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# Long-term in vitro maintenance of neuromuscular junction activity of *Drosophila* larvae

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**Abstract**

The larval *Drosophila* neuromuscular junction (NMJ) has proven to be an excellent system to test fundamental aspects of synaptic transmission, such as relationships among ion channel function, subtypes of glutamate receptors, and the functions of synaptic proteins in the presynaptic compartment. Recent advances in understanding bi-directional communication between nerves and muscles of *Drosophila* are helping uncover developmental as well as maintenance cues that could be applicable to all chemical synapses. The development of HL3 medium makes it possible to record synaptic responses at NMJs for prolonged periods of time. We demonstrate that media commonly used to culture CNS neurons and imaginal disks of *Drosophila* such as Schneider's and M3 completely block glutamatergic synaptic transmission at the NMJ. The depressed postsynaptic excitatory junction potentials (EJPs) partially recover from exposure to such media shortly after switching to the HL3 medium. Preliminary results from NMJs of filleted 3rd instar larvae for 4 days in vitro bathed in a modified HL3 medium show great promise. The resting membrane potential and the EJP amplitudes after 4 days in vitro are normal. These results demonstrate the possibility for chronic studies of developmental regulation in culture, which in some cases are impractical in the whole animal.


*Keywords:* Neurotransmission; Neuromuscular junction; Behavior; Development

## 1. Introduction

There are many advantages of the *Drosophila* NMJ that make it a model system for investigation of synaptic maintenance and development. The muscle fibers are easily identified and there are few innervating motor neurons. In addition, the developmental staging is well characterized (Crossley, 1978; Bate et al., 1993; Broadie and Bate, 1993; Sink and Whitington, 1991; Atwood et al., 1993; Kurdyak et al., 1994; Lnenicka and Keshishian, 2000; Li et al., in press). The most commonly studied *Drosophila* NMJs are on the ventral longitudinal abdominal muscle fibers m6 and m7 (Crossley, 1978), each innervated by only two motor neurons which are referred to as type Ib and Is terminals (Atwood et al., 1993).

The *Drosophila* 3rd instar larva is large enough to allow easy measurement of synaptic potentials and currents over discrete regions of the relatively large motor nerve terminals, permitting one to take full advantage of the system by examining the effects of precise mutations directed at processes involved in neuromuscular synaptic transmission (Keshishian et al., 1993; Wan et al., 2000; Li et al., 2001; Li and Cooper, 2001). Within the organism, there are compensatory mechanisms that mod-

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ulate responses to experimentally induced genetic, hormonal or environmental alterations. Thus, it is hard to maintain consistency over the course of long-term experimental manipulations since unknown factors within the animal that impinge on physiological mechanisms induce uncontrolled variables. To control as many variables as possible in experimental design, investigators have turned to culturing tissues of interest to answer questions concerning developmental and regulatory processes. Our particular interests center on the influences of hormones and neuromodulators which have a role in altering synaptic growth and plasticity at the neuromuscular junction (NMJ) (Ruffner et al., 1999; Neckameyer and Cooper, 1998; Cooper and Neckameyer, 1999; Li et al., 2001; Li and Cooper, 2001). To further investigate the mechanisms of action by particular neuromodulators, or growth-related hormones like ecdysone, we have developed a way to maintain in vitro the intact CNS and longitudinal body wall musculature of *Drosophila* larvae.

The usual media used to culture *Drosophila* salivary glands, imaginal disks, and dissociated neurons from the CNS of larvae are Schneider's and M3 medium. These media were originally designed for insect cell culture, but they do not mimic well the ionic composition of *Drosophila* larval hemolymph (Stewart et al., 1994). A physiological saline based on the composition of larval hemolymph, HL3, preserves synaptic transmission as well as muscular function and integrity (Stewart et al., 1994).

In this paper we compare the effectiveness of different medium formulations to support *Drosophila* 3rd instar larval NMJ survival and activity for up to 4 days in vitro. There are some significant differences among Schneider's, M3, and HL3-based media that could affect the reliability of experimentally determined synaptic parameters: Schneider's and M3 have relatively higher $[K^+]$ than do hemolymph and HL3 saline, moreover Schneider's is high in $Cl^-$ and has double the ratio of $[Na^+]$ to $[K^+]$ (1.4 vs. 3.0) of larval hemolymph. These differences would make it difficult for cells to maintain ion homeostasis in Schneider's. Both Schneider's and M3 media have higher amino acid content than hemolymph. We reasoned that these media might dampen the EJP amplitude due to their excessive L-glutamate and/or aspartate content and thus desensitize receptors for the excitatory neurotransmitter glutamate at the NMJ. We find that medium formulations based on HL3 saline allow prolonged in vitro analysis of NMJ function.

In the absence of any literature describing long-term culture of *Drosophila* NMJs, we describe procedures we have used successfully to maintain functional NMJs for up to 4 days in vitro. In addition, we describe the acute effects of other insect culture media on NMJ function.

## 2. Methods

### *2.1. Fly stocks and staging of larvae*

The common 'wild-type' laboratory strain of *Drosophila melanogaster*, Canton S, was used in these studies. Synchronous egg collections were made over 2-h periods on apple juice–agar plates with yeast paste. The adults were given a 20-min period to expel developmentally advanced embryos maintained within the ovipositor before the 1-h period during which time embryos were collected. The eggs were allowed to hatch and develop at 18 °C with a 12:12 dark–light cycle. The methods used to stage fly larvae have been described previously (Campos-Ortega and Hartenstein, 1985; Li et al., in press). All animals were maintained in vials partially filled with a cornmeal–agar–dextrose–yeast medium. Larvae at the beginning of the 'wandering' phase of the 3rd instar were used in these experiments.

### *2.2. Dissection and in vitro conditions*

Third instar larvae were surface-sterilized with 70% ethanol for 1 min and rinsed three times with sterile deionized water prior to dissection. Dissections included removal of the heart, and viscera leaving filleted larvae, containing only body wall, body wall muscles and CNS as described in Cooper et al. (1995). The larvae were placed in Sylgard covered 35 mm plastic Petri dishes in 2 ml of the various culture media and placed at 19 °C in a dark drawer.

In all dissected preparations, the CNS and the segmental nerves were left intact. After dissection, the preparations were washed three times with the HL3-based minimal medium and the preparations were repined with fine insect pins (type 000, Fine Scientific Tools) in a Petri dish (35-mm diameter) containing hardened Sylgard. The HL3–FCS medium was exchanged with fresh HL3–FCS medium

every 8 h for 4 days. In vitro larvae were examined once for NMJ function between days 1 and 4 of culture.

### 2.3. Anatomy of the neuromuscular junctions

Fixed cultures were used to examine nerve terminal structure using a fluorescent anti-HRP primary antibody to aid in identifying the motor nerve terminals on muscle m6 (Johansen et al., 1989a,b). Composite Z-series images were collected with a Leica TCS NT/SP confocal microscope for illustration.

### 2.4. Electrophysiology

Electrophysiological recordings made from long-term in vitro preparations were performed in the same dish in which they were maintained. The recording arrangement was essentially the same as previously described (Stewart et al., 1994; Neckameyer and Cooper, 1998). Intracellular recordings were made with 30–60 MΩ resistance, 3 M KCl-filled microelectrodes. The electrophysiological values of interest were the resting membrane potential ($R_p$) and the EJP amplitudes elicited by Is and Ib motor nerve terminals in segment 4 of muscle m6. Short trains of four stimuli at 20 Hz were given to determine whether the EJP amplitudes depressed or facilitated. Responses were recorded with a 1 X LU head stage and an Axoclamp 2A amplifier. Electrical signals were recorded to VHS tape (Vetter, 400) as well as online to a PowerMac 9500 via a MacLab/4s interface. All events were measured and calibrated with the MacLab Scope software 3.5.4 version. All experiments were performed at room temperature (19–22 °C).

### 2.5. Solutions and media

The HL3-based minimal medium used in dissections was prepared in the lab from component reagents (Sigma) and contained: 1.0 mM $CaCl_2 \cdot 2H_2O$, 70 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, and 5 mM BES (*N*,*N*-bis[2-Hydoxyethyl]-2-aminoethanesulfonic acid).

Since HL3 by itself is a minimal medium of essential salts, we added gentamicin to help reduce bacterial growth. Initial attempts at culturing the larvae without gentamicin in the media and without pre-washing the larva before dissection in 70% ethanol produced massive blooms of bacteria.

In the acute studies, HL3 dissection medium was completely replaced by a 1:1 mixture of HL3 and Schneider's or M3 (Sigma). At various times after beginning acute studies, the medium was changed back to HL3 to examine recovery of synaptic functions.

Long-term in vitro preparations were maintained in 'HL3–FCS' medium consisting of HL3 plus 2% (final concentration) heat inactivated fetal calf serum (FCS) (Gibco BRL-Life Technologies) and gentamicin (final concentration of 0.01 mg/ml; Gibco BRL-Life Technologies). In some experiments, amphotericin B (2 μg/ml; fungizone, Sigma) was added to control growth of molds.

## 3. Results

When the 4th segmental nerve of filleted and pinned larvae (Fig. 1A) was stimulated, one or both of the Ib and Is axons that innervate muscles 6 and 7 (i.e. m6 and m7, Fig. 1B) responded by producing excitatory junction potentials (EJPs). The terminals of the Is axon contain small varicosities along its length and give rise to large EJPs in the muscle (Fig. 1B and C). The Ib axon has big varicosities on its terminals, but produces smaller EJPs. By varying the stimulus intensity and duration one can selectively recruit either the Is, or Ib, or both axons (Fig. 1C).

To quantify the effects of medium composition on synaptic transmission, a supra-threshold stimulus was given at 0.5 Hz to the segmental nerve while monitoring the amplitude of the composite EJP within m6. Baseline recordings were made in HL3 medium before switching the bathing medium to 1:1 Schneider's:HL3 (Fig. 2B), 1:1 M3:HL3 (Fig. 2C), or, as a control, replacement with fresh HL3 (Fig. 2A). The HL3 was exchanged for 1:1 mixtures of HL3 and Schneider's or M3 media because rapid switching from HL3 to pure Schneider's or M3 produced damagingly strong muscle contractions. Both the Schneider's-HL3 and M3-HL3 mixtures produced a rapid decline of EJP amplitude (Fig. 2). Attempts to wash out depressing effects of Schneider's or M3-HL3 mixtures were highly unsuccessful; the deleterious effects of Schneider's and M3 were largely irreversible. In larvae continuously exposed to either HL3 or HL3–FCS media (Fig. 2D), the EJP responses were maintained without much degradation in

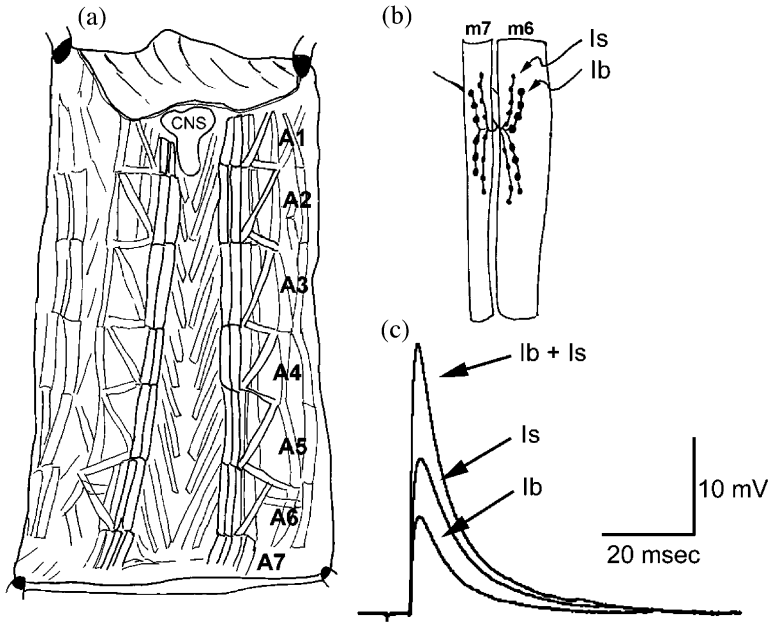


Fig. 1. Schematic diagram of the *Drosophila* larva preparation. (A) The preparation is pinned at four points to keep the preparation taut. (B) An enlargement of segment A4 of the right hand side of the preparation. The ventral abdominal muscles, m6 and m7, are innervated by two excitatory motor nerve terminals contained in one segmental nerve. One of the nerve terminals contains small varicosities (Is) and gives rise to large EJPs. The other terminal has big varicosities (Ib), but produces smaller EJPs. (C) EJPs in muscle were elicited by stimulating axons Is and Ib separately and together, as indicated in m6.

amplitude. In none of the three media, however, were sustained alterations of the resting membrane potential seen.

These acute responses to HL3 substantiate previous findings that HL3 medium maintains synaptic properties (Stewart et al., 1994). We did not examine the effects of the widely used 'Standard' *Drosophila* saline (Jan and Jan, 1976) since it has already been demonstrated to contain ion concentrations far removed from those of larval hemolymph (Stewart et al., 1994); but we do show the composition of the Standard and HL3 media for comparison (Table 1), as well as the compositions of Schneider's and M3 (Table 2). The compositions of HL3–FCS media is in Table 1.

Since HL3–FCS media showed no adverse acute effects on synaptic transmission, its ability to support relatively long-term in vitro maintenance was assessed. Two parameters of healthy tissue were tested: maintenance of synaptic transmission at the NMJ and the resting membrane potential of muscle. Five out of the six larval preparations remained viable, with functional chemical synapses after 4 days in vitro. They maintained pronounced EJPs and facilitated responses upon induction by short stimulus trains (Fig. 3A and B). The occurrence of normal short-term facilitation responses was of special interest since the results suggest that calcium buffering and homeostatic maintenance were preserved in the nerve terminals. Normal facilitated responses of freshly filleted larvae are shown and quantified in Stewart et al. (1994). Of the five preparations that were examined only on the fourth day, the mean amplitudes of the composite Ib and Is EJPs were within the observed range for acute preparations measured in HL3 media (Stewart et al., 1994). In addition, the resting membrane potential of muscle m6 was maintained (Fig. 3C). Stewart et al. (1994) reported that after 2 h of acute exposure to HL3 the resting membrane potential was $-56$ ($\pm 4.5$ mV) for 41 fibers examined which is close to our mean value after days in vitro, however the variation was large. The range of resting membrane values as well as the amplitude of the EJPs for the Ib and Is muscles are consistent with a number of reports in which HL3 was utilized for acute studies (Ruffner et al., 1999; Cooper and Neckameyer, 1999; Li and Cooper, 2001; Li et al., 2001).

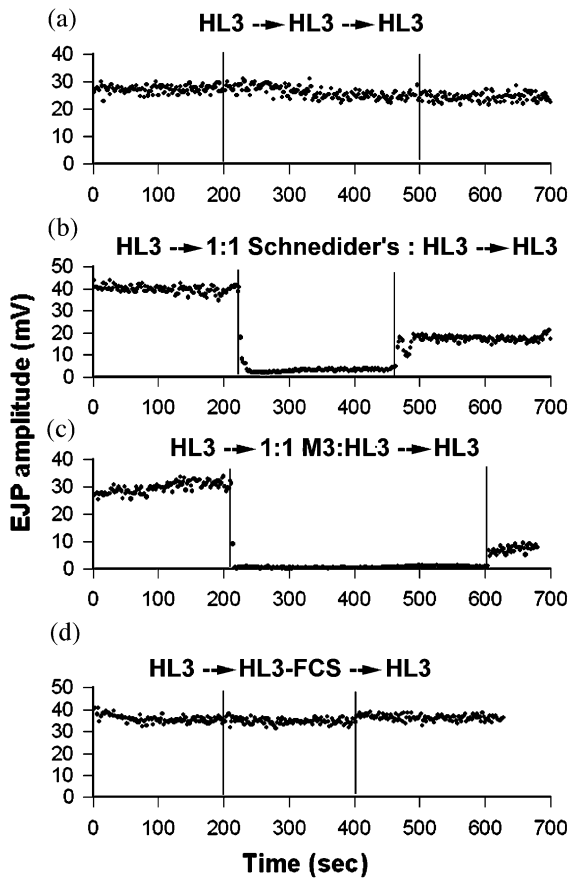


Fig. 2. The acute effects of various media on the EJPs. The fourth segmental nerve was stimulated while the evoked EJPs were recorded in muscle m6 with an intracellular electrode. The composite EJP amplitude of the Ib and the Is motor axons was measured over time during the exposure to the various media. The stimulation frequency was 0.5 Hz. In the HL3 media the responses remained functional for a prolonged period (A). However, when the medium is exchanged for a 1:1 (vol:vol) HL3 and either Schneider's (B) or M3 (C) medium, the amplitude of the EJPs rapidly decreased. The responses did not recover upon returning the bathing media to HL3 alone. The modified HL3 culture medium (HL3–FCS), was also examined for acute effects (D). Neither HL3 nor HL3–FCS media appear to have any adverse acute effects on the EJPs.

One of the original six preparations was contaminated with mold and not examined electrophysiologically. Because fungal contamination of such cultures is likely to be a common problem, we added the anti-fungal agent amphotericin-B to the culture medium of six cultures to determine whether or not it affects larval tissue survival and physiology. Six preparations were fed with amphotericin-containing medium and were examined for physiological responses on the fourth day of culture. Only two of the six appeared to be normal. As early as the second day of culture, muscle fibers in two cultured larvae had turned dark brown, indicating necrosis, and by the third day most muscle fibers had detached from the cuticle. Two other preparations underwent the same progression of fiber death starting on the third day. In the two remaining preparations, the EJP amplitudes ($\sim$25 mV) were within the range of those maintained in the HL3–FCS, amphotericin-free medium for 4 days. We are continuing to test lower concentrations of amphotericin in the medium to determine a level which provides protection against molds but does not harm the larval tissues.

Muscle fiber damage was also apparent within 1 day after driving contraction by stimulating the innervating nerves. This damage occurred in the absence of amphotericin and appeared to be the consequence of fiber detachment after contraction. Five cultures were tested for synaptic responses by stimulation after 1, 2, or 3 days in culture. We observed that on the day following stimulation, fibers within the stimulated segment had often detached from the cuticle but did not turn dark, suggesting that the fibers are not dying as with amphotericin but rather, during culture, attachment of muscle fibers to cuticle is weakened and easily broken. In a series of experiments in which the muscles were not stimulated until the fourth day, muscle fibers became detached by the fifth day, again suggesting that muscle attachment to the cuticle is not maintained well under these culture conditions.

Muscle fibers that had remained intact during 4 days of culture do maintain a normal, healthy

Table 1
Composition of physiological media used for *Drosophila* NMJs

| | Jan and Jan (Standard) | HL3 |
|---|---|---|
| NaCl | 128 | 70 |
| KCl | 2 | 5 |
| $CaCl_2?2H_2O$ | 1.8 | 1.5 |
| $MgCl_2?6H_2O$ | 4 | 20 |
| Trehalose | – | 5 |
| Sucrose | 35.5 | 115 |
| BES or HEPES | 5 | 5 |
| $NaHCO_3$ | – | 10 |
| Osmotic pressure | 300 mosm | 343 mosm |

HL3–FCS is comprised of HL3 with heat inactivated FCS 2% v/v, glucose at 6 mg/ml, gentamycin sulfate at 0.1 mg/ml. Concentrations are in millimeters.

Table 2
Composition of media commonly used for *Drosophila* tissue culture

| | Shields and Sang (M3) | | Schneider's | |
|---|---|---|---|---|
| | gm/L | (mM) | gm/L | (mM) |
| Choline chloride | 0.05 | 0.358 | – | – |
| Oxalacetic acid | 0.25 | 1.893 | – | – |
| $CaCl_2$ | 0.76 | 6.845 | 0.6 | 5.41 |
| NaCl | – | – | 2.1 | 35.93 |
| KCl | – | – | 1.6 | 21.46 |
| $KHCO_3$ | 0.5 | 5.0 | – | – |
| $NaHCO_3$ | – | – | 0.4 | 4.76 |
| Magnesium sulfate (anhydrous) | 2.15 | 17.86 | 1.81 | 15.03 |
| Sodium phosphate monobasic | 0.88 | 7.33 | 0.7 | 5.833 |
| Potassium phosphate monobasic | – | – | 0.45 | 3.31 |
| Glucose | 10.0 | 55.49 | 2.0 | 11.1 |
| BIS–TRIS | 1.05 | 5.02 | – | – |
| Succinic acid | – | – | 0.06 | 0.51 |
| L(-)Malic acid, free acid | – | – | 0.6 | 4.47 |
| α-Ketoglutaric acid, free acid | – | – | 0.35 | 1.84 |
| Fumaric acid, free acid | – | – | 0.06 | 0.52 |
| Yeast extract | 2.0 | (NA) | 2.0 | (NA) |
| β-Alanine | 0.25 | 2.81 | 0.5 | 5.61 |
| L-Alanine | 1.5 | 16.84 | – | – |
| L-Arginine, free base | 0.5 | 2.37 | 0.6 | 2.85 |
| L-Asparagine, anhydrous | 0.3 | 2.27 | – | – |
| L-Aspartic, free acid | 0.3 | 2.25 | 0.4 | 3.01 |
| L-Cysteine, hydrochloride | 0.2 | 1.27 | – | – |
| L-Cysteine, dihydrochloride | – | – | 0.27 | 1.39 |
| L-Cysteine, free base | – | – | 0.06 | 0.50 |
| L-Glutamic acid, $K^+$ salt | 7.88 | 42.53 | – | – |
| L-Glutamic acid, $Na^+$ salt | 6.53 | 38.62 | – | – |
| L-Glutamic Acid | – | – | 0.8 | 5.44 |
| L-Glutamine | 0.6 | 4.11 | 1.8 | 12.32 |
| Glycine, free base | 0.5 | 6.66 | 0.25 | 3.33 |
| L-Histidine, free base | 0.55 | 3.54 | 0.4 | 2.58 |
| L-Isoleucine | 0.25 | 1.91 | 0.15 | 1.14 |
| L-Leucine | 0.4 | 3.05 | 0.15 | 1.14 |
| L-Lysine, HCl | 0.85 | 4.65 | 2.06 | 11.27 |
| L-Methionine | 0.25 | 1.68 | 0.15 | 1.01 |
| L- Phenylalanine | 0.25 | 1.51 | – | – |
| L-Proline | 0.4 | 3.48 | 1.7 | 14.77 |
| L-Serine | 0.35 | 3.33 | 0.25 | 2.38 |
| L-Threonine | 0.5 | 4.2 | 0.35 | 2.94 |
| L-Tryptophan | 0.1 | 0.49 | 0.1 | 0.49 |
| L-Tyrosine, disodium | 0.36 | 1.6 | 0.72 | 3.20 |
| L-Valine | 0.4 | 3.42 | 0.3 | 2.56 |

Note: The listed values are rounded to the 2nd decimal place whereas in some cases more percise values can be obtained in the product information from SIGMA. NA mean not applicable in mM values.

appearance (Fig. 4A). Nerve terminals on these muscle fibers also appear to be normal when viewed with Nomarski optics (Fig. 4B). These synaptic terminals are very similar in appearance to those found in acutely isolated preparations in HL3 medium, reported by Stewart et al. (1994).

## 4. Discussion

*Drosophila* media like Schneider's and M3 are particularly useful for culture of larval salivary glands, imaginal disks, and disassociated CNS neurons (Schneider and Blumenthal, 1978; Echalier, 1976; Mitsuhashi, 1982; Shields and Sang, 1977). But we find that these media do not support long-term organ culture in which neuronal activity is to be examined. The rapid decline of synaptic responses at in vitro maintained larval NMJs is likely due to in glutamate-receptor activation and/or desensitization (Stewart et al., 1994) as a consequence of exposure to the high levels of glutamate and other amino acids contained in the media (Table 2). When CNS neurons are cultured for the purpose of examining developmental processes such as neuron growth and axon elongation

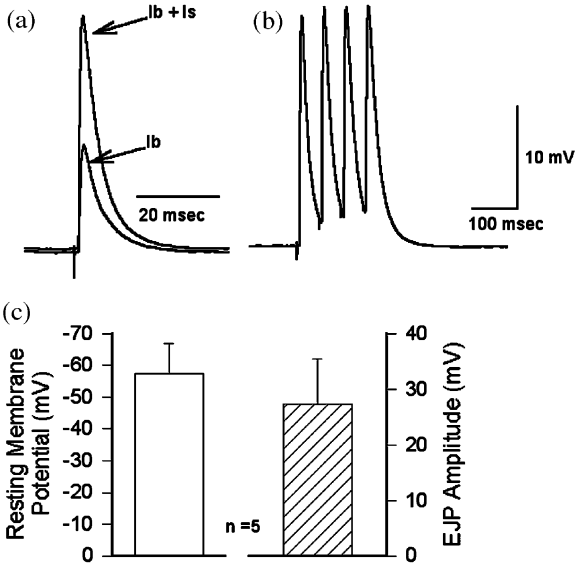


Fig. 3. Representative EJPs on the fourth day of culturing for the Is and the combined Is and Ib reveal that the preparation is viable (A). The response to a short stimulus train (20 Hz, 4 pulses) after 4 days in culture is as robust as preparations acutely dissected (B). Mean values and standard errors of compound EJPs (elicited by stimulating Is and Ib together) and resting membrane potentials in muscle m6 were obtained from five preparations (C). Scale bar: same for both (A) and (B).

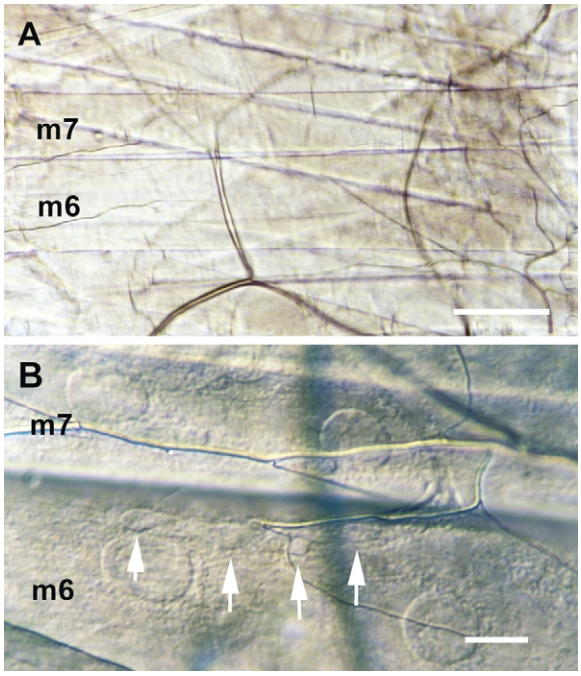


Fig. 4. The integrity of the muscle and NMJs after 4 days in culture (HL3–FCS) can be maintained. The filleted larva is shown with the muscles and innervation intact after 4 days in culture (A). The nerve terminals on muscle m6, viewed with Nomarski optics, appear to be normal in a live preparation (B). The arrow heads identify the varicosities of the terminals. Note the large nuclei within the muscle fiber. Scale bars: 80 μm (A); 15 μm (B).

and target recognition, activation or desensitization of possible glutamate autoreceptors could have very pronounced effects, as shown in crayfish motor neurons (Shinozaki and Ishida, 1992). Various second messenger cascades could be activated by presynaptic metabotropic glutamate receptors. It was noted by Stewart et al. (1994) that the addition of glutamine to HL3, that was modified with a higher potassium concentration, did not show as great of depolarization in the resting membrane potential over time as compared to solutions without glutamine. In addition, Stewart et al. (1994) reports that increasing the $K^+$ concentration to 35 mM in HL3 will result in a depolarization of the membrane potential over a few hours. This may well contribute to the reduction in the EJP amplitudes over time when acute preparations are exposed to M3 or Schneider's media (Table 2). It will be interesting to compare previous studies of *Drosophila* neuron culture using Schneider's and M3 with HL3–FCS. The same concerns we raised might also apply to other insect preparations and culture media currently utilized.

The larvae taken for in vitro maintenance were in the wandering phase of the 3rd instar and the cultures were maintained for up to 4 days at temperatures between 21 and 25 °C. Under these conditions, normally developing larvae would have pupated and the NMJs would be degrading. Yet, the individual EJPs and facilitated responses of the 4-day-cultured preparations appear quite normal, suggesting not only that calcium-handling and voltage-gated ion channels at the nerve terminals are functioning as well as in acute preparations, but also that the developmental program set in motion by hormones during the 3rd instar that results in degradation of 3rd instar larval structures during pupation may be blocked or suspended because of the lack of the hormone producing organs. Thus, long-term larval culture under near-physiological conditions opens up the possibility of investigating NMJ development at earlier times within the 3rd instar and even 1st or 2nd instars. Pathways of regulation of gene expression inducing degradation of muscle and nerve during the pupal stage might also be studied. Another arena in which whole-larva long-term culture could prove useful is in observation of motor neuron terminal growth and differentiation through expression of appropriate GFP fusion proteins.

Plasticity of neural circuitry appears during larval development and its regulation is an important aspect of the transformation of larvae into adults, as well as for growth and development within instars. The levels of the hormones ecdysone and juvenile hormone (and their ratios of one to the other) play significant roles during insect development and differentiation (Truman, 1996; Pak and Gilbert, 1987; Garen et al., 1977; Fahrbach, 1992; Kamimura et al., 1999; Talbot et al., 1993; Truman et al., 1994). Ecdysone, especially, has a large-titer peak in the pupal stage of *Drosophila*, and it is feasible that the high concentration plays a key role in inducing gross alterations in neural circuitry (Truman and Reiss, 1988; Thummel, 1996; Kraft et al., 1998). Similarly, the reduction in juvenile hormone levels, changes in ecdysone/JH ratios, or even effects of cocktails of other hormones have not been fully investigated but may play significant developmental roles. There is little detailed information on subtle acute effects of ecdysone on neuron and muscle development.

However, recent developmental studies with a reduced-ecdysone mutant of *Drosophila* (ecd$^1$/ecd$^1$) demonstrated that the nerve terminals do not grow to their normal length in animals with reduced levels of circulating ecdysone (Li et al., 2001; Li and Cooper, 2001). Since short-term in vitro conditions allow viability of the NMJs one can now address such topics in well-defined conditions. In neuronal cultures, several responses have been shown to depend on ecdysone titer for their manifestations. For instance, cultured *Manduca sexta* motor neurons show varying responses such as apoptosis, axon regression or regrowth at different concentrations of ecdysteroids (Truman and Reiss, 1995). These responses may not be due solely to ecdysone but may in part also be regulated by concentrations of JH and/or other hormones (Cayre et al., 1994, 2001).

Of the currently available insect culture media and physiological salines, HL3 (Stewart et al., 1994) comes closest to the ionic composition of *Drosophila* larval hemolymph. In addition, our observation that addition of FCS to HL3 improves its efficacy in long-term cultures implies that simple modifications to the basic saline, such as addition of essential amino acids, could make it useful for larva culture even without the serum. We intend to use assessment of a physiological function, such as synaptic transmission, as a guide in development of culture media that will more accurately reflect the developmental state of cultured larval tissues.

One problem that we have not yet been able to overcome in this in vitro system is maintaining the strength of muscle fiber attachment to the cuticle. Contractions driven by stimulation of motor nerves generally cause detachment of the muscle fiber from its cuticular attachment as soon as 1 day of culture; detachment is more pronounced at longer culture times. The reasons for this increased fragility of the muscle–cuticle connection in culture are not known. Perhaps the fibers become detached at their anchoring points on the cuticle because the cells making up the endocuticle do not survive in the media we used. Further modifications to the HL3-based media may alleviate this instability.

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