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ORIGINAL PAPER

Analysis of various physiological salines for heart rate, CNS function, and synaptic transmission at neuromuscular junctions in *Drosophila melanogaster* larvae

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Abstract *Drosophila* serves as a playground for examining the effects of genetic mutations on development, physiological function and behavior. Many physiological measures that address the effects of mutations require semi-intact or cultured preparations. To obtain consistent physiological recordings, cellular function needs to remain viable. Numerous physiological salines have been developed for fly preparations, with emphasis on nervous system viability. The commonly used saline drifts in pH and will cause an alteration in the heart rate. We identify a saline that maintains a stable pH and physiological function in the larval heart, skeletal neuromuscular junction, and ventral nerve cord preparations. Using these common assays, we screened various pH buffers of differing concentrations to identify optimum conditions. Buffers at 25 mM produce a stable heart rate with minimal variation in pH. Excitatory junction potentials evoked directly on larval muscles or through sensory-CNS-motor circuits were unaffected by at buffers at 25 mM. The salines examined did not impede the modulatory effect of serotonin on heart rate or neural activity. Together, our results indicate that the higher buffer concentrations needed to stabilize pH in HL3 hemolymph-like saline do not interfere with the acute function of neurons or cardiac myocytes.

Keywords Saline · Physiology · Heart · Synaptic transmission

Abbreviations

BES	5 *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
TRIZMA	Tris(hydroxymethyl)aminomethane
HR	Heart rate
CNS	Central nervous system
EJP	Excitatory junction potential
NMJ	Neuromuscular junction
HL3	Hemolymph-like saline

Introduction

Drosophila melanogaster serves as a playground for examining the genetics of development, physiological function, and animal behavioral (Ganetzky 2000; Bier and Bodmer 2004). With the ease of genetic manipulation, various human disease states are also being modeled in *D. melanogaster* to understand mechanistic processes (Pandey and Nichols 2011; Neckameyer and Argue 2012). Heart disease and cardiac research is increasing in the fruit fly as more becomes known about its anatomy (Lehmacher et al. 2012) and physiology (Gu and Singh 1995; Dasari and Cooper 2006; Cooper et al. 2009; Titlow et al. 2013). The effects

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of ion channel and other mutations on cardiac function are readily approachable with routine genetic manipulations in *D. melanogaster* (Badre and Cooper 2008; Akasaka and Ocorr 2009; Cooper et al. 2009). The genes involved in heart development and the molecular mechanisms of cardiac function are strikingly similar between *D. melanogaster* and humans (Bodmer 1995; Cripps and Olson 2002; Wessells and Bodmer 2004; Nishimura et al. 2011; Na et al. 2013). Functional similarities between man and fly also extend to brain and nervous system function (Strausfeld and Hirth 2013), where fruit flies are commonly used to model neurological disorders (Marsh and Thompson 2006). After discovering that there are limitations in the physiological salines commonly used to study nervous system and cardiac function in larval preparations, our aim was to optimize one of the most commonly used solutions (i.e., hemolymph-like saline, HL3; Stewart et al. 1994).

Several studies have employed salines that mimic *D. melanogaster* hemolymph for the purpose of maintaining physiological function in dissected or cultured nervous system preparations. Very few have actually characterized the molecular makeup of the hemolymph or compared the various types of saline on physiological processes (Gu and Singh 1995; Desai-Shah et al. 2010; Titlow et al. 2013). In an attempt to identify the ionic constituents of larval hemolymph, Stewart et al. (1994) used ion sensitive electrodes to measure the concentration of K^+, Ca^{2+}, and Na^+ in pooled hemolymph samples from 3rd instar *D. melanogaster* larvae. In that study, synaptic transmission at the neuromuscular junction was used as an assay to compare commonly used insect salines, with HL3 being the most effective at maintaining synaptic function in that preparation (i.e., Jan and Jan 1976 saline A; Johansen et al. 1989). Since then a modified HL3 saline has been developed to examine physiological activity over longer durations (Ball et al. 2003). Adding fetal calf serum and antibiotic/antifungal compounds to HL3 maintained motor neuron and muscle viability, though after 24 h the muscle would start to detach from the cuticle. Another version of HL3 (referred to as HL3.1) was designed to optimize physiological function at higher temperatures that are used to restrict the function of temperature sensitive alleles. Reducing the concentration of Mg^{2+} from 20 to 4 mM stabilized synaptic transmission under these conditions, possibly by eliminating a charge screening effect that interfered with excitability (Feng et al. 2004). Schneider's and M3 are salines that are commonly used to culture larval salivary glands, imaginal disks, and disassociated CNS neurons (Echalier 1976; Shields and Sang 1977; Schneider and Blumenthal 1978; Mitsuhashi 1982). At the larval neuromuscular junction, synaptic responses are not stable in these solutions (Ball et al. 2003).

The larval heart is even more sensitive to subtle changes in saline composition. Saline components that are known to influence larval heart function include neurotransmitters (Johnson et al. 1997; Dasari and Cooper 2006; Titlow et al. 2013) and charged ions (Gu and Singh 1995; Desai-Shah et al. 2010). A comprehensive study examining the effect of various ionic concentrations on larval heart rate (HR) determined that a saline similar to HL3 was the most suitable for maintaining cardiac function at pH 7.0 (Gu and Singh 1995). Standard HL3 solution that was designed for neuromuscular junction preparations is at pH 7.2 (Stewart et al. 1994). The difference is subtle but the larval heart is very sensitive to changes in pH, as pH increases HR decreases (Badre et al. 2005; Desai-Shah et al. 2010). This becomes an issue if saline pH drifts throughout the day, which is something we have observed using standard HL3. To address this problem, we experimented with different buffers by testing their ability to stabilize pH for up to 20 h. The most effective buffered salines were then tested on dissected heart and nervous system preparations for physiological effects, and for interference with neuromodulator activity. Together these results provide a reference for selecting between common physiological saline buffers used in dissected *D. melanogaster* larva preparations.

Methods

Fly strain and maintenance

The common 'wild-type' laboratory strain of *D. melanogaster*, Canton S, was used in these studies. Early 3rd instars were developmentally staged so that all specimens were 50–70 h post hatching. Flies were maintained at room temperature (21–23 °C) on a 12-h light: dark cycle in vials partially filled with a cornmeal–agar–dextrose–yeast medium.

Monitoring pH drift in physiological salines

Saline solutions contained standard HL3 quantities of ions and sugars (in mM): 1.0 $CaCl_2 \cdot 2H_2O$, 70 NaCl, 20 mM $MgCl_2$, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose. Four different types of buffers were examined: 5 *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonicacid(HEPES; Note not the base form), tris(hydroxymethyl)aminomethane-HCl (TRIZMA-acid) and tris(hydroxymethyl)aminomethane (TRIZMA-base). Each buffer was examined in various concentrations ranging from 1 to 50 mM. After aeration, the pH of all salines was adjusted to 7.1 with HCl (1 M) or NaOH (1 M) as needed. The saline pH was monitored at room temperature (21–23 °C) using an Accumet model 10 pH meter (Fisher Scientific) and Ag/AgCl glass electrode. Five replicates containing 50 mL of each

solution were recorded at six different time points over the course of 20 h. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Heart rate measurements

The fileted larva preparation for testing saline solutions on cardiac function has been described previously (Gu and Singh 1995; video format in Cooper et al. 2009). Larvae were dissected ventrally and pinned on four corners. Guts and visceral organs were removed, leaving the heart intact and still attached to the rostral and caudal ends of the dermis. Dissection time was 3–6 min. The preparation was allowed to recover in HL3 saline for 3–5 min after dissection. Heartbeats were counted by manual inspection through a dissecting microscope at four different time points: initially after recovery in standard HL3 saline, 1 min after switching the saline to a test saline, 5 min after switching to the test saline, and 10 min after switching to the test saline. To examine the effect of modulation on the heart, a test saline was used that contained 100 nM 5-HT (Dasari and Cooper 2006). Preparations that had a HR of 50 beats/min (BPM) or less after the initial dissection were excluded from further experimentation. We do not know why some rates are lower than 50 BPM, but to avoid using preparations with potential damage during dissection we did not include these preparations.

Intracellular recordings from the neuromuscular junction

Larval dissections were performed as described previously (Stewart et al. 1994, 1996; Ruffner et al. 1999). In brief, the preparations were "fileted" along the mid-dorsal longitudinal axis and pinned flat. The preparation dish consisted of a glass slide with magnetic tape adhered to one side. A hole in the center of the magnetic strip allowed the preparation to be viewed from the bottom with transmitted light. This type of recording dish has been described previously for pinning ganglia isolated from the leech ventral nerve cord (Muller et al. 1981). Excitatory junction potentials (EJPs) were evoked by stimulating the segmental nerve with a glass suction electrode. The stimulator (S-88, Grass) output was passed through a stimulus isolation unit (SIU5, Grass) to alter polarity and gain. Intracellular recordings from muscle 6 or 7 were made with microelectrodes filled with 3 M KCl having a resistance of 30–60 MΩ. Responses were recorded with a 1× LU head stage and an Axoclamp 2A amplifier. Electrical signals were recorded to a computer A/D interface (AD Instruments). All events were measured and calibrated with the Scope software. EJP recordings consisted of an average of 10–20 events. All experiments were performed at room temperature (21–23 °C).

Recording output from a sensory-CNS-motor circuit

Dissections were identical to those described for the neuromuscular junction, the exception being that care was taken to leave as many segmental nerves intact as possible. Stimulations were given to the two segmental nerves innervating the most caudal segments as described previously (Dasari and Cooper 2004). Ten-pulse trains (40 Hz) were delivered at 10 s intervals and the number of evoked EJPs in M6 (segments 3 or 4) served as an index of motor output driven by sensory afferents and the CNS.

Results

pH stability of physiological salines with various buffers

When standard HL3 saline is prepared the pH is slightly acidic and requires titration to the physiologically optimal pH using a strong base (1 M NaOH). Physiological salines should be aerated to enhance cell viability. We found that aerating the standard HL3 and other buffered solutions with a syringe caused the pH to increase (HL3 from 6.55 to 6.65). Therefore, it is wise to aerate the salines prior to adjusting to physiological pH.

Standard HL3 saline was titrated to pH 7.1, and then the pH was monitored for 20 h at room temperature. After 3 h the pH drifted from 7.10 to 7.25 (Fig. 1). After 8 h the pH had drifted up to 7.35, and at 20 h the pH had drifted up to 7.60. Increasing the concentration of BES from 5 to 25 mM markedly reduced the drift. HEPES (25 mM) was slightly less effective at stabilizing pH for the first 3 h. TRIZMA-acid (25 mM) and TRIZMA-base (25 mM) are the least effective

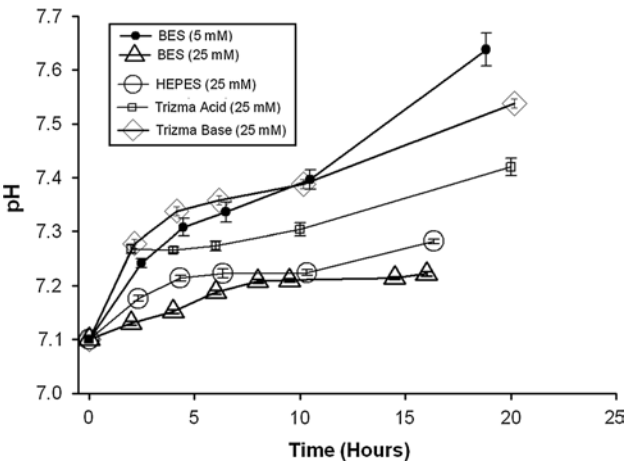

Fig. 1 Physiological salines drift over time. The pH of standard HL3 saline (BES 5 mM, *black circles*) with different buffer concentration (25 mM, *triangles*) or different buffer molecules (*squares*, *circles*, and *rotated squares*) was monitored for up to 20 h

at maintaining pH. Our conclusion is that higher buffer concentrations are needed to stabilize pH for a duration that will accommodate multiple experiments. To determine if the higher buffer concentrations influence cardiac function, nervous system activity and the responsiveness to serotonin (5-HT) we performed an array of physiological experiments.

Larval heart function in HL3 saline with different buffers

Dissected larvae bathed in standard HL3 saline have an average HR of 85 BPM. Exchanging the bath solution with fresh HL3 saline does not significantly alter the HR (Fig. 2a). Exchanging the bath solution with fresh HL3 saline containing serotonin (100 nM) increases HR on average to 160 BPM (Fig. 2a). The values obtained with HL3 saline (BES 5 mM at pH 7.1) and this saline containing 5-HT were used to determine if the other buffers and pH changes had an influence on HR or interfere with serotonergic modulation. Of the nine solutions tested, only TRIZMA-acid (50 mM) had a significant effect on HR. Exchanging HL3 with this solution caused complete cardiac arrest in ten preparations examined. Within the first minute of exchanging saline, the preparations would slow down and then would come to a complete stop by 10 min. Remarkably, preparations with no HR within the 5 min period were revived when TRIZMA-acid (50 mM) solution was exchanged with a TRIZMA-acid (50 mM) containing 5-HT (100 nM) (Fig. 2b). This was disturbing that TRIZMA-acid (50 mM) would stop the heart for such length of time and in some preparations longer than 5 min. TRIZMA-acid (25 mM) also decreased HR acutely, but the rates returned to initial values observed with HL3 BES (5 mM) within 5 min and remained stable. None of the other buffers significantly altered serotonergic modulation of HR as compared to BES 5 mM. Exposure to 5-HT significantly increased HR in each solution tested ($P < 0.05$, non-parametric rank sum).

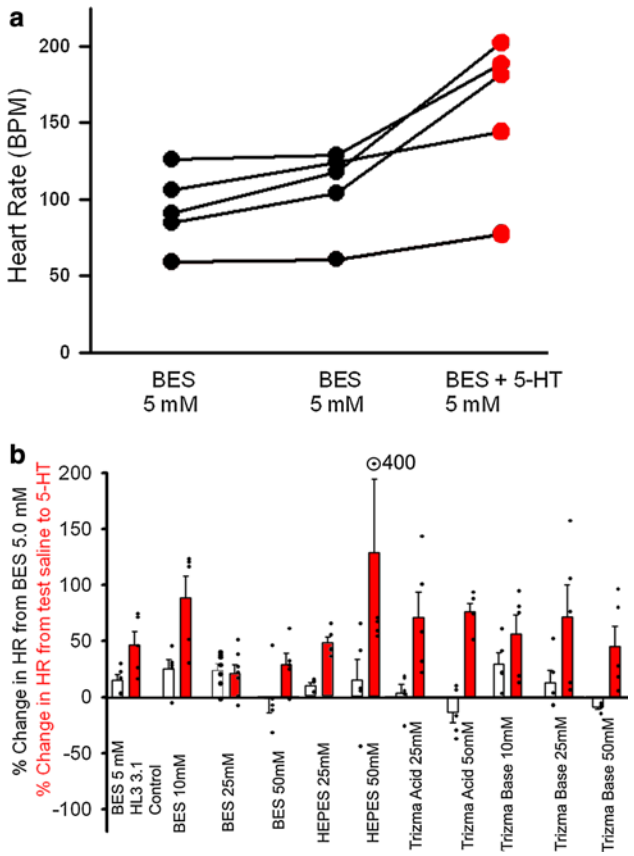

Fig. 2 Examining the effect of various buffers on larval heart rate and serotonergic modulation. (**a**) Representative example of heart rate changes in response to 5-HT using the HL3 (BES 5 mM) saline. Each *line* represents an individual preparation. Exchanging HL3 saline with a solution that contains 5-HT (100 nM) increases heart rate to an average of 160 BPM. (**b**) *Bars* represent the change in heart rate after standard HL3 was exchanged with a test saline containing an alternate buffer (*white bars*). The change in heart rate after exchanging the test saline with one containing 5-HT is also shown (*red bars*). The modulatory effect of 5-HT is not significantly different among the various buffers; however, there is a significant increase by 5-HT within each buffer. Note that at 50 mM TRIZMA-acid significantly reduces heart rate but upon exposure to 5-HT the heart became responsive. The one outlier of the graph is highlighted at 400 %

To address the influence of pH on HR we examined standard HL3 saline at a pH of 7.1 and switched the same saline to pH 7.4. No significant differences were observed. Some preparations had a slight shift in increasing HR while others decreased without a drastic change over time. However, switching from normal HL3 saline (pH 7.1) to pH 7.6 caused an acute decrease in HR ($P < 0.05$ paired t test; Fig. 3). The percent changes are shown with one preparation continuing to decrease even after 10 min, while other preparations started to show a slight increase in HR. A percent

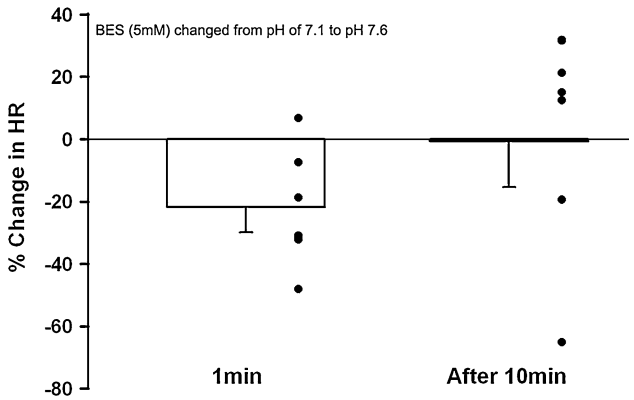

Fig. 3 The effect of alkaline saline on HR. Heart rate (HR) decreases when changing from a saline with BES (5 mM) at pH of 7.1 to pH 7.6 HR ($P < 0.05$ paired t test). After 10 min in pH 7.6 no further significant changes is noted as some preparations increase and some decrease in their HR

change was measured from pH 7.1 to 7.6 for the first minute, and then a second percent change was taken from the first minute at pH 7.6 to the tenth minute at pH 7.6.

Modulation of a sensory-CNS-motor circuit

An electrophysiological assay has been developed to test the fidelity of CNS circuitry that innervates the body wall muscles (Dasari and Cooper 2004). This assay provides an index of CNS integration and output in the context of exogenous neuroactive chemicals (see Fig. 4a for anatomical layout and Fig. 4b for stimulation protocol). A representative evoked response is shown in Fig. 4c. Serotonin (100 nM) has been shown to increase the output of this circuit (Dasari and Cooper 2004). In standard HL3 saline a ten-pulse train (40 Hz) applied to a segmental nerve evokes 54.1 ± 12.0 EJPs in M6. Exchanging HL3 saline (BES 5 mM) with BES (25 mM), TRIZMA-acid (25 mM), or HEPES (25 mM), does not significantly alter the frequency of EJPs (Fig. 4d). To determine if the buffers interfere with neuromodulation in this circuit we exchanged the test solution with a solution containing the test solution and serotonin (100 nM). This caused a significant increase in the number of EJPs following a ten-pulse stimulus for each saline tested (Fig. 4d; $P < 0.05$, non-parametric rank sum). Therefore, none of the buffers

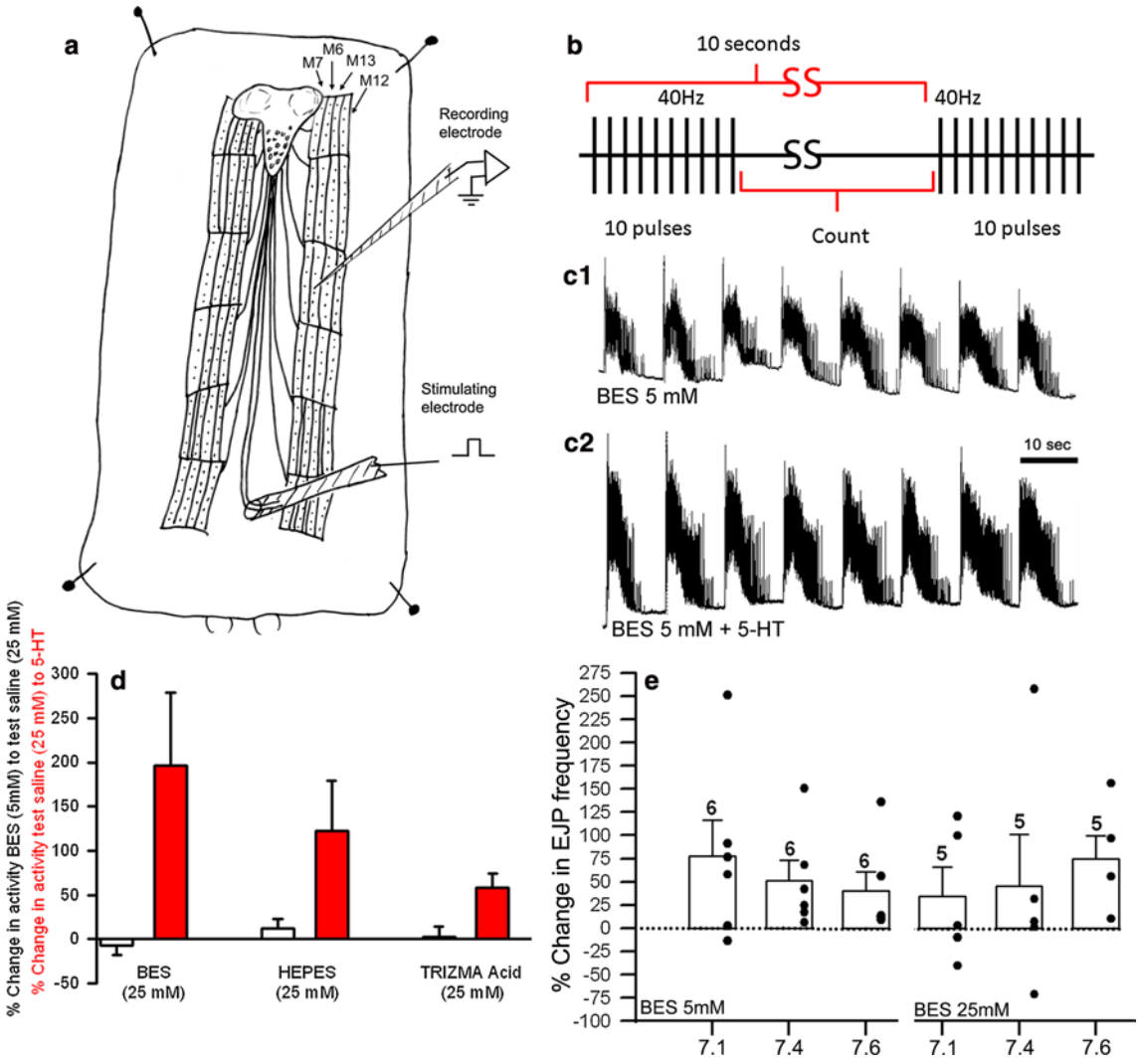

Fig. 4 Testing various buffers on central circuit function. (**a**) Schematic of the preparation for recording neural circuit activity. Electrically excited sensory neurons are stimulated to evoke motor activity that is measured by intracellular recordings in muscle 6. (**b**) Diagram of the stimulus. (*c1*) Representative traces before (*c1*) and after (*c2*) standard HL3 saline was exchanged with a test solution that contained 1 μM serotonin. In the test solution the number of EJPs significantly increased ($P = 0.003$, paired Student's t test, $n = 8$). (**d**) At 25 mM, none of the buffers affected the average number of evoked responses recorded from M6 (ANOVA, mean ± SEM). Serotonergic facilitation was also unaffected by the different buffers. (**e**) Changing the concentration of BES from 5 to 25 mM did not alter the sensory-CNS-motor circuit. Increasing pH from pH 7.1 to 7.1 or to 7.4 or to 7.6 did not cause any significant change for saline with 5 or 25 mM BES

interfere with serotonergic modulation of this sensory-motor circuit.

Next, we sought to determine if various changes in pH and the concentration of the BES buffer altered activity in the neural circuit. Only HL3 saline with 5 or 25 mM BES buffer was examined before and after a change in pH. To control for the effect of changing the bathing solution a change from pH 7.1 to 7.1 was used. The other changes were from pH 7.1 to either 7.4 or 7.6. No significant differences were observed for the pH alterations in either BES 5 or 25 mM (Fig. 4e).

Synaptic function in larval neuromuscular junctions

To determine if TRIZMA-acid, HEPES and BES at 25 mM influences synaptic transmission we recorded evoked EJPs from larval body wall muscles before and after exchanging standard HL3 saline (BES buffer 5 mM) with BES (25 mM), TRIZMA-acid (25 mM), or HEPES (25 mM). The synaptic responses from the Is, Ib or combined Is and Ib were examined before and after exchanging the saline (Fig. 5a). Amplitude and waveforms of EJPs were unaffected by either buffer in single-pulse (Fig. 5b) or during 4-pulse 20 Hz train stimulations (Fig. 5c). No consistent alterations occurred from BES (5 mM) to the other three solutions buffered at 25 mM. This preparation is known to run down in synaptic transmission over a short while (30 min) as observed in the reduction of the EJP amplitudes (Stewart et al. 1994).

Resting membrane potential in the muscle fiber was also unaffected by these treatments. Preparations were only used if the initial resenting membrane potentials were −50 mV or more negative as the muscles were deemed healthy with such values. Increasing the buffer concentrations to 25 mM for BES or TRIZMA-acid or HEPES does not affect HR, nervous system function or synaptic transmission at the NMJ, nor does this concentration interfere with serotonergic modulation of the heart or CNS.

Synaptic transmission at the NMJ was examined at high pH and high buffer concentrations, as was done with the CNS and heart preparations. To account for the effect of changing bath solution potential run down over time, a change from pH 7.1 to 7.1 was used for a baseline comparison. Other changes were from pH 7.1 to either 7.4 or 7.6 in solutions with 5 and 25 mM BES buffer. No significant differences were observed for the pH alterations in either BES 5 or 25 mM (Fig. 5d).

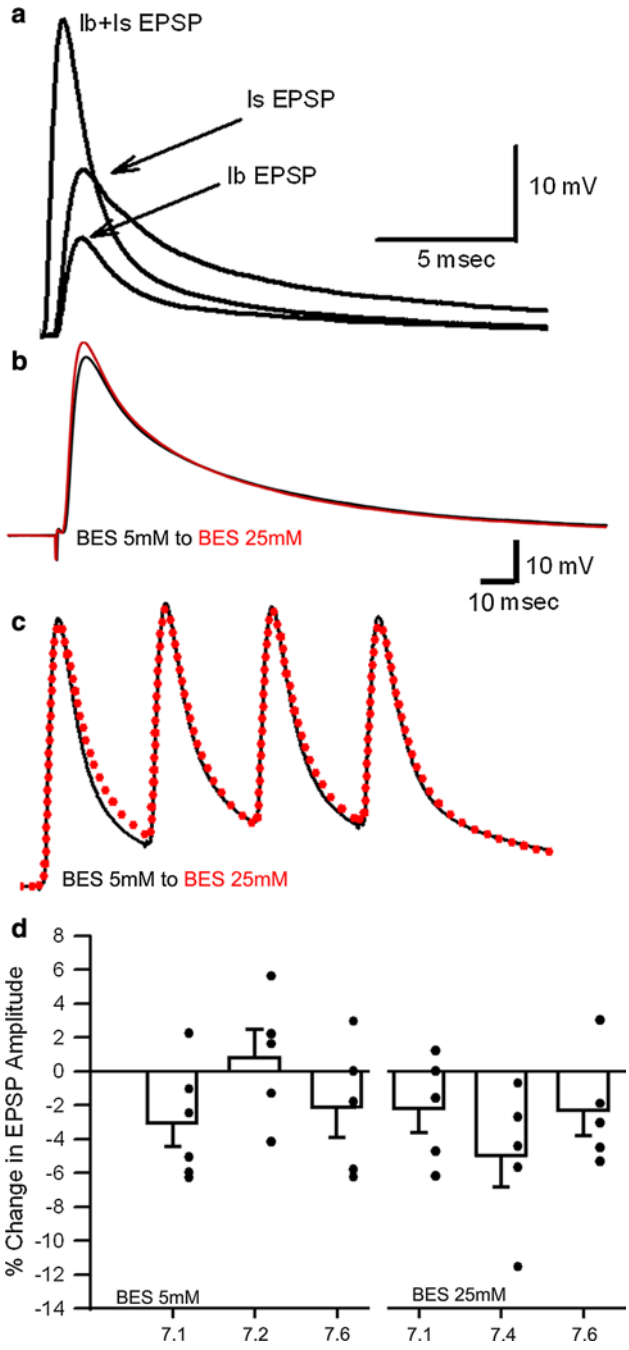

Fig. 5 EJP amplitudes and waveforms at the neuromuscular junction are unaffected by the various salines. (**a**) Representative traces from single EJP responses for the Ib, Is and the combined Ib and Is terminals. (**b**) No differences were apparent in the combined Ib and Is EJPs for BES (25 mM), TRIZMA-acid (25 mM) and HEPES (25 mM) from the prior responses measured in BES (5 mM). Slightly larger or smaller responses can be obtained in the exchanged saline conditions but no consistent trends were observed. (**c**) Trains of four stimuli delivered at 20 Hz produced EJPs that show slight depression in amplitude throughout the train but no significant alterations in the responses due to pH and types of buffers. (**d**) The BES buffer at 5 and 25 mM was specifically examined for alteration in the EJP amplitude when exchanging saline from pH 7.1 to 7.1 or to 7.4 or to 7.6. No significant differences were observed

Discussion

Larval *D. melanogaster* preparations are used for a variety of cardiac and neurophysiological investigations. The

influence of neuromodulators and other biologically active compounds on the function of these systems is an increasing area of research interest, as are developmental and genetic influences. Heart, NMJ, and sensory-CNS-motor circuit experiments require a stable dissection and incubation media. We observed that the pH of the most common physiological saline (HL3) tends to rise from 7.1 to 7.4 over a period of 10 h at room temperature. The semi-intact heart preparation is remarkably sensitive to these pH changes. So to avoid constantly re-titrating the saline, we experimented with different pH buffers to identify optimal saline solutions, and then tested the solutions on two nervous system preparations to confirm their viability.

In comparison to *Drosophila*, acidic conditions slow down HR in rodents (Kapur et al. 2009). Mechanistically this might be accounted for by the low pH resulting in an increase in intracellular Ca^{2+} which promotes repolarization (Kapur et al. 2009). Also, low extracellular pH may reduce activation of a K^+ channel resulting in slower repolarization; thus, slowing down HR (Zhou and Bett 2010). The influence of the HERG K + channel was addressed in *Drosophila* with a heart-specific knockdown of HERG-related fly genes. The knockdown produced various types of arrhythmias (Ocorr et al. 2007). As for the HR increasing in *Drosophila* with acidic conditions, we do not have a definitive mechanism to explain this phenomenon. Speculation that the PMC (plasma membrane Ca^{2+} pump) might be very susceptible to pH in *Drosophila* and that the acidic conditions may remove inactivation of the PMC, as known to occur in basic conditions, would then speed up the pump. Thus, reducing intracellular Ca^{2+} faster could remove inactivation of ryanodine receptor within the ER to allow the Ca^{2+} cycle to increase in overall speed. It is known that reducing the function of the PMC with basic saline (pH 8.8) can slow HR in *Drosophila* by 50 % (Desai-Shah et al. 2010), so as more acidic conditions are obtained there would be a reduced effect on the PMC.

Use of various common buffers to stabilize pH in HL3 saline

HL3 is the saline of choice for investigating electrically excitable cells because it closely resembles the ionic composition of larval hemolymph (Stewart et al. 1994). The electrical properties of larval myocytes in HL3 (Cooper et al. 2009; Desai-Shah et al. 2010) and in Schneider's insect medium (Lalevee et al. 2006) have been described in previous studies. Distinct action potential waveforms and spike amplitudes in different regions of the cardiac tube make it difficult to compare electrical properties from separate studies (Desai-Shah et al. 2010). Given that different EJP waveforms were observed at the larval NMJ using HL3 and Schneider's media, it would be of interest to determine whether cardiac action potentials are affected. One of the key differences between those two media is that Schneider's contains several different amino acids. Piyankarage et al. (2008) found that there are at least 13 different amino acids in larval hemolymph. A modified HL3 saline (HL6) was developed to incorporate amino acids and improve the loading of calcium imaging dyes into the motor nerve terminals (Macleod et al. 2002). The pH of this saline is likely to drift because 5 mM BES is used as a pH buffer. Given the differences in ionic concentrations, it also is likely that HL6 would influence cardiac function. Pharmacological and genetic approaches have shown that a plasma membrane sodium–calcium exchanger, the plasma membrane Ca^{2+} ATPase, and the sarco/endoplasmic reticulum Ca^{2+} ATPase influence HR (Badre and Cooper 2008; Desai-Shah et al. 2010). The delayed rectifier K + current is also involved in larval heart function (Gu and Singh 1995). The likelihood of these salines slowly drifting to more basic levels is that there is a 10 mM bicarbonate buffer in each saline and over time CO_2 is leaving solution, thus reducing the protons ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$, Stone and Koopowitz 1974). In the more heavily buffered salines the bicarbonate buffer plays a smaller role and thus less change is observed by CO_2 dissipation. The HR is known to increase as pH is reduced (Badre et al. 2005). HR continues to increase as pH is reduced from 7.4 to 6.0 and sharply at 5.0. The present study showed the opposite trend for basic saline resulting in an increase in pH. Thus, for conducting physiological studies with the larval heart, pH monitoring is critical over the extent of the experimentation. In addition, the use of CO_2 to anesthetize adult flies within a culture chamber in which one obtains larva for experimentation, the larvae will have a depressed HR due to CO_2 in the hemolymph despite a reduction in pH (Badre et al. 2005).

Increasing the concentration of the HL3 pH buffer (BES) is an obvious approach to stabilize pH. Increasing the (BES) from 5 to 25 mM significantly reduces the amount of pH drift over a period of 10 h without influencing HR, NMJ function, or the sensory-CNS-motor circuit function. The increased buffer concentration slightly increases osmolarity and $[Na^+]$ because the solution has to be adjusted to physiological pH with a small amount of NaOH (1 M). HEPES and TRIZMA-acid at 25 mM are also effective at stabilizing pH, with the caveat of having to increase osmolarity and $[Na^+]$ using more NaOH to bring the pH to 7.1. TRIZMA-acid at 50 mM is the only buffer that influenced HR initially. The Tris(hydroxymethyl)aminomethane molecule does not appear to be cardioactive per se, as TRIZMA-base (50 mM) did not cause cardiac arrest. TRIZMA-acid is just the crystalline hydrochloride salt of TRIZMA-base. The additional protons should increase HR, and chloride flux does not affect the cardiac rhythm in this system as

far as we are aware. Also the adjustment of TRIZMA-base (50 mM) to pH 7.1 should have resulted in the same compound as TRIZMA-acid (50 mM) at pH 7.1 and behaved similarly in stopping the heart. 5 *N,N*-bis(2-hydroxyethyl)-2-Aminoethanesulfonic acid and HEPES buffers (25 mM) were superior to the TRIZMA compounds at stabilizing pH in HL3 solution, making these buffers ideal for several hours of experimentation. HEPES is already being used in adult heart and nervous system preparations (Wang et al. 2003; Fink et al. 2009). For further confirmation that the buffers do not interfere with molecular pathways in larval myocytes and neurons we measured the effects of an exogenous neuromodulator.

Electrodes used to measure pH can also be problematic. The standard Ag/AgCl pH electrode can drift over time and they are sensitive to temperature changes. However, the wet Ag/AgCl wires which are chloride coated appear to be fairly stable for 8 h (Suzuki et al. 1998; Park et al. 2003). Some pH electrodes are sensitive to the buffer tris(hydroxymethyl) aminomethane (Tris) which is suggested to be due to tris and the linen fiber junction of the reference electrode (Ryan 1969; Durst and Staples 1972; Illingworth 1981; Sigma 1996). The pH electrode used in our studies is considered not to be Tris sensitive according to the manufacturer.

Modulation of heart rate and synaptic transmission in different fly salines

Monoamines, peptides and neurotransmitters are present in hemolymph where they act as hormones to modulate various organ systems (Zornik et al. 1999). Modulation of larval HR by monoamines has been characterized pharmacologically (Johnson et al. 1997; Dasari and Cooper 2006; Titlow et al. 2013) and with the use of transgenic receptors that respond to synthetic compounds (Becnel et al. 2013). These studies have shown that HR is modulated by canonical GPCR signal transduction cascades. Given the robust stimulatory effect of serotonin on larval HR in both intact and semi-intact preparations, we used exogenous serotonin to test for interactions between the different buffers and modulation. None of the buffers interfered with serotonin's stimulatory effect on HR in dissected preparations. Remarkably, even the arrested hearts incubated in TRIZMA-acid (50 mM) were stimulated by serotonin.

The question remains as to why the HR drops so substantially when the larvae are dissected and exposed to saline (Desai-Shah et al. 2010 see Fig. 2a1 vs b1). This can be due to many variables that are hard to control for, such as the numerous modulators that are bathing the intact heart and the limited composition of the salines. However, to examine the effects of single variables like a pharmacological compound or modulator it is best to have the heart in a well-defined saline environment. Extrapolation of the in situ to in vivo conditions needs to be taken with care as different results may be obtained. Why such variation in the HR is observed in the freshly dissected preparations remains to be determined. The HL3 *Drosophila* saline that was originally described used trehalose as an energy source and sucrose as an osmotic balance (Stewart et al. 1994). So we kept the concentration of trehalose and sucrose as described; however, it would be interesting to determine if glucose or other sugars would work as well or better for long term maintenance. We have not tested Schneider's and M3 on the heart but since they are not suitable for CNS and NMJ studies we don not see the need to continue using those media for *Drosophila* studies.

Impact and future directions

Visual inspection of HR is a very basic assay used to quantify the effect of pharmacological and genetic alterations. With more advanced analytical techniques that optically distinguish systolic/diastolic phases of the cardiac cycle and diameter (Fink et al. 2009; Tsai et al. 2012) this preparation will be used more frequently to model heart disease and investigate genetics of cardiac function. The NMJ preparation is already well established for answering questions related to development of motor nerve terminals (Li et al. 2002; Xing et al. 2005), receptor pharmacology of post synaptic receptors (Marrus et al. 2004; Bhatt and Cooper 2005; Guerrero et al. 2005; Qin et al. 2005; Lee et al. 2009; Frank 2013), and modulation of synaptic efficacy (Ruffner et al. 1999; Dasari and Cooper 2004; Dasari et al. 2007, 2009). Use of these preparations will continue to grow as optogenetic techniques and genetic manipulation of neuronal activity (Venken et al. 2011) become more efficient. Stable saline solutions will be an important variable to control in these experiments.

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