Electrophysiological Analysis of Synaptic Transmission in Central Neurons of *Drosophila* Larvae

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Rohrbough, Jeffrey, and Kendal Broadie. Electrophysiological analysis of synaptic transmission in central neurons of *Drosophila* larvae. *J Neurophysiol* 88: 847–860, 2002; 10.1152/jn.01010.2001. We report functional neuronal and synaptic transmission properties in *Drosophila* CNS neurons. Whole cell current- and voltage-clamp recordings were made from dorsally positioned neurons in the larval ventral nerve cord. Comparison of neuronal Green Fluorescent Protein markers and intracellular dye labeling revealed that recorded cells consisted primarily of identified motor neurons. Neurons had resting potentials of −50 to −60 mV and fired repetitive action potentials (APs) in response to depolarizing current injection. Acetylcholine application elicited large excitatory responses and AP bursts that were reversibly blocked by the nicotinic receptor antagonist dtC (d-tubocurarine). GABA and glutamate application elicited similar inhibitory responses that reversed near normal resting potential and were reversibly blocked by the chloride channel blocker picrotoxin. Multiple types of endogenous synaptically driven activity were present in most neurons, including fast spontaneous synaptic events resembling unitary excitatory postsynaptic currents (EPSCs) and sustained excitatory currents and potentials. Sustained forms of endogenous activity ranged in amplitude from smaller subthreshold “intermediate” sustained events to large “rhythmic” events that supported bursts of APs. Electrical stimulation of peripheral nerves or focal stimulation of the nervous system, enabling genetic and molecular approaches in *Drosophila* CNS, has been used extensively to elucidate cellular and molecular mechanisms of synaptic development and transmission throughout development and thus well-characterized. *Drosophila* central neuronal synapses by comparison are small, complex in number and organization, and seemingly incomparable to detailed electrophysiological study and thus almost completely uncharacterized. Nevertheless, the limitations, both real and perceived, of such an exclusive approach have become increasingly apparent as genetic and behavioral screens continue to reveal additional classes of “learning and memory” genes (Boynton and Tully 1992; Grotewiel et al. 1998; Pinto et al. 1999; Skoulakis and Davis 1996) which when mutated produce similar altered transmission phenotypes at the NMJ (Broadie et al. 1997; Rohrbough et al. 1999, 2000).

A long-standing irony for *Drosophila* neurophysiologists is that despite the large body of related genetic, behavioral, and electrophysiological studies, examinations of synaptic transmission and activity-dependent plasticity have relied almost exclusively on the NMJ. In particular, questions of central neurotransmission and processing underlying synaptic and behavioral modulation remain largely unaddressed in the CNS. The reasons for this paradox are readily apparent. The NMJ is large and highly accessible to morphological and functional studies throughout development and thus well-characterized. *Drosophila* central neuronal synapses by comparison are small, complex in number and organization, and seemingly incomparable to detailed electrophysiological study and thus almost completely uncharacterized. Nevertheless, the limitations, both real and perceived, of such an exclusive approach have become increasingly apparent as genetic and behavioral screens continue to reveal additional classes of “learning and memory” genes (Boynton and Tully 1992; Grotewiel et al. 1998; Pinto et al. 1999; Skoulakis and Davis 1996) which when mutated produce similar altered transmission phenotypes at the NMJ (Broadie et al. 1997; Rohrbough et al. 1999, 2000).

An alternative approach to recording intracellularly from central neurons has been to study properties of differentiated *Drosophila* neurons in culture. Embryonic neurons and cleavage-arrested “giant neurons” have proved especially useful in defining the development of excitability and the function of genes encoding various ion channels in a heterogenous neuronal population (O’Dowd 1995; O’Dowd and Aldrich 1988; Saito and Wu 1991; Sole and Aldrich 1988; Tsunoda and Salkoff 1995a,b). However, after nearly two decades of *Drosophila* culture studies, spontaneous synaptic transmission between neurons has only recently been reported (Lee and

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**INTRODUCTION**

*Drosophila* is a uniquely suited model for studying the nervous system, enabling genetic and molecular approaches in combination with assays of neuronal and synaptic structure and function. The neuromuscular junction (NMJ) of the larva and adult has been used extensively to elucidate cellular and molecular mechanisms of synaptic development and transmission (Kawasaki et al. 1998, 2000; Koh et al. 1999; Marek et al. 2000; Renger et al. 2000; Wu et al. 1999). Likewise, *Drosophila* behavioral mutants have provided valuable insights into plasticity pathways, particularly Ca$^{2+}$ and cAMP-dependent signaling pathways, important for learning and memory (Connoly et al. 1996; Davis 1996; Davis et al. 1995; Dubnau and Tully 1998; Joiner and Griffith 1999; Yin and Tully 1996).

Synaptic studies directly support the role of these genes and pathways in morphological and functional plasticity mechanisms (Davis et al. 1996; Griffith 1997; Renger et al. 2000; Wang et al. 1994; Zhong and Wu 1991; Zhong et al. 1992). Most importantly, the molecular mechanisms underlying fundamental aspects of synaptic transmission, plasticity, and behavior in *Drosophila* are highly conserved across species (Koh et al. 2000).

A long-standing irony for *Drosophila* neurophysiologists is that despite the large body of related genetic, behavioral, and electrophysiological studies, examinations of synaptic transmission and activity-dependent plasticity have relied almost exclusively on the NMJ. In particular, questions of central neurotransmission and processing underlying synaptic and behavioral modulation remain largely unaddressed in the CNS. The reasons for this paradox are readily apparent. The NMJ is large and highly accessible to morphological and functional studies throughout development and thus well-characterized. *Drosophila* central neuronal synapses by comparison are small, complex in number and organization, and seemingly incomparable to detailed electrophysiological study and thus almost completely uncharacterized. Nevertheless, the limitations, both real and perceived, of such an exclusive approach have become increasingly apparent as genetic and behavioral screens continue to reveal additional classes of “learning and memory” genes (Boynton and Tully 1992; Grotewiel et al. 1998; Pinto et al. 1999; Skoulakis and Davis 1996) which when mutated produce similar altered transmission phenotypes at the NMJ (Broadie et al. 1997; Rohrbough et al. 1999, 2000).

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O’Dowd 1999, 2000; Yao et al. 2000) and reliable evoked neuronal transmission has so far not been demonstrated. Notably, however, intracellular recordings of voltage-gated currents and endogenous synaptic activity in identified embryonic motor neurons have recently been achieved in normal and mutant conditions (Baines and Bate 1998; Baines et al. 1999, 2001), suggesting the feasibility of in vivo neuronal synaptic recordings. Our long-term interest in the genetic regulation of synaptic plasticity and learning/memory mechanisms depends on developing tractable in situ central preparations in Drosophila amenable to functional synaptic recordings and, ultimately, to detailed plasticity analyses. Here we report whole cell current and voltage recordings from an accessible group of identified motor neurons in the larval CNS. These neurons display robust and repetitive firing on depolarization, respond to appropriate excitatory and inhibitory neurotransmitters, and exhibit both endogenous and evoked synaptic activity driven largely by excitatory cholinergic synaptic input. These detailed recordings from Drosophila central neurons provide a basis for future analyses of central synaptic transmission and plasticity in Drosophila.

METHODS

Fly stocks

Drosophila stocks were maintained at 25°C on standard cornmeal medium supplemented with dry yeast. All experiments were performed on mature “wandering” third instar larvae. The embryonic lethal abnormal visual system (elav) neural promoter (Yao and White 1981) drives expression of GFP in the larval CNS. These neurons display robust and repetitive firing on depolarization, respond to appropriate excitatory and inhibitory neurotransmitters, and exhibit both endogenous and evoked synaptic activity driven largely by excitatory cholinergic synaptic input. These detailed recordings from Drosophila central neurons provide a basis for future analyses of central synaptic transmission and plasticity in Drosophila.

Examination of neuronal GFP markers and neuronal identification

Five prominent superficial dorsal neurons were readily visualized near the midline in each hemisegment of the ventral nerve cord (VNC) of elav-GFP larvae. In the Drosophila embryo, five similarly positioned dorsal motor neurons, aCC and RPI-4, have been identified by retrograde cell labeling of the postsynaptic muscle targets (Baines et al. 1999; Landgraf et al. 1997). Similar neuronal labeling studies in mature larvae have recently indicated that the identity of two of these five neurons (aCC and RPI-3) is consistent with that in the embryo. Interestingly, the identity and motor projections of the remaining three neurons differ in the embryo and larval VNC (Hoang and Chiba 2001; L. Griffith, personal communication). However, all are glutamatergic motor neurons serving similar functions in driving larval muscle contraction and locomotion. In early stages of this study, we routinely examined GFP fluorescence both immediately after dissection and again after enzymatic and mechanical treatment of the VNC, to confirm that exposed cell neurons were from this identified subgroup (see following text). Location and projections of larval central cholinergic neurons were examined in a GAL4 line (Cha-GAL4 UAS GFP) expressing GFP under control of the choline acetyltransferase (Cha) promoter, kindly supplied by Dr. P. Salvaterra.

Neuronal electrophysiological recordings

Larvae were secured at the head and tail to silicone elastomer (Sylgard)-coated coverslips in low Ca2+ (0.2 mM) recording saline, using surgical histoacryl glue (Histoacryl blue, B. Braun, Emmenbrucke, Switzerland). Glue was applied to the substrate via glass patch electrode-type pipettes (WPI 1B100F-4 capillaries). Larvae were dissected open dorsally, and the cuticle was glued flat as described previously (Rohrbough et al. 1999) to expose the dorsal aspect of the CNS and ventral ganglion. Because the larval CNS is loosely tethered and moves freely, thin ribbons of glue were applied across the preparation anterior and posterior to the CNS to help immobilize and reduce random or perfusion-related movement of the structure. In some cases, the entire CNS with a small amount of attached tissues was cut free and glued directly to the cover slip. No differences in results were found between these approaches. Dorsal neuronal cell bodies in the ventral nerve cord were exposed with a combination of focal protease application and manual pressure, similar to the approach described by Baines and Bate (1998) for embryonic neuronal recordings. The CNS sheath material was drawn by suction for several minutes into the tip of a large-diameter patch pipette (20–50 μm) containing 0.5–1% protease (type XIV, Sigma) in recording saline. Gentle positive and negative pressure was alternated under visual control to visibly rupture the sheath and to further clear away overlying material and free underlying cell bodies. This procedure was usually repeated once or twice in adjacent areas, and the preparation was then washed with fresh recording saline. In most cases, several soma accessible to a recording pipette could be distinguished under Nomarski optics. In practice, seals were readily formed on cleanly exposed soma, while stable whole cell recordings were successfully achieved for ~50% of attempts of patched cells. Each preparation was limited to one or two successful recordings.

Standard whole cell voltage- and current-clamp recordings were made at 18–20°C, except where noted in the following text. Functional neuronal identity was confirmed for all cells by two criteria: a negative resting potential greater than ~30 mV, although the majority of recorded cells had resting potentials of ~45 to ~65 mV (~52.9 ± 7.5 (SD) mV, n = 78) and action-potential (AP) firing in response to intracellular injection of depolarizing current. In some cases, AP firing was confirmed in voltage-clamp mode by the presence of characteristic biphasic regenerative action potential currents at depolarizing holding potentials (~40 to ~30 mV). In earlier stages of this study, approximately 10% of patched cells failed these criteria, and these were discarded from analysis. To facilitate comparison of results in current-clamp recordings, membrane potential was adjusted if necessary to ~30 to ~60 mV with DC current. APs were elicited with square 200-ms current pulses, applied in increasing 20-pA increments. Firing threshold was determined from the inflection point on the rising phase of the voltage response at minimal suprathreshold current. AP amplitude was measured from threshold to the peak, and AP duration as the interval between the threshold potential on the rising and falling phase. Values from several APs were averaged for each neuron. AP firing frequency and adaptivity was determined from 800-ms current pulses 20–40 pA greater than the minimal suprathreshold current.

Voltage-clamp recordings were made at a holding potential of ~60 mV except where indicated. One or more categories of endogenous or evoked synaptic transmission events was evident in all neurons. Fast spontaneous excitatory postsynaptic currents (EPSCs) were recorded for 10–40 s shortly after break-in. Sustained or “rhythmic” endogenous activity and analogous sustained evoked responses were recorded continuously for ~30 min. In neurons exhibiting sustained forms of endogenous synaptic activity (~40% of those examined), electrically evoked sustained synaptic responses were also reliably observed. A large suction pipette (~20 μm tip) filled with external saline was used to deliver stimuli (3–40 V, ~1–10 ms) to the lateral surface of the CNS, usually anterior to the soma, or to an anterior peripheral nerve or nerve stump near its exit from the CNS. In
endogenously active neurons, CNS or nerve stimulation effectively generated analogous forms of activity, with either approach producing similar responses. In many cases, both current and voltage recordings of synaptic activity were made from the same neurons. In those neurons (~60%) not exhibiting endogenous sustained activity, fast EPSCs could be successfully evoked in ~80% of trials by focal stimulation of the neuropil. Examination of Lucifer-yellow-filled motor neurons showed that prominent arborizations, presumably representing the dendritic regions, were located either ipsi- or contralateral to the soma. Saline-filled pipettes (5–10 μm tips) were impaled into the neuropil either ipsi- or contralateral to recorded soma, approximately one segment anterior or posterior to the expected location of the dendritic region.

Experiments to examine blockade of synaptic transmission in shibire conditional mutants were performed on a temperature-controlled stage (Dagan, Minneapolis, MN). Expansion of the recording chamber/substrate and preparation movement precluded reliable neuronal recordings while varying bath temperature between normal and restrictive (29–34°C) levels. Control and shibire preparations were therefore first incubated for 10–30 min at restrictive bath temperature (29–34°C) prior to recording at the stable restrictive temperature. Whole cell recording lifetime and quality were nevertheless limited at temperatures >25°C, and evoked synaptic currents at 29–34°C often exhibited substantial rundown over multiple trials. Data at elevated temperature were limited to initial sEPSC amplitude and frequency, and maximum evoked EPSC amplitude.

Electrophysiological data were filtered (0.5–2 kHz), digitized to disk (5–10 kHz), analyzed, and exported for display using PClamp6 acquisition and analysis hardware and software (Axon Instruments, Burlingame, CA), the IGOR analysis programs (WaveMetrics, Lake Oswego OR), and standard spreadsheet and graphics software. Quantified data are presented as mean ± SE.

**Solutions and drugs**

Normal external recording saline contained (in mM) 140 NaCl, 3 KCl, 2 CaCl₂, 4 MgCl₂, 5 HEPES, 10 sucrose, and 2 NaOH (pH 7.2). The patch solution contained (in mM): 140 K-Acetate, 2 MgCl₂, 0.1 CaCl₂, 10 HEPES, 1.1 EGTA, 2 Na₂-ATP, and 6 KOH (pH 7.2). In experiments examining pharmacology of agonist responses and endogenous activity, normal external saline, saline without added Ca²⁺, and saline containing picrotoxin (0.5–1.0 mM) or t-tubocurarine (0.5 mM; Sigma), were exchanged by perfusion of the recording chamber (~1.5 ml volume). Otherwise, under control conditions the recording chamber was not perfused. Viable recordings of endogenous activity were obtained for ≤30 min both with and without bath perfusion, though recording resolution and synaptic current amplitudes tended to gradually decline with longer recording times. AcH and GABA (Sigma) were applied iontophoretically to the soma of recorded cells via sharp microelectrodes containing a 100 mM solution of agonist in pH 4–5 to favor agonist ejection by positive charge. l-Glutamate (2 mM in normal external saline) was focally applied via pressure pipette (~1 μm tip). The potassium-based intracellular solution was used for all recordings so that neuronal resting potentials and excitability properties were minimally altered. No other drugs or ion channel blockers were added to the external saline in effort to maintain neuronal firing and endogenous and evoked syntactically driven activity.

**Intracellular dye fills and fluorescence imaging**

Lucifer yellow (dipotassium salt, 1–2 mg/ml; Molecular Probes, Eugene OR) was added to the patch solution in most recordings from wild-type and non-GFP mutant animals to visualize the position and morphology of recorded cells. Lucifer yellow-labeled preparations were fixed for 15–30 min in 4% paraformaldehyde following the recording and mounted in Vectashield medium. Images of dye labeling were collected on the same or following day, using a Zeiss Axioscope microscope equipped with a SPOT camera and image-acquisition software, or a Zeiss confocal microscope. Presentation figures showing neuronal GFP expression and Lucifer yellow labeling were constructed with Adobe Photoshop.

**RESULTS**

**Larval CNS preparation and identification of motor neurons**

The *Drosophila* larval ventral nerve cord (VNC) contains ~200 neurons per hemisegment, including 30–35 identified primary motor neurons (Landgraf et al. 1997). Most neuronal cell bodies are relatively inaccessible within the VNC, which is ensheathed by glia and connective tissue, and difficult to identify (Fig. 1A). However, five large (~10–15 μm soma diameter) neurons are prominently clustered in each hemisegment in a superficial location near the dorsal midline, as visualized by confocal imaging of panneuronal GFP expression in the dorsal VNC (Fig. 1B). Comparison of embryonic and larval motor neurons which have been unambiguously identified by dye-labeling their motor terminals indicates that these neurons likely correspond to the identified aCC and RP3 embryonic neurons (Baines et al. 1999; Landgraf et al. 1997), as well as three other larval type I motor neurons (Hoang and Chiba 2001; L. Griffith, personal communication). This group of neurons was targeted as being potentially accessible to detailed electrophysiological recording with minimal disruption of the surrounding architecture, providing an opportunity to investigate neuronal excitability and synaptic transmission properties in the larval CNS.

Whole cell recordings from acutely exposed cells consistently revealed robust neuronal firing properties, responses to neurotransmitter agonists, and functional endogenous synaptic responses consistent with a motor neuron identity (see following text). In most experiments with non-GFP animals, recorded neurons were loaded with Lucifer yellow dye via the recording pipette. Visualization of the intracellular fluorescence following the recording confirmed the neuronal morphology of recorded cells, including axonal projections and extensively branched arborizations presumably representing dendritic regions (Fig. 1C). While poor dye filling or fluorescence signal/background prevented an unambiguous confirmation of motor neuron identity in some preparations, a labeled axon leaving the neuropil either ipsilateral or contralateral to the soma was present for 67% (38 of 57) of filled neurons.

The segmental motor neurons function to drive larval locomotion. Although neither specific excitatory nor inhibitory presynaptic inputs to these neurons are identified, endogenous activity in embryonic *Drosophila* motor neurons requires excitatory cholinergic transmission (Baines and Bate 1998; Baines et al. 1999). We examined the cellular location and processes of cholinergic neurons in a GAL4 UAS line expressing GFP selectively in cholinergic neurons (Cha-GAL4 UAS GFP). Cholinergic processes are found extensively throughout the VNC neuropil, projecting both longitudinally and transversely and clearly overlapping extensively with neuropil areas occupied by the branched dendritic-like arbors of motor neurons. In addition, numerous axonal swellings and varicosities are resolvable along cholinergic processes which potentially represent synaptic structures (Fig. 1D). We have previously shown similar structures localize GFP-labeled synaptotagmin and n-synaptobrevin and thus likely represent functional synaptic contacts (Rohrbough et al. 2000).
control neurons and present in a minority (16%) of cells with less negative (-45 mV) RMP. By contrast, in the hyperexcitable K+ channel double mutant ether-a-go-go Shaker (eag Sh), sAPs from RMP of -50 to -60 mV were present in 88% of neurons (n = 8; Fig. 2C and Table 1). These mutants also exhibit increased spontaneous motor axon AP firing and elevated endogenous synaptic transmission at larval neuromuscular junctions (Ganetzky and Wu 1983; and data not shown). Consistent with the increased incidence of sAPs, eag Sh neurons had significantly more negative threshold for AP firing in response to depolarizing current injection (-40 ± 2 mV; P < 0.0002 vs. control).

All neurons fired multiple APs in response to 200- to 800-ms suprathreshold depolarizing current pulses (Fig. 2, A and B). The majority of neurons in both control (23 of 34) and eag Sh mutant larvae (6 of 7) exhibited adaptive AP firing (Fig. 2B), characterized by a decrease in AP frequency during prolonged pulses, while the remaining generated APs at a fairly invariant, or tonic, frequency (Fig. 2A). “Adaptive” firing neurons (Zhao and Wu 1997) were classified as those for which the interval between the last two APs in an 800-ms pulse was >40% greater than that between the initial two APs. Firing patterns remained consistent from trial to trial in individual neurons. Neither tonic nor adaptive firing category could be correlated specifically with morphological differences or with motor neuron identity. Neuronal electrical excitability properties are summarized in Table 1.

Responses to excitatory and inhibitory neurotransmitters

Neurons in vivo responded to focal applications of three putative neurotransmitters: acetylcholine (ACh), GABA, and glutamate. Brief (1–2 ms) iontophoretic ACh application elicited robust excitatory responses in all cells assayed (Fig. 3A). ACh-evoked current and depolarizing potential responses had amplitudes of 215 ± 49 pA (VHI -60 mV; n = 7) and 44 ± 3 mV (from RMP; n = 6), respectively. ACh-evoked depolarizations were invariably accompanied by bursts of APs on the rising phase and peak of the response. ACh responses were reversibly blocked by the nicotinic ACh receptor antagonist, d-tubocurarine (dtC, 0.5 mM; Fig. 3A).

All cells assayed responded to iontophoretically applied GABA. GABA responses in different neurons varied in amplitude and duration and often exhibited a biphasic appearance, but were predominantly inhibitory and mediated by chloride currents (Fig. 3B). GABA elicited potential responses reversing near RMP, and currents that clearly reversed at negative holding potentials (-56 ± 3 mV) in six of nine neurons assayed, in reasonable agreement with an ionic dependence on Cl-. GABA responses were also reversibly blocked by the Cl- ion channel blocker, picrotoxin (PTx, 0.5 mM; n = 4). In three of nine neurons, GABA elicited depolarizing responses of 16 mV from RMP and one or more APs (not shown); however, these “excitatory” responses were substantially slower and weaker than both ACh-evoked responses and endogenous spontaneous excitatory synaptic potentials (see following text).

Glutamate application (2 mM in normal bath saline, 100- to 500-ms pressure ejection) also evoked inhibitory responses (Fig. 3C) that were especially prolonged (2–5 s) but otherwise strongly resembled inhibitory GABA responses. Glutamate responses clearly reversed at -50 to -60 mV (-55 ± 2 mV;
TABLE 1.  
Electrical excitability properties in larval motor neurons

<table>
<thead>
<tr>
<th></th>
<th>RMP, mV</th>
<th>AP Current, pA</th>
<th>AP Threshold, mV</th>
<th>AP Amplitude, mV</th>
<th>AP Duration, ms</th>
<th>AP Frequency, Hz</th>
<th>Percent Neurons With sAPs, %</th>
<th>AP Firing Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−54 ± 2</td>
<td>63 ± 6</td>
<td>−29 ± 1</td>
<td>19 ± 1</td>
<td>4.1 ± 0.1</td>
<td>43 ± 3</td>
<td>16</td>
<td>68% adaptive</td>
</tr>
<tr>
<td>(n = 38)</td>
<td>(−36 to −72)</td>
<td>(20–140)</td>
<td>(−44 to −17)</td>
<td>(6–36)</td>
<td>(2.5–6.2)</td>
<td>(18–75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eag sh</td>
<td>−51 ± 3</td>
<td>18 ± 2*</td>
<td>−40 ± 2*</td>
<td>17 ± 2</td>
<td>3.7 ± 0.2</td>
<td>35 ± 6</td>
<td>88</td>
<td>83% adaptive</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(−44 to −68)</td>
<td>(10–20)</td>
<td>(−47 to −35)</td>
<td>(11–21)</td>
<td>(2.7–4.4)</td>
<td>(15–62)</td>
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</table>

Values are means ± SE, with range of values indicated in parentheses. Control recordings consisted of embryonic lethal abnormal visual system (elav) Green Fluorescent Protein (GFP) and wild-type larvae. Asterisks indicate mutant values significantly different from control (P < 0.005).  
Resting membrane potential (RMP) measured shortly after establishing whole cell configuration.  
Minimal depolarizing current (200-ms duration, 20 pA increments) sufficient to generate action potential (AP) firing.  
Determined from inflection point on rising phase of AP.  
Measured from AP threshold to AP peak.  
Interval between threshold potentials on the rising and falling phase of AP.  
During first 200 ms for current step 20–40 pA greater than the minimal suprathreshold level.  
% of neurons firing APs spontaneously from RMP in the absence of DC current pulses.  
Adaptive firing criteria was defined as an increase in AP interval by >1.4-fold between the first and last pairs of APs during an 800-ms depolarizing current step.  
sAPs, spontaneous APs.

n = 7) in both current and voltage recordings and were reversibly blocked by PTx (1 mM, n = 2). Glutamate also prevented AP generation when applied to neurons during normally suprathreshold depolarizing current steps (n = 2; data not shown). These motor neurons thus express Cl−-permeable GABA- and glutamate-gated receptors in addition to nicotinic ACh receptors.  

Endogenous excitatory synaptic currents and potentials in vivo

Neurons examined in this study exhibited several categories of endogenous synaptic activity. Immediately detectable in virtually all cells following patch rupture were fast events resembling unitary spontaneous excitatory synaptic currents (sEPSCs) (5–50 pA) or synaptic potentials (1–10 mV) in current and voltage recordings, respectively. Events with ≥10-pA peak amplitude were well resolved and had rapid rise times (~1 ms) and brief duration (<25 ms; Fig. 4A), closely resembling fast cholinergic sEPSCs reported in Drosophila cultured neurons (Lee and O’Dowd 1999, 2000) and in embryonic motor neurons in vivo (Baines et al. 1999). Spontaneous currents with fast rise times and much slower decays resembling GABAergic inhibitory postsynaptic currents (IPSCs) recorded in culture (Lee and O’Dowd 1999) were rarely observed. Fast sEPSC amplitude and frequency in control neurons averaged 14.1 ± 2.0 pA and 4.1 ± 1.4 Hz, respectively (n = 18), but varied widely among individual neurons.  
sEPSC amplitude and frequency were correlated overall (r = 0.83, Fig. 4A). Because all our recordings were made in TTX-free saline, spontaneous presynaptic AP firing may contribute to larger and more frequent sEPSCs in some neurons as found in the embryo (Baines and Bate 1998) and in cultured embryonic neurons (Lee and O’Dowd 1999).

Differing strikingly from fast synaptic events was the presence in approximately 40% of neurons (21 of 50 wild-type and elav GFP control neurons) of endogenous currents and potentials with much larger amplitudes and longer durations (Figs. 4B and 5; Table 2). We termed the largest and most prominent class of sustained activity spontaneous “rhythmic” currents (SRCs) or potentials (SRPs). These events appeared to represent a form of periodic endogenous excitatory motor output, supporting a burst of APs on the prolonged response in both current and voltage recordings (Figs. 4B and 5). SRCs averaged 445 ± 66 pA in amplitude and 663 ± 59 ms in duration and occurred at a frequency of 2.4 min−1 (range of 0.3–6 min−1) in active control neurons analyzed (n = 15; Table 2). Active neurons also typically exhibited a range of smaller (20–90 pA), noisier currents that we termed “intermediate” sustained currents (Figs. 4B and 5A, Table 2). Intermediate currents often appeared to be composed of faster sEPSC-like
currents visible as multiple peaks on the slower summed response (Figs. 4B and 5A). Similar fast current peaks could also be observed in the initial component of SRCs (Fig. 5A), suggesting these sustained endogenous events represent a continuum of a common form of excitatory synaptic drive.

When current and voltage recordings were compared for the same active neurons, it appeared that intermediate sustained currents produced spontaneous potentials ranging from subthreshold depolarizations to those which were clearly excitatory, generating one or more APs (Fig. 4, B and C). In general, endogenous sustained currents exceeding 50–100 pA in amplitude were effectively excitatory, leading to AP firing. This observation is consistent with the result that focally evoked ACh currents, which averaged 215 pA in amplitude, invariably produced sustained large, rhythmic-like excitatory potentials indicative of overall synaptic drive.

Robust, SRC-like sustained evoked responses tended to be generated when preceded by quiescent or “refractory” intervals of ∼10 s, whereas evoked responses shortly following a large endogenous sustained event more often had reduced amplitudes (Fig. 5A, bottom). We found no obvious correlation between motor neuron morphology or identity revealed by dye labeling and differences in evoked response characteristics, or the presence of sustained endogenous (i.e., rhythmic and intermediate) excitatory activity. The similarity between various forms of spontaneous and stimulation-evoked activity suggested they share a common synaptic basis for their generation, though the levels of endogenous excitatory rhythms and different levels of voltage-gated current activation contribute to the range of response amplitudes and frequencies recorded in the cell body.

**Evoked excitatory synaptic responses in vivo**

An important finding was that electrical stimulation directly evoked or triggered excitatory responses similar to endogenous forms of activity. In neurons displaying endogenous sustained activity, direct stimulation of the lateral surface of the ventral ganglion (Fig. 5) or of a peripheral nerve with a suction electrode elicited analogous sustained excitatory responses supporting APs as well as smaller intermediate-like currents with variable amplitude (Fig. 5 and Table 3). The strength of evoked sustained responses from trial to trial was correlated with stimulation intervals and with the recent occurrence of large endogenous events. Robust, SRC-like sustained evoked responses tended to be generated when preceded by quiescent or “refractory” intervals of ∼10 s, whereas evoked responses shortly following a large endogenous sustained event more often had reduced amplitudes (Fig. 5A, bottom). We found no obvious correlation between motor neuron morphology or identity revealed by dye labeling and differences in evoked response characteristics, or the presence of sustained endogenous (i.e., rhythmic and intermediate) excitatory activity. The similarity between various forms of spontaneous and stimulation-evoked activity suggested they share a common synaptic basis for their generation, though the levels of endogenous excitatory rhythms and different levels of voltage-gated current activation contribute to the range of response amplitudes and frequencies recorded in the cell body.

**Spontaneous and evoked excitatory activity is mediated by cholinergic synaptic input**

The hypothesis that the activity observed in these neurons requires synaptic transmission for its generation is supported by pharmacological data. First, because synaptic transmission

![Figure 3](http://jn.physiology.org/)

**Figure 3.** Agonist responses in motor neurons in vivo. **A:** excitatory acetylcholine (ACh) responses are mediated by nicotinic receptors. Current responses in voltage-clamp recording (V_m = −60 mV, left) and voltage response from resting membrane potential (RMP; −55 mV, right) elicited by 1-2 ms iontophoretic ACh application in 2 different control neurons. ACh-evoked depolarizations are typically accompanied by APs on the rising phase and peak of the response. ACh responses are reversibly blocked by bath perfusion of the nicotinic receptor antagonist, d-tubocurarine (dTC, 0.5 mM; left). **B:** inhibitory GABA responses. **B1:** iontophoretic GABA application (25 ms, ↓) produces a prolonged hyperpolarizing response (left) from RMP of −50 mV, in contrast to clearly excitatory endogenous spontaneous potentials in the same neuron (right). **B2:** GABA-evoked currents reverse in the range of −50- to −60-mV holding potential in voltage-clamp recordings (left) in most neurons and are reversibly blocked by the chloride channel blocker picrotoxin (PTx, 0.5 mM, right), consistent with a GABA-gated Cl⁻ conductance. **C:** inhibitory glutamate responses. **C1:** top: prolonged hyperpolarizing response from RMP to focal glutamate application (2 μM in bath saline, 500-ms pressure ejection). Endogenous excitatory potentials were occurring spontaneously in the same neuron. **Bottom:** voltage response evoked by a 200-ms glutamate application in another neuron. Membrane conductance increases during glutamate response as evidenced by decrease in the amplitude of voltage deflections produced by repetitive 20-pA hyperpolarizing steps. **C2:** glutamate-evoked current responses reverse in the range of −50 to −60 mV (left) and are reversibly blocked by PTx (1 mM, right).
FIG. 4. Fast spontaneous excitatory postsynaptic currents (sEPSCs) and sustained endogenous excitatory synaptic activity exhibited by motor neurons. A, left: 1-s continuous traces of fast sEPSCs of varying amplitude and frequency in 2 different wild-type neurons. Fast synaptic currents of 10–50 pA were present in all control recordings and are likely to be nicotinic sEPSCs, as they were not observed in the presence of D-tubocurarine. Twenty consecutive sEPSCs from each neuron are shown superimposed (middle), with the average sEPSC for each neuron (right). Right: sEPSC amplitude is positively correlated with frequency (r = 0.83; wild-type recordings at 19–21 and 29–32°C), consistent with larger sEPSCs being mediated by presynaptic action potentials. B and C: endogenous sustained excitatory currents (B, ~60 mV V_H) and potentials (C, at RMP) in continuous voltage- and current-clamp recordings, respectively, from the same neuron. *, responses elicited at 20-s intervals by ACh application. Right: traces of representative individual events. Spontaneous events are categorized as 1) large sustained “rhythmic” currents and potentials (SRCs and SRPs) that generate action potentials and 2) smaller sustained “intermediate” currents and potentials. Portions of the rhythmic responses are shown at 4 times expanded time scale (→). ACh-evoked current and voltage responses (3) are shown for comparison.

requires presynaptic Ca^{2+} influx, we recorded from the same neurons in normal 2 mM Ca^{2+} and 0 Ca^{2+} external saline. All forms of endogenous and evoked activity were gradually abolished in 0 Ca^{2+} saline and partially or completely recovered after returning to normal saline (Fig. 5A). Second, both endogenous and evoked activity was largely or completely abolished in the presence of dTC (0.5 mM, Fig. 5B), indicating the observed activity is dependent on excitatory cholinergic transmission. The time course of pharmacological block, and especially recovery, was quite slow in these experiments, typically requiring recordings of 20–30 min. This is most likely because the synaptic regions within the neuropil remained relatively inaccessible to changes in the external medium, in contrast to the pharmacological block of agonist responses elicited from the exposed neuronal soma. In particular, blockade of synaptic activity by dTC was difficult to reverse; while ~5 min exposure reliably blocked most activity, only partial recovery was observed after >10-min wash (Fig. 5B).

Alterations in sEPSCs and evoked EPSCs in shibire conditional synaptic mutant and dance learning and synaptic plasticity mutant

The prominent sustained forms of synaptically driven endogenous activity described in the preceding text were present in similar subsets (25–45%) of recorded neurons in each of several mutant genotypes examined, including eag Sh (2 of 8 neurons), the conditional shibire^{ts1} (shi^{ts1}) mutant at permissive temperature (4 of 11 neurons), and the behavioral and synaptic plasticity mutant dance^{1} (5 of 11 neurons) (Table 2 and data not shown). The overall amplitudes, frequencies, and other features of endogenous activity in active neurons were similar to those in wild-type and elav GFP control preparations (Table 2). For this reason and because endogenous sustained activity was not observed in over half of the neurons in these preparations, we undertook to more directly examine mutant transmission properties by experimentally driving fast synaptic transmission. Focal electrical stimulation of the neuropil with a small suction electrode (5- to 10-μm tip, see METHODS) evoked fast EPSCs resembling aggregate sEPSCs with ~85% success (30 of 35 cells). Fast EPSCs were most efficiently and reproducibly evoked (≥50-pA EPSC amplitude, ~20-V stimulation) when postrecording examination of the dye-filled cell showed that the stimulation pipette was located closely adjacent to the presumed dendritic region (Fig. 6A). In such cases, fast EPSCs could be elicited in all-or-none fashion (Fig. 6B), indicating the stimulation was effectively exciting presynaptic APs.
Using this approach, we examined sEPSCs and directly evoked fast EPSCs in the temperature-sensitive synaptic mutant shi<sup>ts1</sup>, in which block of synaptic vesicle (SV) endocytosis at restrictive temperature results in SV depletion and loss of transmission (Ikeda et al. 1976; Keonig and Ikeda 1979; Pooldry and Edgar 1979; Salkoff and Kelly 1978) (Fig. 7A). At

![Endogenous activity and evoked responses require external Ca<sup>2+</sup> and are blocked by t-tubocurarine. A: endogenous and evoked activity is Ca<sup>2+</sup>-dependent. Portions of continuous records (left) showing endogenous sustained excitatory currents, and sustained excitatory currents evoked by electrical stimulation of the CNS (↓), in control 2 mM Ca<sup>2+</sup> saline (a), after ~10 min perfusion with 0 Ca<sup>2+</sup> saline (b) and ~7 min following return to 2 mM Ca<sup>2+</sup> saline (c). Right: traces show representative endogenous SRC and intermediate currents (1 and 2), and stimulus-evoked currents (3) for control and recovery periods. Boxed area of SRC trace is shown at twofold expanded scale; note fast synaptic currents on the rising phase of the large sustained event. All types of activity were eventually abolished in 0 Ca<sup>2+</sup> saline and recovered in normal saline. The recording shown in this example exhibited above average recovery. Fast AP-mediated currents initially present on large spontaneous and evoked events (present in a) typically became less pronounced or disappeared (as in c) over the course of prolonged recordings. Scales: 200 ms; 200 pA for large responses (1 and 3); 100 pA for intermediate currents (2); 100 pA and 100 ms for inset portion of trace 1. B: endogenous and evoked activity requires cholinergic synaptic transmission. Portions of continuous records (left) of endogenous currents and currents evoked by CNS stimulation (↓) in normal saline (a), following 3- to 4-min perfusion with normal saline containing 0.5 mM dtC (b), and following ~9-min wash (c). Right: traces show individual events for each period of the experiment as in A; both spontaneous and evoked currents were nearly completely abolished in dtC and partially recovered after wash. ●, residual activity in dtC. Incomplete recovery from dtC blockade was typical in these recordings.

**TABLE 2. Summary of endogenous sustained excitatory synaptic activity in larval motor neurons**

<table>
<thead>
<tr>
<th>Incidence Sustained Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Frequency of Sustained Activity&lt;sup&gt;b&lt;/sup&gt;, min&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>SRC Amplitude&lt;sup&gt;c&lt;/sup&gt;, pA</th>
<th>SRC Duration&lt;sup&gt;d&lt;/sup&gt;, ms</th>
<th>Int’d Current Amplitude&lt;sup&gt;e&lt;/sup&gt;, pA</th>
<th>Int’d Current Duration&lt;sup&gt;d&lt;/sup&gt;, ms</th>
<th>SRP Amplitude&lt;sup&gt;f&lt;/sup&gt;, mV</th>
<th>SRP Duration&lt;sup&gt;d&lt;/sup&gt;, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>elaV GFP, wild-type</td>
<td>21/50 (42%)</td>
<td>2.4 ± 0.3</td>
<td>445 ± 66</td>
<td>663 ± 59</td>
<td>60 ± 5</td>
<td>307 ± 17</td>
<td>34 ± 8</td>
</tr>
<tr>
<td>eag sh</td>
<td>2/8 (17–7.8%)</td>
<td>293 ± 129</td>
<td>879 ± 352</td>
<td>31 ± 15</td>
<td>372 ± 18</td>
<td>372 ± 18</td>
<td>41 (n = 1)</td>
</tr>
<tr>
<td>dnc&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5/11 (45%)</td>
<td>3.0 ± 0.4</td>
<td>460 ± 118</td>
<td>477 ± 115</td>
<td>51 ± 8</td>
<td>277 ± 80</td>
<td>41 (n = 1)</td>
</tr>
</tbody>
</table>

For columns 2–8, values are means ± SE, with range of values indicated in parentheses. <sup>a</sup> Percentage of recorded cells with large (AP-producing) or intermediate sustained forms of excitatory synaptic activity. <sup>b</sup> Average frequency of endogenous sustained events in active neurons. <sup>c</sup> Spontaneous “rhythmic” sustained currents producing APs. <sup>d</sup> Duration from event initiation to 80% decay from peak amplitude. <sup>e</sup> Intermediate sustained currents (non-AP producing).<sup>f</sup> Spontaneous “rhythmic” potential amplitude in active neurons.
permissive temperatures (19–21°C), wild-type and shi<sup>ts1</sup> sEPSCs and evoked EPSCs were indistinguishable. After 10–30 min at restrictive temperature (29–32°C), transmission in shi<sup>ts1</sup> larvae ranged from a nearly complete absence of sEPSCs or evoked EPSCs to sustained levels of transmission in the normal range (Fig. 7B). Mean sEPSC frequency (0.8 ± 0.3 Hz; n = 7) was reduced by >75% compared with wild-type and shi<sup>ts1</sup> at permissive temperature. Because of the large range of sEPSC frequencies (0.3–20 Hz) recorded under normal conditions, this decrease was barely statistically significant (P = 0.05 vs. wild-type and P = 0.07 vs. shi<sup>ts1</sup> controls at 19–21°C; Welch t-test). However, the reduction in shi<sup>ts1</sup> sEPSC frequency was accompanied by a significant reduction in sEPSC mean amplitude and variability (P < 0.01 vs. wild-type and P < 0.05 vs. shi<sup>ts1</sup> control at 19–21°C). In five of seven shi<sup>ts1</sup> recordings at 29–32°C, evoked transmission failed, or generated EPSCs with greatly reduced amplitude (<20 pA). Moreover, in no case did we observe sustained endogenous synaptic activity in mutant animals at 29–32°C. However, robust EPSCs (>95 pA) were evoked in two of seven shi<sup>ts1</sup> neurons, indicating that even prolonged incubation at restrictive condition did not eliminate or substantially reduce transmission in all mutant neurons. Overall, shibire mutant EPSC amplitude (39 ± 20 pA, n = 7; shi<sup>ts1</sup> at 29–32°C) was decreased by ~60% compared with wild type (100 ± 24 pA, n = 7; P = 0.07) and shi<sup>ts1</sup> control (89 ± 18 pA, n = 13; P = 0.08) at 19–21°C (Fig. 7B). Taken together, these results further indicate the direct synaptic basis of the endogenous and evoked activity observed in central neurons.

We likewise examined sEPSCs and evoked EPSCs in dunce<sup>l</sup> (dnc) cAMP phosphodiesterase mutants (Byers et al. 1981), which have elevated cAMP levels and short-term behavioral memory defects (Davis 1996). Functionally, dnc mutants have altered evoked synaptic responses and activity-dependent plasticity defects at the larval NMJ (Zhong and Wu 1991) and elevated sEPSC frequency in embryonic cultured neurons (Lee and O’Dowd 2000). In dnc larval motor neurons, we also found sEPSC frequency (11.4 ± 2.4 Hz, n = 7) to be increased by threefold compared with wild-type (P < 0.04), though mean sEPSC amplitude was unchanged. Exceptionally large fast evoked EPSCs (>200 pA) were recorded in three of eight dnc neurons, and overall mutant-evoked EPSC amplitude (157 ± 46 pA, n = 8) was increased, albeit insignificantly, by >50% compared with wild-type (P > 0.30; Fig. 7B).

**DISCUSSION**

The full advantage and potential of the *Drosophila* genetic system as a model for brain function and plasticity lies in extending detailed synaptic studies to the CNS. To achieve this objective, we must first characterize endogenous and evoked synaptic activity exhibited by identified central neurons in vivo. Our results significantly strengthen the promise that direct functional assays of central synaptic transmission in *Drosophila* are feasible and provide a path toward future assays of behaviorally relevant central synaptic transmission and plasticity mechanisms.

**Neuronal identity and heterogeneity in basic excitability properties**

This study focused on a small subpopulation of neurons clustered dorsally and near the midline of the larval VNC. Previous cell-labeling studies indicate that these consist primarily of type I glutamatergic motor neurons having either ipsi- or contralateral axons terminating on body wall muscles, though the soma location and/or muscle target of several neurons appear to undergo changes between embryonic and mature larval stages (Baines et al. 1999; Hoang and Chiba, 2001; Landgraf et al. 1997; L. Griffith, personal communication). Our intracellular dye labeling during neuronal recordings revealed ipsi- or contralaterally projecting motor axons in 67% of targeted neurons. An even greater majority of recordings were probably from motor neurons, though a minor subset may have been from interneurons.

Coordinated larval locomotion is driven by patterned bursts of APs delivered to the NMJ (Broadie and Bate 1993; Cattaert and Birman 2001). The ability to sustain trains of all-or-none APs is a thus a predicted property of motor neurons. During prolonged depolarizations, the majority of neurons exhibited adaptive firing, characterized by a slight to marked decrease (but not termination) in AP frequency, while a minority exhibited tonic firing at relatively constant frequency. In cultured *Drosophila* giant neurons, similar tonic and adaptive firing patterns have been previously described in substantial detail (Zhao and Wu 1997). However, cultured *Drosophila* embryonic and giant neurons exhibit considerably greater heterogeneity in AP firing properties, including 40–50% without all-or-none APs (O’Dowd 1995; Saito and Wu 1991; Zhao and Wu 1997). Because we were able to target a limited population of motor neurons in their normal environment, this result is not unexpected. It should be noted that because we could not distinguish specific motor neuron identities, heterogeneity in firing patterns recorded in vivo may result from cell-autonomous differences in excitability properties. Alternatively, heterogeneity in firing patterns among motor neurons in vivo may also be influenced by intrinsic variables such as different patterns of impinging synaptic input.

**Functional neuronal responses mediated by nicotinic ACh receptors and Cl<sup>-</sup>-permeable GABA and glutamate receptors**

Larval motor neurons in vivo respond to focal application of ACh, GABA, and glutamate. ACh responses are strongly ex-
citatory, generating sustained depolarizations supporting bursts of APs, and are mediated by nicotinic receptors as evidenced by their blockade by dTC. Responses to GABA as well as glutamate are inhibitory and mediated primarily by Cl−-channel currents, on the basis of similar reversal potentials near the 

![Image](http://jn.physiology.org/)

**FIG. 6.** Fast EPSCs evoked by focal neuropil stimulation. A: image of a wild-type larval CNS preparation following successful recording of fast evoked EPSCs in a motor neuron that did not exhibit endogenous sustained excitatory activity. Neuronal morphology is visualized by Lucifer yellow, introduced via the recording pipette (not shown), following recording and brief fixation. The position of the stimulating suction electrode (stim) placed in the neuropil is depicted. Electrode placement either slightly anterior or posterior to the dendritic region increased the success of directly evoking fast synaptic currents in recorded neurons. Electrode placement in the neuropil contralateral to the primary dendritic region was less effective or in some cases failed to evoke responses. B: evoked EPSC (25-V, 2-ms stimulation at 20°C) recorded from the neuron shown in A: stimulation artifact. EPSC amplitude remained consistent for several minutes at low (2 min−1) stimulation frequency. C: all-or-nothing EPSCs (2–5 V, 2 ms at 19°C) recorded from a dnc−/− mutant motor neuron (confirmed by dye filling, not shown). The stimulation electrode was placed in the lateral neuropil slightly posterior to the dendritic region. Stimulation at 3 and 4 V resulted in failure, whereas 5 V stimulation resulted in reproducible fast EPSCs. Inset: sEPSC recorded in same neuron for comparison.

![Image](http://jn.physiology.org/)

**FIG. 7.** Alterations in sEPSCs and evoked EPSCs in shi−/− conditional synaptic mutants and dnc−/− cAMP pathway mutants. A: schematic depiction of functional alterations expected for shi−/− and dnc−/− mutant synapses. In shibire, block of SV endocytosis at restrictive temperature results in depletion of synaptic vesicles and loss of transmission (left). In dnc−/− (right), increased SV mobilization from SV reserve pool (Kuromi and Kidodoro 2000) is expected to increase spontaneous transmission frequency.

B: sEPSC frequencies (top), sEPSC amplitudes (middle), and evoked EPSC amplitudes (bottom) recorded in wild-type, shi−/−, and dnc−/− neurons at normal (19–21°C) and restrictive (29–32°C) temperature. At restrictive temperature, shi−/− sEPSC frequency is reduced compared with wild type at 19–21°C, and shi−/− sEPSC amplitude is significantly reduced compared both to shi−/− and wild type at 19–21°C. sEPSC frequency in dnc−/− is significantly elevated compared with wild type. Mean evoked EPSC amplitude is insignificantly decreased by ~60% in shi−/− versus wild type (P = 0.07) and shi−/− (P = 0.08) at 19–21°C. Evoked EPSC amplitude vs. wild type is insignificantly increased by >50% in dnc−/− mutants (P > 0.30 vs. wild-type).
−55 mV and block by PTx. Although these focal agonist applications are unlikely to have substantially activated synaptic receptors in the neuropil, these neurons express functional receptors appropriate for excitatory cholinergic input, and potentially two inhibitory classes of synaptic input.

ACh and GABA are proposed as the major excitatory and inhibitory central transmitters, respectively, in insects. In Drosophila, this conclusion is supported primarily by the abundance and distribution of neurotransmitter and receptor expression revealed immunohistochemically as well as by the targeting of reporter molecules to neurons of a specific transmitter class (Aronstein et al. 1996; Gorczya and Hall 1987; Jonas et al. 1994; Nassel 1996; Schuster et al. 1993; Yasuyama and Salvaterra 1999). Behaviorally, the targeted block of cholinergic synaptic function results in adult paralysis (Kitamoto 2001). Functionally, embryonic dorsal VNC neurons in vivo respond to ACh from 16 h of development (Baines and Bate 1998). Likewise, pharmacological and targeted genetic disruption of cholinergic function in the adult giant fiber system has identified cholinergic inputs onto identified motor neurons (Gorczya and Hall 1984; Trimarchi et al. 1999).

Responses to GABA and glutamate, as well as various other putative neurotransmitters, have been reported in Drosophila embryonic and adult neuronal cultures as well as in other insect neurons. However, functional responses to these neurotransmitters have not to our knowledge been previously recorded in Drosophila neurons in vivo. PTx-sensitive GABA receptors are expressed by cultured embryonic Drosophila neurons (Lee and O’Dowd 1999). Inhibitory glutamate receptors are well documented in neurons of arthropods and other invertebrates (Cleland 1996; Culy et al. 1996b), and expression of the Drosophila homologue of this receptor family in oocytes produces a functional glutamate-gated chloride current (Culy et al. 1996a). Additionally, several Drosophila homologs of vertebrate N-methyl-D-aspartate (NMDA)- and non-NMDA-type glutamate receptor channels are expressed in the developing and adult Drosophila CNS (Littleton and Ganetzky 2000). Thus while it is not particularly surprising to find glutamate-activated inhibitory responses in Drosophila neurons, a role for excitatory glutamatergic transmission and glutamate receptor-mediated forms of synaptic plasticity appears likely to exist in the Drosophila CNS.

Endogenous and evoked cholinergic synaptic transmission in vivo

Spontaneous forms of excitatory cholinergic synaptic activity similar to those exhibited by larval neurons in vivo have also been demonstrated in Drosophila embryos and in cultured embryonic neurons. Fast sEPSCs mediated by nicotinic ACh receptors are recorded in the majority of cultured embryonic neurons making intercellular contacts (Lee and O’Dowd 1999, 2000). In the embryonic VNC, identified dorsal motor- (aCC and RP2) and interneurons (pCC) exhibit infrequent (2–3 min−1) fast spontaneous EPSCs (≤25 pA) after 16 h of development (Baines and Bate 1998), and sustained inward currents (≥200 pA) supporting APs after 19–20 h (Baines et al. 1999, 2001). Both types of embryonic activity require external Ca2+ and are reduced or eliminated by mutations or genetic constructs that block AP firing, deplete synaptic vesicle supplies, or conditionally eliminate cholinergic transmission selectively during development (Baines and Bate 1998, 1999, 2001).

Larval sEPSCs and sustained excitatory responses thus represent more robust versions of embryonic activity (e.g., Fig. 2 in Baines et al. 1999), having greater amplitudes and frequency, but similar pharmacological dependence on both Ca2+ and cholinergic transmission. Excitatory cholinergic transmission thus represents the predominant form of functional synaptic activity so far detected in Drosophila neurons in vivo and in culture. In larval motor neurons, this activity takes the form of fast unitary currents, as well as prominent sustained transmission episodes that may result from synchronized activity in multiple presynaptic cholinergic inputs. The latter type of input is sufficient to trigger a sustained burst of postsynaptic AP firing that is propagated to the NMJ (Cattaert and Birman 2001) and supports muscle contraction.

A significant advance of this work is the demonstration of intracellularly recorded, evoked excitatory responses at Drosophila central synapses. Several lines of evidence indicate that both endogenous and evoked forms of activity are mediated by common cholinergic synaptic pathways. First, properties of evoked responses and endogenous forms of activity present in the same neuron are closely analogous. In those neurons exhibiting endogenous sustained events, electrical stimulation tended to trigger similar sustained responses; in other neurons, faster EPSCs were generated that resembled summed sEPSCs. Second, the generation of both endogenous and evoked sustained events is similarly influenced by recent activity, with an interval of several seconds required between successive responses. Finally, both forms of activity exhibit indistinguishable cholinergic pharmacology. It should be noted that sustained endogenous activity was absent in roughly half of the recorded neurons in our preparations and clearly represents a remnant of normal rhythmic motor output. It was recently reported that in larvae with CNS and peripheral nerves intact, blockade of NMDA-type glutamate receptors inhibits centrally generated endogenous rhythmic output recorded at the NMJ (Cattaert and Birman 2001). Thus while cholinergic input supplies the major excitatory synaptic drive to motor neurons, additional forms of central transmission are likely to shape normal frequency and pattern of motor rhythms.

Our current knowledge regarding the identity of presynaptic partners, and the location of functional synaptic sites on these neurons remains limited and circumstantial. Cholinergic neurons are located centrally primarily in the lateral VNC but are also present peripherally. Longitudinal and horizontal cholinergic axons with numerous varicosities are found extensively throughout the central neuropil, overlapping spatially with the putative dendritic arbors of motor neurons. Because such central axonal varicosities are known to localize presynaptic vesicle proteins (Rohrbough et al. 2000), the formation of synaptic contacts between these overlapping processes is likely. When labeled neurons were examined following the physiological recording, it was clear that fast synaptic responses were effectively and reproducibly evoked when we were able to apply stimulation to the neuropil adjacent to the primary dendritic structure. This suggests cholinergic inputs are formed by both ascending or descending longitudinal axons; however, the number of inputs to each motor neuron, and the specific pattern (ascending vs. descending, ipsilateral vs. contralateral) of individual cholinergic projections remains in question.
Endogenous and evoked synaptic currents that we were able to clearly resolve and characterize were depolarizing or clearly excitatory. Although these motor neurons express functional GABA and glutamate receptors that mediate similar slow inhibitory Cl⁻ conductances, we detected no clear evidence of GABA- or glutamate receptor-mediated inhibitory synaptic currents. GABAergic IPSCs with fast rise times and slow (>20 ms) decay kinetics are prominent in a minority of cultured embryonic neurons (Lee and O’Dowd 2000). One possibility is that IPSCs were undetected due to low frequency or to insignificant amplitude at negative holding potentials with the ionic conditions used. A second possibility is that slow inhibitory transmission is present at a low levels sufficient to modulate the frequency of excitatory motor output. The identification of specific GABAergic and glutamatergic inputs, and the role of noncholinergic forms of transmission to these motor neurons, remain to be addressed in future work.

Altered excitability and synaptic transmission properties in eaq Sh, shibire, and dnc mutants

Analysis of neuronal excitability and central synaptic transmission in mutants promises new insights into cellular mechanisms of behavior that have previously been addressed primarily at the glutamatergic NMJ or in neuronal cultures. Here we examined three classical Drosophila mutants; the K⁺ channel mutant eaq Sh, the conditional dynamin mutant shi ts¹, and the short-term memory mutant, dnc. Mutant eaq Sh motor neurons exhibit reduced AP threshold and increased spontaneous firing, confirming the hyperexcitable neuronal firing phenotype recorded from larval peripheral nerves and NMJs (Ganetzky and Wu 1993). Access to identified central neurons thus offers the potential to assess the consequence of altered neuronal excitability on central synaptic transmission.

Transmission is reduced at restrictive temperature in shi ts¹ mutants, confirming genetically that endogenous and evoked forms of central activity are synaptic mediated. At larval NMJs, several hundred evoked release events at restrictive temperature are necessary to deplete shi ts¹ terminals of releasable synaptic vesicles (Delgado et al. 2000). Despite the small vesicle pools predicted at central synapses, spontaneous and evoked EPSCs persist at restrictive temperature in shi ts¹ larvae. Thus at least in the absence of significant ongoing levels of evoked activity, even prolonged (tens of minutes) block of synaptic vesicle endocytosis does not necessarily eliminate central transmission. The rapid (<2 min) adult behavioral paralysis when shi ts¹ expression is selectively targeted to cholinergic neurons (Kitamoto 2001) may result from disruption of coordinated transmission levels in active motor circuits rather than complete block of evoked release.

The cAMP-dependent pathway is involved in behavioral and functional synaptic modulation in both Drosophila and vertebrate systems (Davis et al. 1995). At the larval NMJ, dnc mutants have increased evoked quantal release, and decreased Ca²⁺- and activity-dependent synaptic facilitation (Zhong and Wu 1991). The dnc transmission defects are correlated with increased mobilization of synaptic vesicles into the releasable pool but a decreased supply of reserve vesicles accessible during and following high-frequency activity (Kuromi and Kidokoro 2000). At central dnc mutant synapses, we observe a marked increase in sEPSC frequency, and a parallel though statistically insignificant increase in evoked EPSC amplitude by >50%. The elevated dnc sEPSC phenotype in vivo is consistent with that in cultured dnc mutant neurons (Lee and O’Dowd 2000), suggesting increased probability of spontaneous and evoked presynaptic vesicle release at dnc mutant cholinergic synapses. The ability to record focally evoke and record fast transmission at Drosophila central synapses thus offers the first opportunity to examine and test models of activity-dependent synaptic modulation in the CNS of these and other behavioral learning and memory mutants.

Several clear goals remain to extend both the experimental power and specificity of future studies. The first is to improve methods to consistently identify the same larval neuron(s) among this accessible dorsal population. This would be most readily achieved by identifying cell-specific neuronal GAL4 UAS-GFP reporter constructs allowing specific neuron(s) to be distinguished by GFP fluorescence prior to physiological recordings. A related second goal is to more fully define and refine stimulation protocols to allow analysis of specific evoked synaptic inputs or aggregate pathways onto these identified visible targets. A third related goal is to use the GAL4-UAS-targeted mutagenesis and expression strategy to selectively disrupt or rescue presynaptic neuronal excitability or transmission properties. Extending our present work to more detailed examination of synaptic structure, function, and modulation in behavioral and plasticity mutants is our primary short-term objective.

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REFERENCES


SYNAPTIC TRANSMISSION IN DROSOPHILA CENTRAL NEURONS


