presence of presynaptic dense body or T-bar. The length of the active zone was defined as the maximal length of the widened synaptic cleft associated with a presynaptic vesicle cluster. Mean vesicle density, within 200 nm of the active zone was calculated from three centrally located serial sections of each synapse. The outside diameters of synaptic vesicles were measured at magnification of 280,000× to 365,000×. Only vesicles touching the presynaptic membrane were included in the estimate of docked vesicles.

GABAergic terminals were defined as those in which there were multiple gold particles in 3 serial sections within 200 nm of the presynaptic active zone in sections processed for GABA staining. The mean density of gold particles in GABA positive terminals was 51 μm^{-2} ($n = 14$). The mean density of gold particles in the terminals classified as non-GABA synapses was 3 μm^{-2} ($n = 13$), 18-fold lower than in the GABA positive synapses.

RESULTS

Dissociated cell cultures were prepared from brains of late stage pupae when the major anatomical structures of the adult brain, including the antennal lobes and mushroom bodies, are in place. The optic lobes were removed and the central brain region from a single pupa was plated in each culture. As we have shown previously, neurons in these cultures regenerate processes that grow and branch over the first week in culture (Su and O'Dowd, 2003).

Punctate Expression of Markers for Presynaptic Proteins On Cultured Neurons

Previous studies have shown that markers for presynaptic proteins, including Synapsin Syn and Bruchpilot (BRP), exhibit a punctate pattern of staining on neuritic processes of *Drosophila* neurons that differentiate in culture from embryonic neuroblasts (Schmidt et al., 2000; Kuppers-Munther et al., 2004). To determine if these synaptic proteins are expressed in a similar pattern on pupal neurons, cultures were fixed at 1, 2, 4, and 7 days *in vitro* (DIV). To visualize the complexity of the regenerating processes at these different time points, cultures were stained with a neuron specific anti-HRP antibody. By 1 DIV many neurons have regenerated short processes and there are some regions where neurites between adjacent cells overlap [Fig. 1(A,D)]. Processes continue to grow and branch, forming neuritic plexuses that increase in density between 1 and 4 DIV [Fig. 1(A–F)]. Co-labeling with antibodies that recognize the presynaptic protein Syn reveals a punctate pattern of staining. The density of puncta is low at 1 DIV [Fig. 1(A)] and there is a dramatic increase in puncta density associated with the increasing complexity of the neuritic plexuses that develops between 2 and 4 DIV [Fig. 1(B,C)]. A similar developmental pattern of expression of immunofluorescent puncta is observed when cultures are stained with Nc82, an antibody recently shown to recognize the presynaptic BRP (Wagh et al., 2006) [Fig. 1(D–F)]. There is no obvious change in the number of puncta between 4 and 7 DIV (data not shown). While some of the punctate expression of presynaptic markers is seen at sites of physical contact between two or more neurites [Fig. 1(C,F)], there are also puncta on neuronal somata and neurites at locations that are not in contact with other processes [Fig. 1(G,H)]. This suggests that pupal *Drosophila* neurons

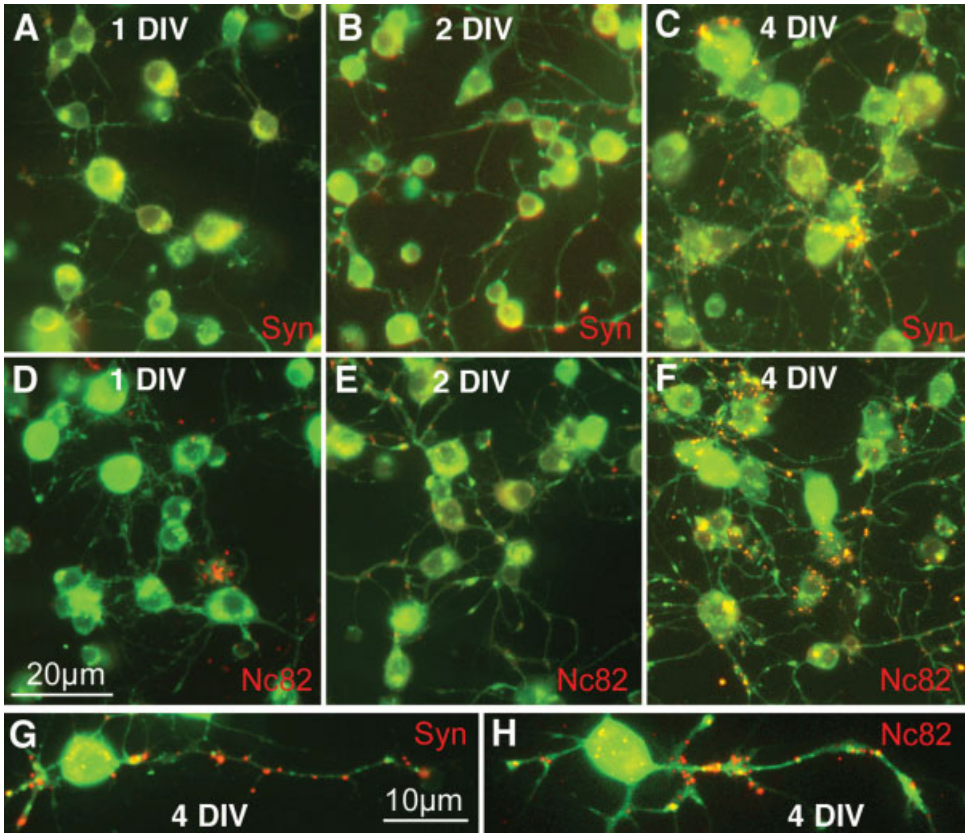

Figure 1 Punctate expression of presynaptic proteins is developmentally regulated in cultured pupal neurons. A–F: Neurites are clearly visible as early as 1 DIV and form extensively overlapping neuritic plexuses by 4 DIV as revealed by staining with anti-HRP antibodies (Green). A–C: Staining with antibodies against the presynaptic protein Synapsin (Syn, Red) reveals a punctate pattern of expression on neurites that is rarely seen at 1 DIV but is prevalent in the neuritic plexuses by 4 DIV. D–F: Staining with the Nc82 antibody against the presynaptic protein Bruchpilot (Nc82, Red) shows a developmental expression pattern similar to Syn. G,H: Isolated neurons at 4 DIV also show punctate expression of Nc82 and Syn suggesting that localized expression of presynaptic proteins does not require a postsynaptic target cell.

can form presynaptic specializations in cell culture, and a least some do not require contact with a postsynaptic target cell.

Cultured Neurons Form Both Chemical Synapses and Orphan Presynaptic Specializations

A previous study showed that cultured embryonic *Drosophila* neurons also form presynaptic specializations on outgrowing processes but they found no ultrastructural evidence of postsynaptic specializations characteristic of mature synapses between adjacent processes (Kuppers-Munther et al., 2004). To determine if pupal neurons are capable of forming chemical synapses with both pre- and postsynaptic specializations, as well as presynaptic specializations in the absence of postsynaptic target cells, the tissue was examined at the ultrastructural level. In light of the similar pattern of expression of markers for presynaptic proteins between 4 and 7 DIV and previous studies showing the incidence of recording synaptic currents did not increase after 3–4 DIV (Su and O'Dowd, 2003) all cultures were fixed and processed for EM as described in the methods at 4 DIV.

Images were obtained from 19 cultures, each made from the brain of a single animal. At low magnification, it is possible to identify regions of high neurite density (Fig. 2). Since the blocks were sectioned parallel to the horizontal surface of the culture, many of the neurites are seen in longitudinal section with cytoplasmic microtubules running parallel to the long axis (Fig. 2). There are large numbers of mitochon-

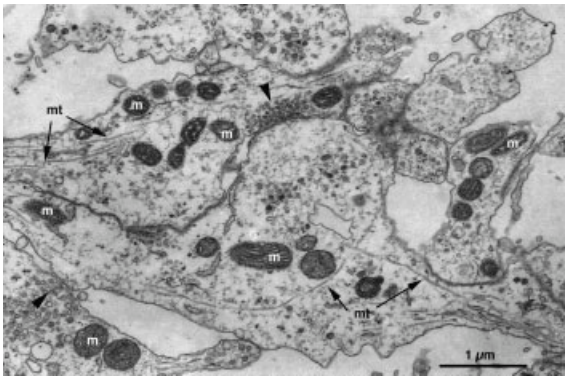

Figure 2 Low power electron microscopic image of a region of a culture at 4 DIV containing a high density of neuronal processes. Neuronal processes cut in longitudinal section are identified by microtubules (mt) running parallel to the long axis in an electron lucent cytoplasm. Large numbers of mitochondria (m) and small vesicles are present within the cytoplasm of most processes. Some vesicle clusters are associated with synapses formed between neurites (arrowheads). There is no evidence of glial processes based on the absence of electron dense profiles either surrounding or between the neurites. This and all subsequent electron micrographs are from cultures fixed at 4 DIV.

dria in the neurites as well as small membrane bound vesicles, some organized in clusters associated with cross sections through synapses (Fig. 2). The absence of electron dense processes surrounding the neuron cell bodies or neurites, suggests that there are few, if any, glial cells in these cultures. This is consistent with the light level anti-HRP antibody staining (Fig. 1) indicating that the majority of the cells that survive and regenerate processes in the defined media are neurons, similar to the neuron enriched cultures derived from embryos grown in defined medium (O'Dowd, 1995).

Analysis of the cultures at higher magnification demonstrates the presence of chemical synapses with many features similar to mature insect synapses *in vivo* including a cluster of vesicles embedded in a loosely organized matrix of electron dense material, associated with a cleft of 10–25 nm separating a pre- and postsynaptic process (Fig. 3). Electron dense material is present within the cleft and is also concentrated on the intracellular surface of the opposing postsynaptic membrane. The presynaptic terminals generally have one or more mitochondria, typical of *Drosophila* central and peripheral synapses *in vivo* where they are involved in the energy intensive process of synaptic transmission (Guo et al., 2005; Hollenbeck, 2005; Verstreken et al., 2005). A subpopulation also contained multivesicular bodies (MVB) [Fig. 3(A)]. Interestingly, all of the chemical synapses observed were between neurites, even though there are neurite-cell body contacts in the cultures, different from the situation *in vivo* where synapse bearing neuritic segments are spatially separated from the cell bodies. Although, we cannot exclude the possibility of a low incidence of chemical synapses on the cell bodies, these data suggest that pupal neurons cultured in defined medium are capable of forming structurally identifiable chemical synapses and there is some specificity governing target choice *in vitro*.

In many regions of the adult insect brain a single presynaptic element contacts multiple postsynaptic targets (Prokop and Meinertzhagen, 2006). In contrast, most synapses in culture (53/61) are monadic with a single pre- and postsynaptic element [Fig. 3(A)]. Dyadic synapses, with a single presynaptic terminal forming synapses on two distinct postsynaptic targets [Fig. 3(B)] are present but found at lower frequency (8/61). Thus, the culture system is permissive for formation of higher order synapses and it seems likely that it is the monolayer structure of the cultures, which limits the probability of contacts between multiple neurites at single sites, rather than inability of the neurons to form the more complex microcircuits typical of the brain neuropil.

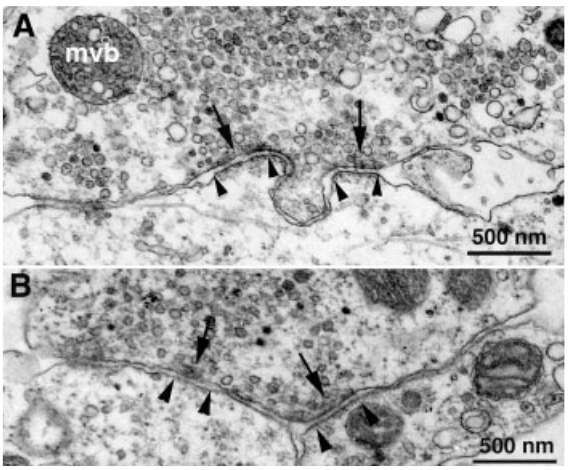

Figure 3 All chemical synapses in culture were found between neurites. A: Cross section of a single pre- and postsynaptic process. There are two active zones (arrows), each defined by a cluster of presynaptic vesicles embedded in a loosely organized matrix of electron dense material associated with a synaptic cleft, and a postsynaptic density (between arrow heads). Additional vesicles embedded in diffusely organized electron dense material and a multivesicular body (mvb) are also present in this region. B: Cross section through a dyadic synapse in which a single presynaptic element forms synapses with two distinct postsynaptic processes.

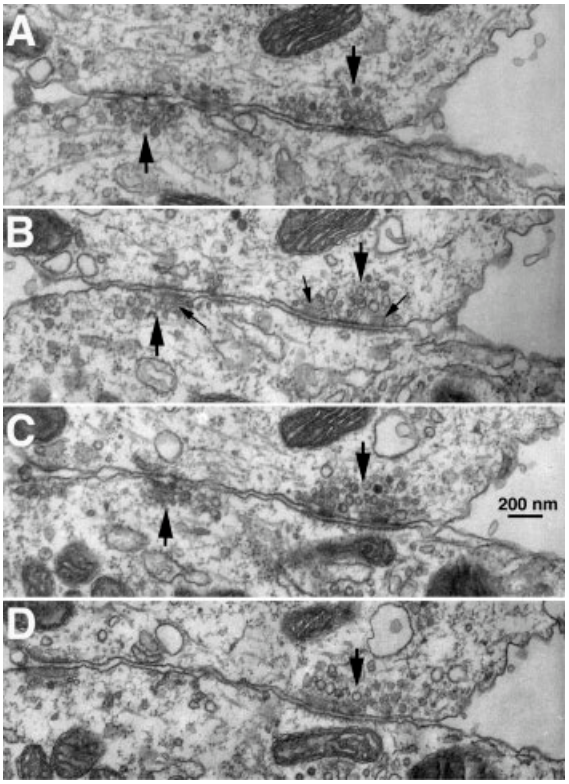

Figure 4 Reciprocal chemical synapses between two neurites cut in cross section. Four ultrathin serial sections. A: Two active zones with presynaptic vesicle clusters facing in opposite directions (large arrows). B: The presynaptic densities appear as T-bars in both synapses in this section. There is one at the left synapse and two at right synapse (small arrows). C: Presynaptic density of the right synapse is more elliptical in shape at this plane. D: Only the right synapse is captured in this plane of section.

Typical of insect central synapses *in vivo*, the relatively loosely organized matrix of electron dense material associated with the vesicle cluster in the presynaptic active zone is punctuated by one or more regions of highly organized electron dense material directly adjacent to the presynaptic membrane (Fig. 4). These regions can appear as T-bars or elliptical structures in cross sections through presynaptic active zones [Fig. 4(B,C)]. Synapses of opposing polarity can occur within single regions of contact between two neurites (Fig. 4). The presence of reciprocal synapses, similar to those reported in both mushroom bodies and antennal lobes in the adult insect brain (Leitch and Laurent, 1996; Distler et al., 1998), present future opportunities to investigate genetic factors that influence bidirectional information flow between two cells.

Whereas the majority of synapses were cut in cross-section, some presynaptic terminals cut tangential to the plane of the section were also observed. These were identified by the presence of presynaptic densities with multiple branches within large vesicle clusters [Fig. 5(A, inset)]. All of the tangential sections that contained one or more vesicle clusters with distinct active zones were found in the first few sections of the block [Fig. 5(B)]. These sections are at the interface between the basal substrate and the cells. The absence of a synaptic partner in the prior sections support the conclusion from our light level analysis indicating that presynaptic specializations can assemble without a postsynaptic target.

Vesicle clusters contain vesicles that are grouped into three subclasses based on morphology and size. The most abundant class, present in all of the synaptic terminals in culture, are clear core vesicles with a diameter range of 20–45 nm, typical of vesicles containing classic neurotransmitters *in vivo* [Fig. 6(A)]. Most are round but some are pleomorphic [Fig. 6(B)]. Many synapses also have a smaller number of granular vesicles characterized by electron dense regions within the core. Most granular vesicles have two or more small, dense regions in the central core [Fig. 6(A)]. A minority have a distinct centrally located density, similar to classic dense core vesicles that typically contain neuromodulatory substances. The third vesicle class is low in abundance, electron lucent, but typically larger than the clear core vesicles with a diameter range of 35–80 nm [Fig. 6(A)]. Organelles similar to the larger electron lucent vesicles and MVBs seen in culture are found at synapses *in vivo* where they are thought to be early and late endosomal compartments, respectively (Sunio et al., 1999; Kramer, 2002; Wucherpfennig et al., 2003).

Vesicles touching the presynaptic membrane in the active zone are considered morphologically docked [see Fig. 7(C)]. Occasional omega profiles, presumably vesicles in the process of fusion, are also present in the active zones [Fig. 6(B)], consistent with a previous electrophysiological study demonstrating that the neurons exhibit spontaneous synaptic activity in culture (Su and O'Dowd, 2003). Synapses with both clear core and granular vesicles suggest potential release of both classic neurotransmitters and neuromodulators at synapses formed in culture.

GABAergic and Non-GABA Synapses Have Quantitatively Distinct Vesicle Populations

Synapses were examined in cultures processed for post-embedding immunocytochemistry using an anti-GABA primary and a gold-labeled secondary anti-

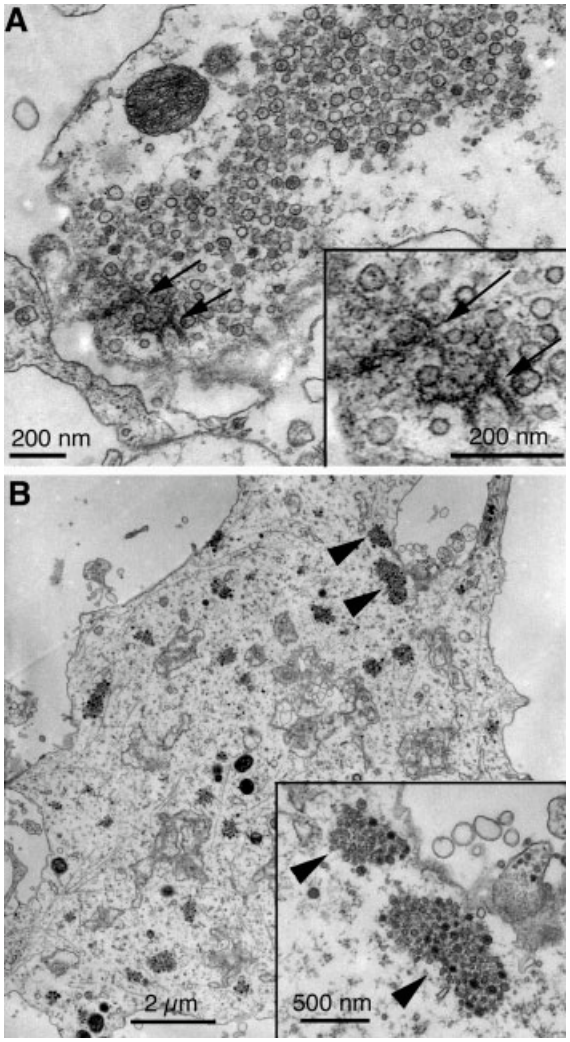

Figure 5 Presynaptic specializations form in the absence of postsynaptic targets. A: Tangential section of a neuronal process at the substrate-cell interface. A large cluster of synaptic vesicles embedded in a diffusely organized electron dense material. A region of highly organized electron dense material (black arrows) surrounded by synaptic vesicles identifies a putative presynaptic active zone in tangential section that has formed in the absence of a postsynaptic target cell. Inset is higher magnification view of highly organized electron density. The multiply branched structure is consistent with the variability in the number and shape of focal regions of high electron density associated with the presynaptic membrane observed in cross sections through active zones. B: Tangential section of a neuronal process at the substrate-cell interface with multiple vesicle clusters representing organization of many presynaptic specializations in the absence of a postsynaptic target cell. Inset is higher magnification view of two of the vesicle clusters in upper right corner (arrowheads).

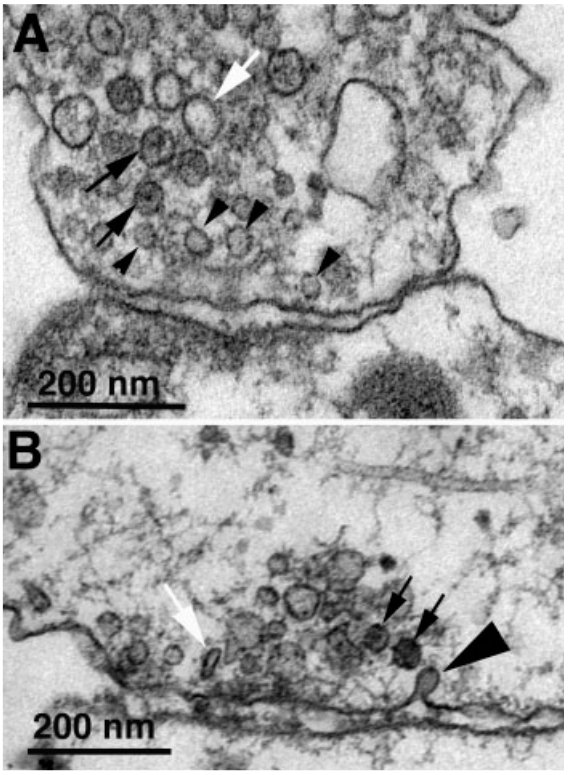

Figure 6 Vesicle populations at most synapses are heterogenous with three morphologically distinct groups. A: An active zone region that contains all three vesicle classes. Small clear core vesicles (arrow heads) are clustered around a T-bar. There are also granular (black arrows), and large electron lucent (white arrow) vesicles. B: Granular vesicles at a second synapse are more similar to classic dense core vesicles (black arrows). Omega profile in the active zone (large black arrow head). The majority of vesicles are round but some are pleomorphic (white arrow).

body. GABAergic synapses were identified by the presence of gold particles in the presynaptic terminal [Fig. 7(B, inset) see methods for details]. Quantitative information was obtained from 3 or more ultrathin serial cross sections through GABAergic ($n = 14$) and non-GABA ($n = 13$) synapses in 6 cultures, prepared from brains of 6 different Canton-S wildtype animals.

The length of the presynaptic active zone ranges from 200 to 800 nm; the mean is similar in GABAergic and non-GABA synapses (Table 1). Vesicles at the GABAergic synapses are generally clear core and small in diameter [Fig. 7(A–C)]. In contrast, at a typical non-GABA synapse there is more heterogeneity in vesicle size and morphological type [Fig. 7(D–F)]. The mean vesicle diameter and mean density of different vesicle types at each synapse were determined from analysis of all vesicles within 200 nm of the presynaptic membrane in 3 cross sections. Although overall vesicle density and number of docked vesicles

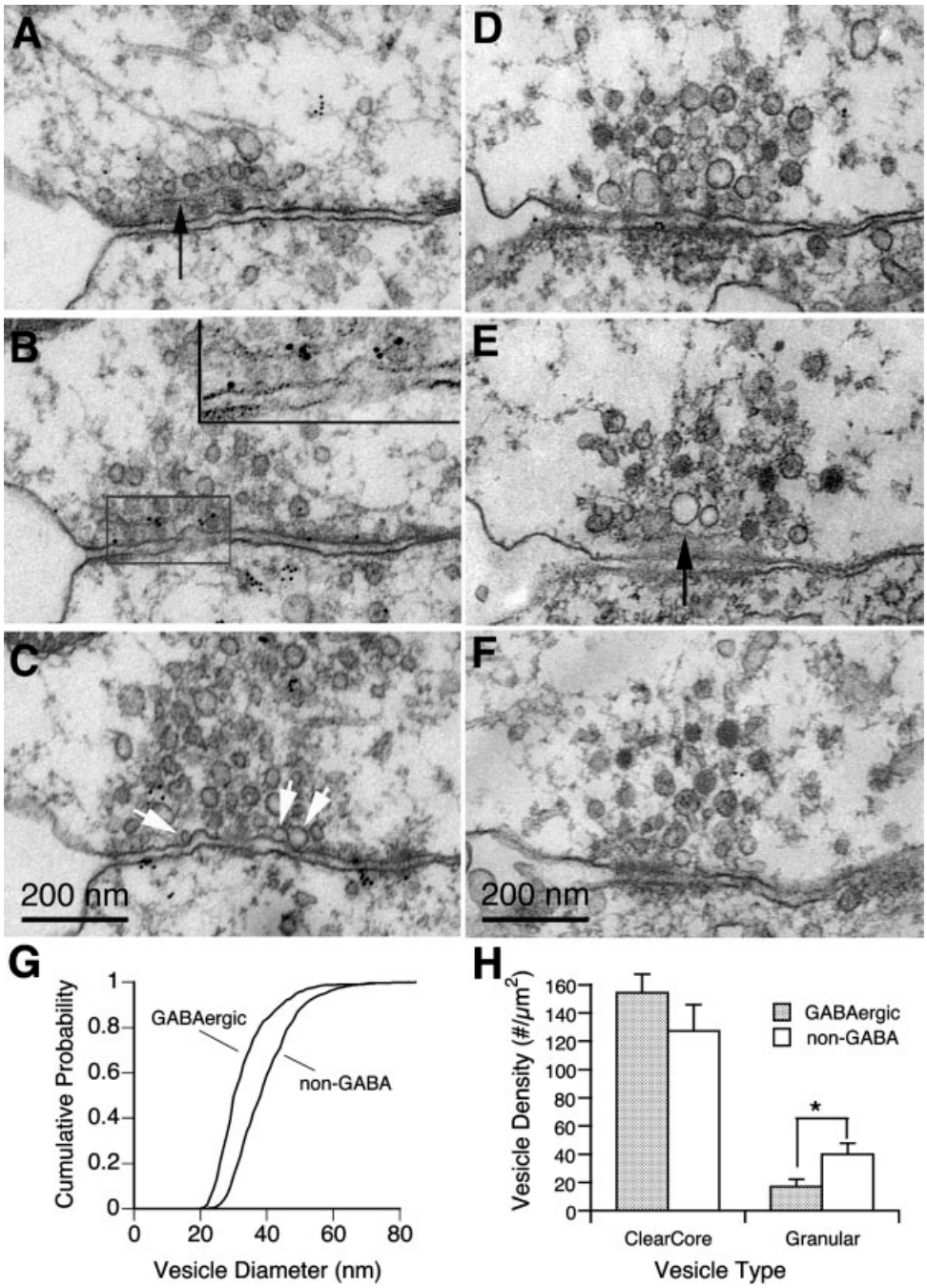

Figure 7 Comparison of vesicle populations in GABAergic and non-GABA synapses. A–C: Cross sections through an active zone identified as GABAergic based on presence of gold particles (see inset in B for higher magnification) in each of three serial sections following post-embedding immuno-gold labeling for GABA. Vesicle population in this terminal is relatively homogenous, consisting predominantly of small and clear core vesicles, and the postsynaptic density is sparse. Synapse contains a T-bar structure (black arrow). Docked vesicles in C are indicated by white arrows. D–F: Serial cross sections through the active zone of a non-GABA synapse. Vesicle population is more heterogeneous with a greater range in size, more granular vesicles intermixed with the clear core vesicles, and a prominent postsynaptic density. Non-GABA synapses also contain T-bar structures (black arrow). G: Cumulative probability curves reveal a narrower distribution and smaller median size of vesicles located within 200 nm of the presynaptic membrane at GABAergic vs non-GABA synapses. $p < 0.001$, Kolmogorov-Smirnov two sample test. Curves constructed from 14 GABAergic (1116 vesicles) and 13 non-GABA synapses (1279 vesicles). H: GABAergic synapses ($n = 14$) have a significantly lower density of granular vesicles than non-GABA synapses ($n = 13$) within 200 nm of the presynaptic membrane. * $p < 0.05$, Student's *t*-test, unpaired.

Table 1 Properties of GABAergic and Non-GABA synapses

	Active Zone Length (μm) Mean ± SEM	Total Vesicle Density (number/μm^2) Mean ± SEM	Docked Vesicle Number per μm Mean ± SEM	*n*
GABAergic	0.453 ± 0.03	174 ± 12	2.6 ± 0.4	14
Non-GABA	0.488 ± 0.05	174 ± 23	2.3 ± 0.4	13

The mean density of all vesicles, within 200 nm of the active zone, and the mean density of docked vesicles were calculated by averaging the means determined from three centrally located serial sections from each synapse.

is similar in GABAergic and non-GABA synapses (Table 1), the vesicle populations are quantitatively different. The size distribution is narrower and the mean vesicle diameter is smaller in GABAergic versus non-GABA synapses [Fig. 7(G)]. In addition, the density of granular vesicles is significantly lower in GABAergic synapses [Fig. 7(H)], suggesting that co-release of neuromodulatory substances, likely to be contained in these vesicles, is lower in these synapses. Large electron lucent vesicles, presumably early endosomes, are present but in low abundance in both synapse types. Finally, the postsynaptic density at GABAergic synapses [Fig. 7(A–C)] is generally sparser than at non-GABA synapses [Fig. 7(D–F)].

Gap Junctions Form Adjacent to and Independent of Chemical Synapses

In addition to chemical synapses, gap junction profiles have been described at a number of locations in the adult *Drosophila* CNS including between photoreceptor terminals in the optic lamina (Shimohigashi and Meinertzhagen, 1998), the giant fiber system (Blagburn et al., 1999), and the antennomechanosensory center (Sivan-Loukianova and Eberl, 2005). At 21% (13/61) of the chemical synapses imaged in 3 or more serial sections in culture, there is an adjacent site of very close contact between the pre- and postsynaptic cell. These are defined as gap junction profiles (putative electrical synapses) when the separation between apposing membranes is less than 2 nm [Fig. 8(A, inset)]. These did not exhibit septa characteristic of junctions formed by the giant fiber system (Blagburn et al., 1999).

Gap junctions are associated with both non-GABA [Fig. 8(A,B)] and GABAergic [Fig. 8(C)] synapses. The mean gap junction length, determined from synapses fully included in at least one of three or more serial sections, is 384 ± 75 nm ($n = 8$). In addition to the closely apposed pre- and postsynaptic membranes, additional compartments are observed within 10 nm of most gap junctions (11/13). Some associated membrane compartments are large in diameter [Fig. 8(A)]. However, the majority are elongated fingers of smooth membrane that run the length of the electrical junctions [Fig. 8(B,C)]. They are on one or both sides of the electrical junction. Some are clear extensions of smooth endoplasmic recticulum (sER) based on continuity with the rough endoplasmic recticulum (rER) that extends into the cytoplasmic region of the process [Fig. 8(B,C)]. Membrane delimited compartments that are part of the endoplasmic recticulum have been described at chemical synapses in the adult *Drosophila* visual system. These appear as long thin cisternae, of unknown function, that underlie the postsynaptic membrane in lamina cells L1 and L2 at the photoreceptor tetrads (Prokop and Meinertzhagen, 2006). In the cultured neurons, the membrane delimited compartments are present at the gap junctions but not the chemical synapses.

Gap junctions and associated membrane compartments in the cultured neurons are also present between neurites in regions that lack adjacent chemical synapses [Fig. 9(A)]. Finally, gap junction profiles can form between some adjacent neuronal somata in cell culture [Fig. 9(B1,B2)]. While this has not been reported *in vivo*, ensheathing glial processes typically separate neuronal somata in the insect brain (Hahnlein and Bicker, 1996). The lack of glial cells in culture allow direct contacts between the plasma membranes of neuronal somata in regions of high-plating density. Thus, glial cells may be important in regulating where gap junction form in the adult nervous system.

DISCUSSION

The ability of pupal neurons to form synapses *in vitro*, whose morphological features are, in many respects, similar to synapses in the adult brain, expands the use of these cultures to the exploration of genetic and environmental factors that influence the structure and function of CNS synapses. Since the cultured neurons are regenerating in conditions very different from their counterparts in the adult brain,

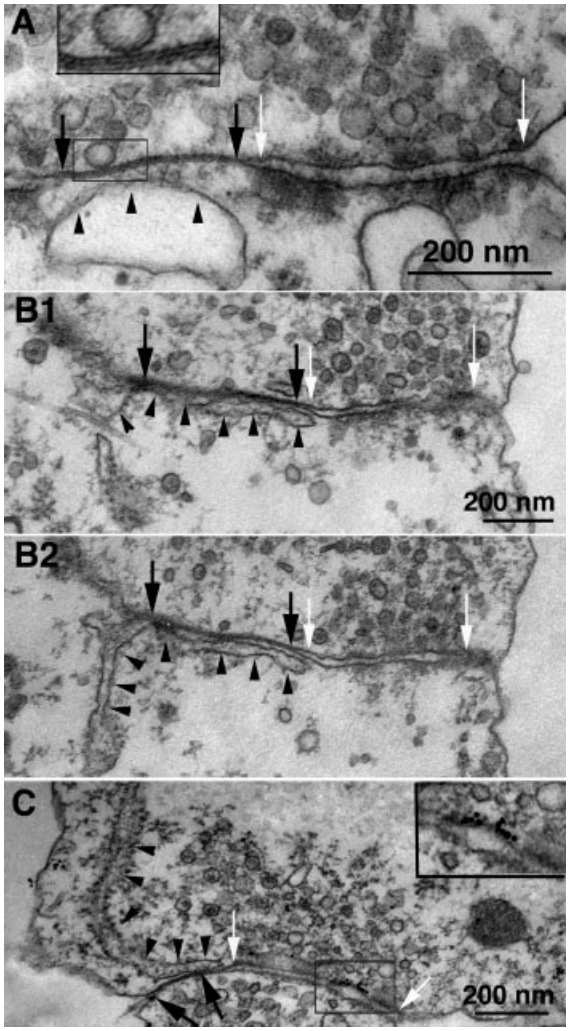

Figure 8 Gap junctions can form adjacent to non-GABA and GABAergic chemical synapses. A: A gap junction profile (delimited by black arrows) adjacent to a non-GABA synapse. Inset is higher magnification of small region of gap junction characterized by a gap of less than 2 nm between pre- and post-synaptic cell that often appears as three parallel electron dense lines. The active zone of the chemical synapse is located between the white arrows. An internal membrane compartment is associated with the gap junction (small black arrowheads). B1, B2: Serial cross sections through a second non-GABA synapse with an adjacent gap junction. The associated membrane compartment is a finger of sER closely apposed to the gap junction along its whole length. This is continuous with rER that extends into the cytoplasmic region of the postsynaptic process as seen in B2. C: GABAergic synapse (delimited by white arrows) adjacent to a gap junction (delimited by black arrows). The associated membrane compartment is a finger of sER (small black arrowheads) that is continuous with a stretch of rER extending into the cytoplasmic region of the synaptic terminal. Inset is magnification of boxed region showing gold particles identifying this as a GABAergic synapse following immunoEM.

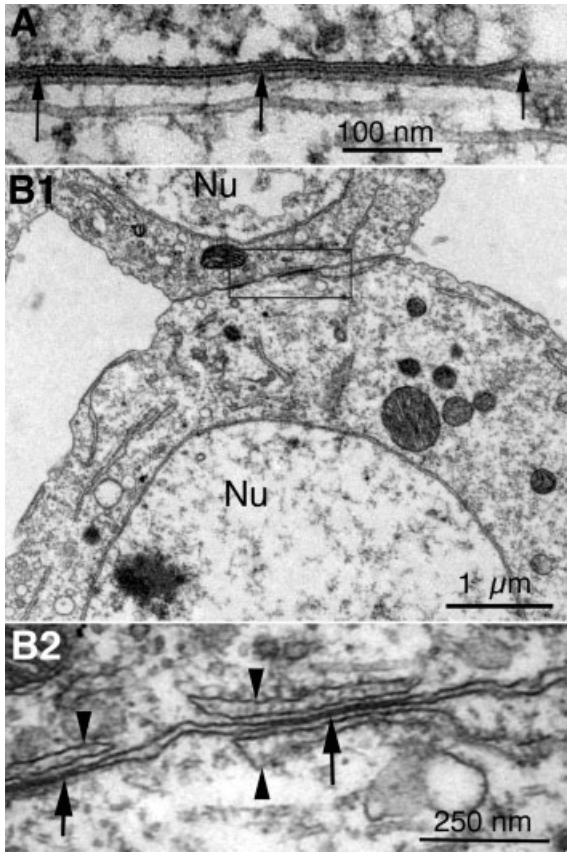

Figure 9 Gap junctions can form between neuronal processes and neuronal somata in the absence of adjacent chemical synapses. A: High magnification view of a gap junction between two neuronal processes. B1: Low power view of two adjacent neuronal cell bodies. Nucleus (Nu). B2: High magnification view of the region of contact between the two cells indicated by the box above. Two gap junctions (arrows) are visible in this region. Closely associated membrane delimited compartments (arrowheads) are present on one (left) or both (right) sides of the gap junctions.

comparison of synapses *in vivo* and *in vitro* allows identification of both intrinsic and extrinsic regulators. The advantage of the pupal brain cultures over embryo cultures is the potential to examine synaptic structure and function in neuronal populations known to mediate complex behaviors in the adult, that can be identified using specific Gal4 drivers (Su and O'Dowd, 2003).

In the adult *Drosophila* brain, chemical synapses form between neuronal processes but, in contrast to vertebrates, are generally not found on the cell bodies (Prokop and Meinertzhagen, 2006). This can be attributed in part to structural segregation: the neuronal processes, consisting of a primary axon and secondary dendritic extensions, are located in the central neuropil, with the somata located in the outer cortical

region. In culture, all of the chemical synapses identified at the ultrastructural level are also between the neuritic processes even though there is no strict separation between the neurites and the cell bodies. This indicates that although the neurons are developing in very different conditions in culture versus in the brain, at least some level of target specificity is retained. This could be explained by neurites being attracted preferentially to other neurites and/or the cell bodies being less responsive to signals that trigger postsynaptic differentiation.

Single cholinergic boutons in the *Drosophila* mushroom body exhibit both pre- and postsynaptic specializations, consistent with the idea that single neurites can serve both axonal and dendritic functions in the *Drosophila* CNS (Yasuyama et al., 2002). This structural organization is retained in culture as some of the neurites are presynaptic to one process and postsynaptic to another. Single regions of contact between two neurites with synapses of opposing polarity demonstrate that the cultured neurons also retain the ability to form reciprocal synapses, such as those previously described in the insect antennal lobe (Distler et al., 1998). It seems likely that feedback inhibition in the *Drosophila* antennal lobe (Wilson and Laurent, 2005) is mediated by reciprocal synapses, as in the mammalian olfactory bulb (Schoppa and Urban, 2003). Thus, these cultures provide an opportunity to explore regulation of activity in reciprocally connected neurons that would be much more difficult to access *in vivo*.

The structure of presynaptic electron dense foci associated with the active zone of most synapses varies between species and between different synapses within the same species (Zhai and Bellen, 2004). In *Drosophila*, presynaptic electron densities known as T-bars, are characteristic of active zones of photoreceptors, NMJs, and some central synapses in the adult brain (Prokop and Meinertzhagen, 2006). Electron dense structures, appearing as T-bars or elliptical densities surrounded by vesicles are also a typical feature of cross sections through the active zones in the cultured neurons. The ability of the cultured neurons to form T-bars is consistent with the punctate expression of an antibody against BRP, a synaptically localized protein that has recently been shown to be involved in T-bar formation, vesicular release, and clustering of calcium channels at chemical synapses *in vivo* (Atwood, 2006; Kittel et al., 2006; Wagh et al., 2006).

The majority of tangential sections through active zones are at the interface between the basal substrate and the cultured neurons, with no postsynaptic component in the prior sections. In addition, there is punctate expression of presynaptic proteins on isolated neurites. Together these data demonstrate that cultured pupal neurons, similar to embryonic *Drosophila* neurons in culture (Kuppers-Munther et al., 2004) and motoneurons *in vivo* (Prokop et al., 1996), can assemble presynaptic active zones in the absence of a postsynaptic target cell. It seems possible that at least some of these are functionally active sites of vesicular release of neurotransmitter based on demonstration of spontaneous acetylcholine secretion from neurites and growth cones of embryonic "giant" *Drosophila* neurons (Yao et al., 2000). It will be interesting to determine if orphan presynaptic sites in the pupal neurons are functional and also possibly mobile, similar to functional orphan release sites in mature cultures of dissociated hippocampal neurons that are formed from and recruited to existing synaptic sites (Krueger et al., 2003).

Previous functional studies demonstrate two classes of spontaneously active functional synaptic connections in neurons cultured from the late stage pupal brain: the most frequently observed are cholinergic and the rest are GABAergic (Su and O'Dowd, 2003). The GABAergic synapses, identified by immuno-gold labeling in this study, are characterized by the presence of a relatively homogeneous population of small diameter clear core vesicles, similar to the vesicles described at central GABAergic synapses in locust (Watson and Schurmann, 2002). On the basis of the functional studies, it is likely that many of the non-GABA synapses are cholinergic. Ultrastructural features of the non-GABA synapses are also consistent with this interpretation. First, similar to central cholinergic synapses in insect brains, the average diameter of the clear core synaptic vesicles is larger in the non-GABA synapses (Watson and Schurmann, 2002). In addition, the postsynaptic density is prominent in most cross sections through presumptive cholinergic synapses in culture, consistent with cholinergic synapses seen in *Drosophila* mushroom bodies (Yasuyama et al., 2002). However, preliminary immunofluorescent antibody staining indicates that a small percentage of the neurons are serotonergic and dopaminergic. Thus additional studies will be required to positively identify cholinergic synapses.

In addition to the clear core vesicles, a small number of granular vesicles are found within 200 nm of many of the active zones in the cultured *Drosophila* neurons. A few of the granular vesicles are classic dense core vesicles. However, the majority do not have single central dense region but instead have granular inclusions, similar in morphology to vesicles that appear in synapses in the adult *Drosophila* brain

(Reist et al., 1998). It is not possible on the basis of morphology to determine the contents of granular vesicles but it seems likely that they contain neuromodulators given the wide-spread distribution of neuropeptides and biogenic amines in interneurons in the insect brain (Watson and Schurmann, 2002; Nassel and Homberg, 2006). The presence of granular vesicles in both types of synapses suggests that neuropeptides are co-transmitters at both GABAergic and cholinergic synapses in the cultured *Drosophila* neurons.

The cultured pupal neurons also appear to retain the ability to form mixed electrical–chemical synapses based on the presence of gap junction profiles, immediately adjacent to the active zone of some chemical synapses. The relatively high abundance of mixed synapses in culture may be related to the finding that all innexins with the exception of *inx4* are expressed a high levels throughout the central brain of mid- to late stage pupae (Stebbings et al., 2002), the tissue from which the cultures are prepared. Although preliminary studies in our lab indicate that a subpopulation of pupal neurons in culture exhibit synchronized spontaneous calcium transients in the presence of synaptic current blockers, additional experiments will need to be done to determine if gap junctions are involved.

Two types of gap junctions have been described in the giant fiber synapse in the adult *Drosophila* thoracic ganglion. One encoded by the *shak-B innexin* gene, characterized by regularly spaced septa and a single row of vesicles on the presynaptic side. A second class does not have septa and is not eliminated in the *shak-B2* mutants. (Blagburn et al., 1999; Godenschwege et al., 2006). The gap junctions in the pupal cultures have no septa and are more similar in structure to the second class of gap junctions at the giant fiber synapse. Therefore, it seems likely that the gap junction structures in the cultured pupal neuron are encoded by one of the other neuronally localized *Drosophila* innexin genes such as *inx5, 6, or 7* (Stebbings et al., 2002; Phelan, 2005).

Another striking feature of the gap junctions in the cultured neurons are closely associated membrane compartments. These appear to be extensions of the endoplasmic recticulum, similar to postsynaptic cisternae that are a feature of the chemical synapses formed by L1 and L2 in the *Drosophila* visual system (Meinertzhagen and O'Neil, 1991). Similar compartments are also seen in images of *shakB2* resistant gap junctions (Blagburn et al., 1999), but they are not described in detail so it is not clear if these are a consistent feature of the giant fiber synapse. While the function of the cisternal compartments is not known, their close proximity to gap junctions in cultured neurons suggests they may play a role in buffering calcium during gap junction communication.

The presence of gap junctions between the processes of cultured neurons are in line with previous studies showing that gap junction profiles have been found in several other neuropil regions in the nervous system of adult *Drosophila* (Shimohigashi and Meinertzhagen, 1998; Sivan-Loukianova and Eberl, 2005). However, unexpectedly, gap junctions are also formed between cell bodies of the neurons in culture. This has not been reported *in vivo* where neuronal cell bodies are physically separated from each other by intervening glial processes (Hahnlein and Bicker, 1996). The cultured neurons lack ensheathing glial cells allowing contact between plasma membranes of adjacent cell bodies. This suggests that glial cells in the *Drosophila* CNS, in addition to their important role in neuronal survival (Shepherd, 2000), formation of the blood brain barrier, and likely involvement in neurotransmitter homeostasis (Daneman and Barres, 2005; Parker and Auld, 2006), may also regulate where gap junctions form.

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