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Comparative Biochemistry and Physiology Part B 122 (1999) 199–210

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Dopaminergic modulation of motor neuron activity and neuromuscular function in *Drosophila melanogaster*

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Received 20 August 1998; received in revised form 28 October 1998; accepted 23 November 1998

Abstract

Dopamine is found in both neuronal and non-neuronal tissues in the larval stage of the fruit fly, *Drosophila melanogaster*, and functions as a signaling molecule in the nervous system. Although dopaminergic neurons in the central nervous system (CNS) were previously thought solely to be interneurons, recent studies suggest that dopamine may also act as a neuromodulator in humoral pathways. We examined both application of dopamine on intact larval CNS-segmental preparations and isolated neuromuscular junctions (NMJs). Dopamine rapidly decreased the rhythmicity of the CNS motor activity. Application of dopamine on neuromuscular preparations of the segmental muscles 6 and 7 resulted in a dose-responsive decrease in the excitatory junction potentials (EJPs). With the use of focal, macro-patch synaptic current recordings the quantal evoked transmission showed a depression of vesicular release at concentrations of 10 μM. Higher concentrations (1 mM) produced a rapid decrement in evoked vesicular release. Dopamine did not alter the shape of the spontaneous synaptic currents, suggesting that dopamine does not alter the postsynaptic muscle fiber receptiveness to the glutaminergic motor nerve transmission. The effects are presynaptic in causing a reduction in the number of vesicles that are stimulated to be released due to neural activity.

Keywords: Drosophila; Dopamine; Neuromodulation; Synaptic

1. Introduction

Neuromodulators are known to function as important signaling molecules in animals, and can alter activity of the central and peripheral nervous systems [25,42,43]. One neuromodulator in particular, serotonin (5-HT), is involved in the behavioral expression of dominance and aggression in widely evolutionarily diverged species; the role of 5-HT in aggression has been explored in crustaceans [31,45,51] and humans [10,11,30,50]. In *Drosophila melanogaster*, 5-HT modulates heartbeat [26,33], and voltage dependence of delayed rectifier and *Shaker* potassium channels [24]. Dopamine and 5-HT have been identified in fly heads, which suggests that neural activity is regulated by these compounds.

Little is known about the neuromodulatory roles of dopamine. It has been shown to act as a neurotransmitter in *Drosophila*, modulating female sexual receptivity and habituation, a form of learning [36,37]; however, it is also required for the normal development of both gonadal and other tissues [35]. We propose dopamine plays a functional role in behavioral modulation, neuroendocrine activity, and in development. Functional roles for dopamine in a variety of insects is well substantiated [21]; for example, the dopamine receptor densities in the brain of the honey bee are altered during development [29] and in relation to certain behaviors in bees [49]. The cloning and functional characterization of dopamine [22] and octopamine [23] receptors from *Drosophila* have been described. Local-

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0305-0491/99/$ - see front matter
PII: S0305-0491(98)10160-8

ization and characterization of these receptors at the NMJ will hopefully be forthcoming.

To understand the neuromodulator effects of 5-HT and dopamine in *D. melanogaster*, we examined the synaptic efficacy of *D. melanogaster* motor neurons at the neuromuscular junctions of high- and low-output terminals during the third instar larva stage by recording evoked and spontaneous quantal currents for quantal analysis. With this approach, one can assess pre- as well as post-synaptic differences induced by the addition of particular neuromodulators. Our results suggest a functional role for dopamine as a neuromodulator at the neuromuscular junction.

2. Methods

2.1. Chemicals

Serotonin hydrochloride (5-HT), dopamine hydrochloride and physiological salts were obtained from Sigma. The 5-HT or dopamine solution was made the day of experimentation. Anti-serotonin antibody was purchased from Incstar (ICN) and secondary antibodies (anti-rat IgG and anti-rabbit IgG, conjugated to fluorescein) were purchased from ICN.

2.2. Animals and dissection

The wild-type Canton S fly strain was raised at 19–20°C on standard cornmeal-agar-dextrose-yeast medium. Only wandering third instar larvae were used for the physiological studies because of the ease in dissection. The larval dissections were performed as previously described [48]. In brief, the preparations were slit along the mid-dorsal longitudinal axis and pinned flat. The preparation dish consisted of a glass slide (VWR) with magnetic tape (Best Buys retail outlet) adhered to one side. A hole in the center of the magnetic strip allowed the preparation to be viewed with transmitted light. Dissecting pins (Fine Scientific Tools, WA) were bent and glued to paper clips. The paper clips are easily maneuvered on the magnetic tape to hold the filleted preparation in place. This type of recording dish has been previously described for utilization of pinning ganglia isolated from the leech ventral nerve cord [34].

2.3. HPLC analysis

Quantification of dopamine and serotonin levels in second instar, early third and mid-third instar *Drosophila* larvae was accomplished via high pressure liquid chromatography. Larvae were quick frozen in liquid nitrogen and homogenized in 0.1 M perchloric acid (3 μl/mg of tissue). The chromatographic system consisted of an ESA pump (model 580), a manual injector (model 9125), and an HR-80 3 μm particle size column. Detection and quantification were accomplished using a Coulochem detector (ESA model 5200A), an analytical cell (model 5011, channel 1 set at −50 mV and channel 2 set at 300 mV), and a guard cell (model 5020, set at 400 mV). The mobile phase consisted of 75 mM NaH_2PO_4, 1.5 mM SDS, 100 μl/l triethylamine, 15% acetonitrile, 12.5% methanol. Under these conditions, dopamine eluted at 6 min 30 s, and serotonin eluted at 11 min. A standard concentration curve of dopamine and 5-HT was run in addition to the samples; 9–10 independent analysis were determined for all stages.

2.4. Immunocytochemistry

Immunocytochemistry was performed as previously described [38]. Briefly, larvae were filleted as described above, or their central nervous systems were hand-dissected in phosphate-buffered saline (PBS). Tissues were fixed for 2 h at room temperature in 4% formaldehyde in PBS and washed extensively in PNT (1 × PBS, 0.1% BSA, 0.1% Triton X-100). Primary antibody was added as indicated below and incubated overnight, followed by extensive washing in PNT. Tissues were then incubated for 4–6 h at room temperature in secondary antibody, washed extensively in PNT, and viewed with an epi-fluorescent microscope after mounting in 5% *n*-propyl gallate/20 mM sodium carbonate (pH 9.5)/80% glycerol.

A polyclonal anti-serotonin antibody was used at a 1:300 dilution. The affinity-purified anti-*Drosophila* tryptophan-phenylalanine hydroxylase antibody, which recognizes both serotoninergic and dopaminergic neurons, was used at a 1:250 dilution [40]. Antibody to *Drosophila* tyrosine hydroxylase (DTH) was generated as follows and used at a 1:500 dilution: a PCR-generated 1.5-kb fragment with *Nde* I linkers containing only the DTH coding region was subcloned into the *Nde* I site of the *E. coli* expression vector pET11a (Stratagene). This construct was transformed into BL21/DE3 cells. Log-phase cells were induced with 1 mg/ml IPTG and allowed to grow at 37°C for 3 h. The cells were harvested by centrifugation and the pellet was resuspended in 1 × protein electrophoresis buffer and loaded onto a preparative 10% SDS-PAGE gel. The proteins were transferred to nitrocellulose; the DTH band was visualized using Ponceau S stain, excised with a scalpel, minced, and injected into rats with the appropriate adjuvant.

2.5. Electrophysiology

The recording arrangement and solutions are essentially the same as previously described [46,47]. The

physiological saline contained (in mM): 1.0 $CaCl_2.2H_2O$, 70 NaCl, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 5 BES (*N*,*N*-bis[2-Hydoxyethyl]-2-aminoethanesulfonic acid). All experiments were performed at room temperature (19–22°C). The entire bathing solution was exchanged rapidly (< 1 min) three times with saline containing either 5-HT or dopamine. Following the 5-HT or dopamine application, the preparations were rinsed with saline, or saline containing 5 mM Ca^{2+}, to determine the recoverability of the preparation from the observed synaptic depression.

Intracellular recordings were made using microelectrodes filled with 3 M KCl (30–60 mΩ). Responses were recorded with a $1\times$ LU head stage and an Axoclamp 2A amplifier. Focal, macro-patch recordings were made with a 10-μm diameter fire polished glass electrode placed directly over nerve terminals that were viewed under a $40\times$ water immersion lens (Nikon, NA 0.55). By grossly mis-aligning the light condenser on the Nikon Optiphot microscope, an image of the nerve terminals as viewed under Nomarski optics is visible (suggestions by Dr Len Kaczmarek, Yale). In order to record synaptic currents, a $0.1\times$ LU head stage was used. Electrical signals were recorded to VHS tape (Vetter, 400) as well as on-line to a PowerMac 9500 via a MacLab/4s interface. All events were measured and calibrated with the MacLab Scope software 3.5.4 version. Averages of 1000 traces of evoked currents were made to obtain an overall average as presented for the Ib and Is terminals. Recordings presented of the EJPs consisted of an average of 10–20 events. The average in each of the five preparations was used to calculate the mean $\pm$ standard error of the mean (S.E.). All evoked recordings were made at 0.5 Hz stimulation frequency. CNS derived activity to motor neurons was measured by monitoring the frequency of EJPs elicited within muscles m6 and m7 with intracellular recordings.

2.6. Synaptic currents

The *D. melanogaster* NMJ preparation was used to assess the direct role of 5-HT and dopamine as neuromodulators on the high- and low-output synapses among terminals innervating a given muscle fiber type. Using the standard techniques of macro-patch recording and quantal analysis to measure synaptic transmission at these sites, one can assess the effects on transmission by determining the quantal parameters *m* (mean quantal content), *n* (number of release sites), and *p* (probability of release at a site), in the absence and presence of single neuromodulators.

The quantal measures and analysis are as previously described [44]. In brief, averages of 1000 traces of evoked currents were made to obtain an overall average as presented for the Ib and Is terminals. All recordings were made at 0.5 Hz stimulation frequency. A photograph was taken after determining the terminal region to record for focal macropatch recordings. The calibrated photograph was then traced to scale in order to indicate the recorded terminal location. In each trace a trigger artifact was visualized, which was used as a reference point to measure the time to evoked responses. Evoked and spontaneous events were analyzed to determine mean quantal content, *m*, as previously described in detail [13]. Mean quantal content as determined by two approaches was implemented for all the synaptic current recordings. Measurements of the maximum peak for each evoked event, including failures, provided an average evoked peak. This value was then divided by the mean peak amplitude of spontaneous events to provide the mean quantal content determined by the peak amplitude (m_{peak}) approach [20]. It should be noted that the times of peak evoked events were varied, and thus the point in time at which the measurements were made was allowed to shift to obtain the true peak in each evoked response. The area under the trace or charge (pA × ms) of the evoked events and failures was similarly divided by the mean charge of the spontaneous events to provide the mean quantal content (m_{charge}) by the charge approach. Histograms of the evoked events were made for each trace within a recorded period, and for any spontaneous events throughout the recording, to determine if shifts in peak and charge distributions occurred upon the addition of neuromodulator and high Ca^{2+} containing solution.

3. Results

3.1. HPLC analysis

Quantification of dopamine and 5-HT in whole larvae revealed different levels of expression. Systemic larval dopamine levels decrease from approximately 0.7 μg/ml in second instar to less than 0.4 μg/ml in the mid-third instar (larvae were homogenized in 3 μg/ml of perchloric acid, see Section 2, Fig. 1A). However, tyrosine hydroxylase levels are known to peak at the hatching/first instar and late third instar/pupariation boundaries [39]. Systemic 5-HT levels, although substantially lower than dopamine, increase during this time period from 0.06 to 0.10 μg/ml (Fig. 1B). Since this analysis quantified biogenic amine levels in the whole animal, one cannot exclude the possibility of regional differences within the CNS or hemolymph.

The presence of detectable dopamine and 5-HT levels, and their immunocytochemical localization to cell bodies within the larval body wall as well as within the CNS (see below), suggested that these molecules could act as modulators to affect signaling at the neuromuscular junction. Both dopamine and 5-HT can be detected in the larval hemolymph (Neckameyer and

Cooper, unpublished observations), suggesting that the circulating biogenic amines could have effects on distant targets. Our results demonstrate that the neuromuscular junctions we tested showed only alterations in activity with changes in dopamine but not 5-HT levels.

3.2. Immunocytochemistry

Several catacholaminergic and indolaminergic cells were visualized within the body wall of filleted third instar larvae using antibodies raised against serotonin, *Drosophila* tyrosine hydroxylase, and *Drosophila* tryptophan-phenylalanine hydroxylase (DTPH) (Fig. 2). These same antibodies recognized neurons within the larval CNS in the previously described stereotypic patterns for these biogenic amines (Fig. 2A,D; see also [9,40]). There are distinct and non-overlapping clusters of dopaminergic and serotonergic neurons in brain lobes. Within the larvae ventral ganglion, there exists medial unpaired neurons and bilaterally symmetrical dorsolateral neurons. The serotonergic neurons within the ventral ganglion are paired, bilaterally symmetrical cells distinct from the dopaminergic neurons. The larval brain shown in Fig. 2A was left intact after the fillet. Fig. 2B depicts the cells fluorescing with the same antibody in the body wall. The DTPH antibody recognizes both dopaminergic and serotonergic neurons [41]; the following cells seen in Fig. 2E. It is likely that these cells do release dopamine and 5-HT into the hemolymph that can alter the state of the CNS or NMJ activity.

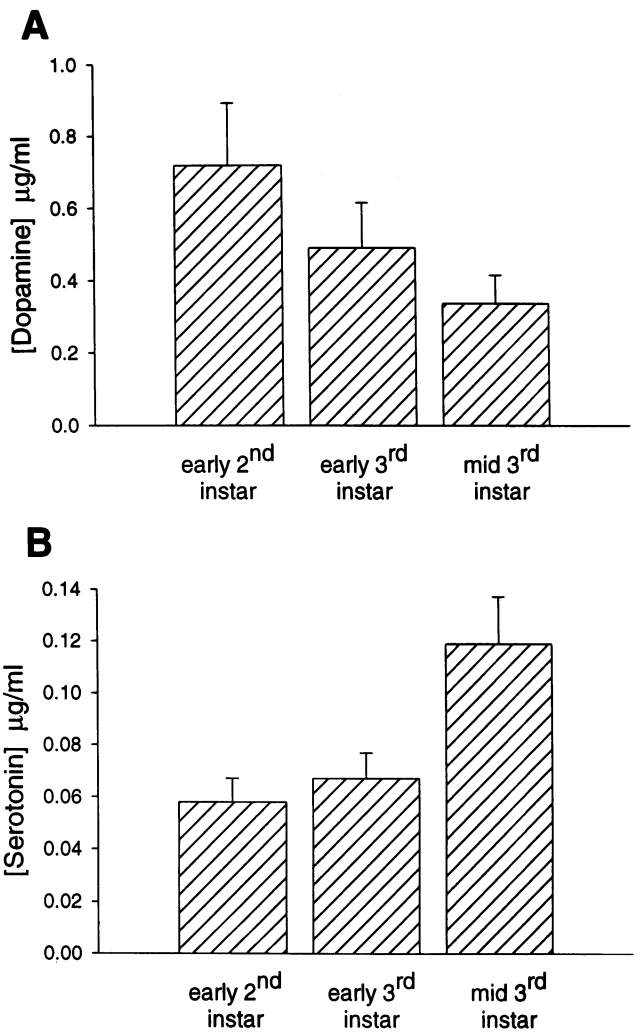

Fig. 1. HPLC analysis. (A) Quantification of dopamine levels in whole *Drosophila* larvae. (B) Quantification of serotonin levels in whole *Drosophila* larvae. Second, early third, or mid-third instar were homogenized in 0.1 M perchloric acid and subjected to HPLC analysis. Means and S.E. of the mean (bars) are shown.

3.3. Electrophysiology

The muscles are easily identifiable in the third instar larvae which provides reproducible preparation to examine the influence of various compounds on neuromuscular transmission (Fig. 3A,B). Muscles m6 and m7 are of particular interest, as they are both innervated by two distinctly different types of motor neurons [5], one which makes 'small' terminal boutons (Is), and another which makes 'bigger' boutons (Ib). The Is terminal produces a large EJP in the muscle fiber, whereas the Ib produces a smaller response (Fig. 3C). They are thus also referred to as high- and low-output terminals and the differences in the Is and Ib responses have been previously well characterized [5].

3.3.1. Central motor pattern activity

In preparations in which the CNS connections to the segments are left intact, en passant suction electrodes can be used to monitor the electrical activity in the motor nerve roots. The electrical activity measured is the motor neuron output to that particular segment. In the majority of cases, the activity profile in freshly dissected preparations is rhythmic. This can be altered by bath application of various modulators or by exchanging the bath with a solution of different ionic composition. To examine the effects of dopamine on motor output from the CNS, baseline recordings in saline were taken from a segmental root and are shown in Fig. 4A. The activity is rhythmic with short bursts of activity followed by a silent period (Fig. 4B), then tonic activity until another burst occurs. The extent of the post-burst silent periods among preparations varies while recording in saline, but within a preparation the depression becomes less prominent during the dopamine exposure. Upon application of 10 µM dopamine the bursting duration increased within 2 min, and after 5 min the bursting activity almost completely disappeared, leaving only a steady firing frequency (Fig. 4C). The absolute firing frequencies varied among preparations, but the trends shown in Fig. 4 were the

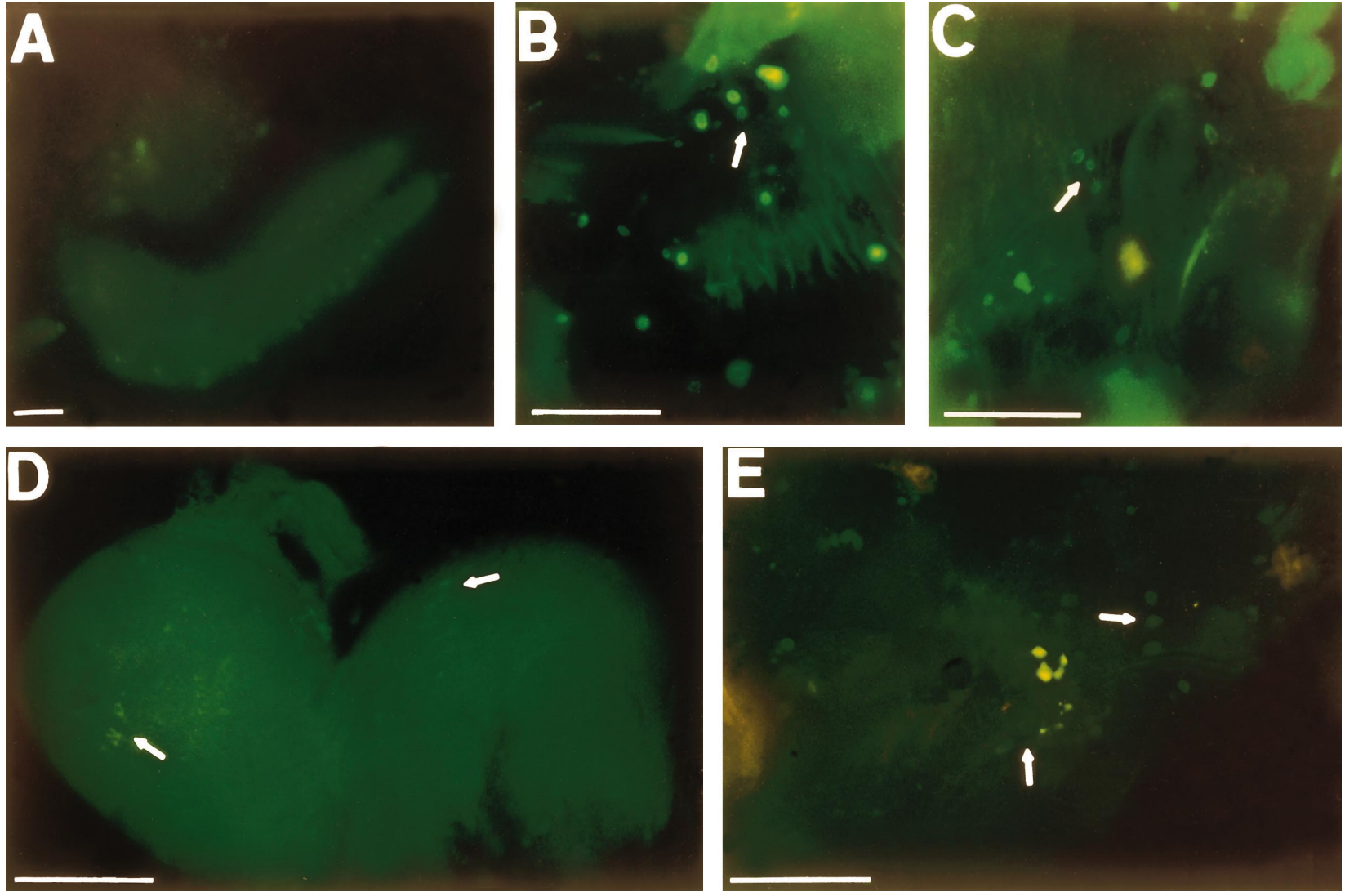

Fig. 2. Immunoreactivity of catecholaminergic and serotonergic neurons in the CNS and cells in the body wall of the third instar larvae. (A) α-serotonin immunoreactivity of a brain attached to a larval carcass. The stereotypic pattern of paired bilaterally symmetric serotonergic cells within is observed. (B) The same antibody used to visualize cells within the larval body wall. (C) α-DTPH immunoreactivity within the larval body wall. This antibody recognizes both dopaminergic and serotonergic neurons. (D) α-DTH immunoreactivity in larval brain lobes. Although the signal is weak, the expected clusters of neurons are seen. (E) The anti-DTH antibody was used to visualize catecholaminergic cells within the larval body wall. Arrows indicate clusters of immunoreactive cells. Scale bars, 50 μm.

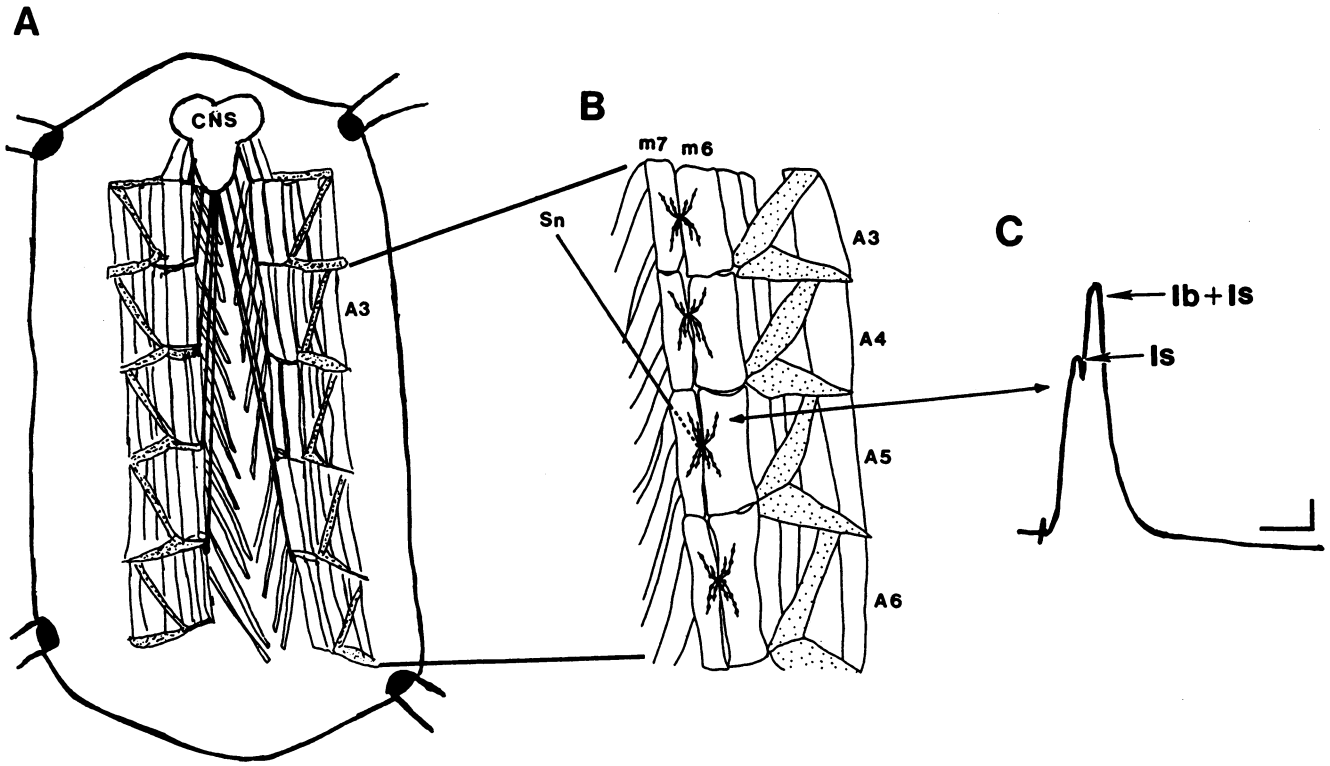

Fig. 3. Schematic diagram of the *Drosophila* larva preparation. A, The preparation is pinned in four points to keep the preparation taut. B, An enlargement of segments A3 to A6 of the right-hand side of the opened preparation. The ventral abdominal muscle, m6 is innervated by two excitatory motor nerve terminals contained in one segmental nerve (Sn). One of the nerve terminals contains small varicosities (Is) and gives rise to large excitatory junction potentials EJPs. The other terminal has big varicosities (Ib), but produces smaller EJPs. C, A composite response of both Is and Ib EJPs is shown. The Is has, in this particular recording arrangement, a shorter latency than the Ib response. Scale bars, A 200 μm, B 100 μm, C 10 mV and 20 ms.

same for each of the preparations. No significant alterations in the activity could be observed with exchanging the bathing medium to one containing 100 nM, 1μM or 10 μM 5-HT.

3.3.2. Excitatory junctional potentials (EJPs)

Dopamine, but not 5-HT, has a direct effect on modulating synaptic transmission at the neuromuscular junction. This was assessed in preparations in which the roots are severed from the CNS and the roots are placed into a suction electrode for stimulation. When recruiting both the Ib and Is motor neurons, the amplitude of the EJPs was measured while the preparations were bathed in saline, and during the exchange to saline containing various concentrations of biogenic amine. At a concentration of 1 mM dopamine, the combined (Ib and Is) EJP amplitudes show a rapid reduction which can partially be recovered when the dopamine is washed away by exchanging the medium back to a dopamine-free saline (Fig. 5A,B).

3.3.3. Excitatory junctional currents (EJCs)

To examine in more detail the reasons for the reduction in the EJPs in the presence of dopamine, focal, macropatch-recordings were made directly over varicosities of either the Is or Ib visualized terminals. The advantage of the current recordings over the intracellular EJP measures is that the evoked as well as the spontaneous events are easily measured over discrete regions of the terminals allowing the Ib and Is responses to be examined separately. In addition, by measuring the amplitude or the charge of the evoked and spontaneous currents, the effects of dopamine on presynaptic terminals versus the postsynaptic muscle is revealed. The amplitude and charge of the spontaneous or mini-excitatory junctional currents (mEJCs) are readily measurable in the current recordings. The average of 1000 EJCs when bathed in saline, or during the exposure to dopamine, shows the overall effects on evoked events. This average response is useful for determining the overall quantal response. To observe the gradual alterations in evoked release over time while exposed to various concentrations of dopamine, the peak amplitude of the EJCs in two preparations were plotted (Fig. 6). In two typical preparations (Fig. 6A,B), the exposure to 10 μM dopamine elicited a rapid and slight enhancement in the amplitude of the EJC. Although the enhancement was short lived, it occurred for both Is and Ib terminals. The enhancement was followed by depression of release. The rate in the

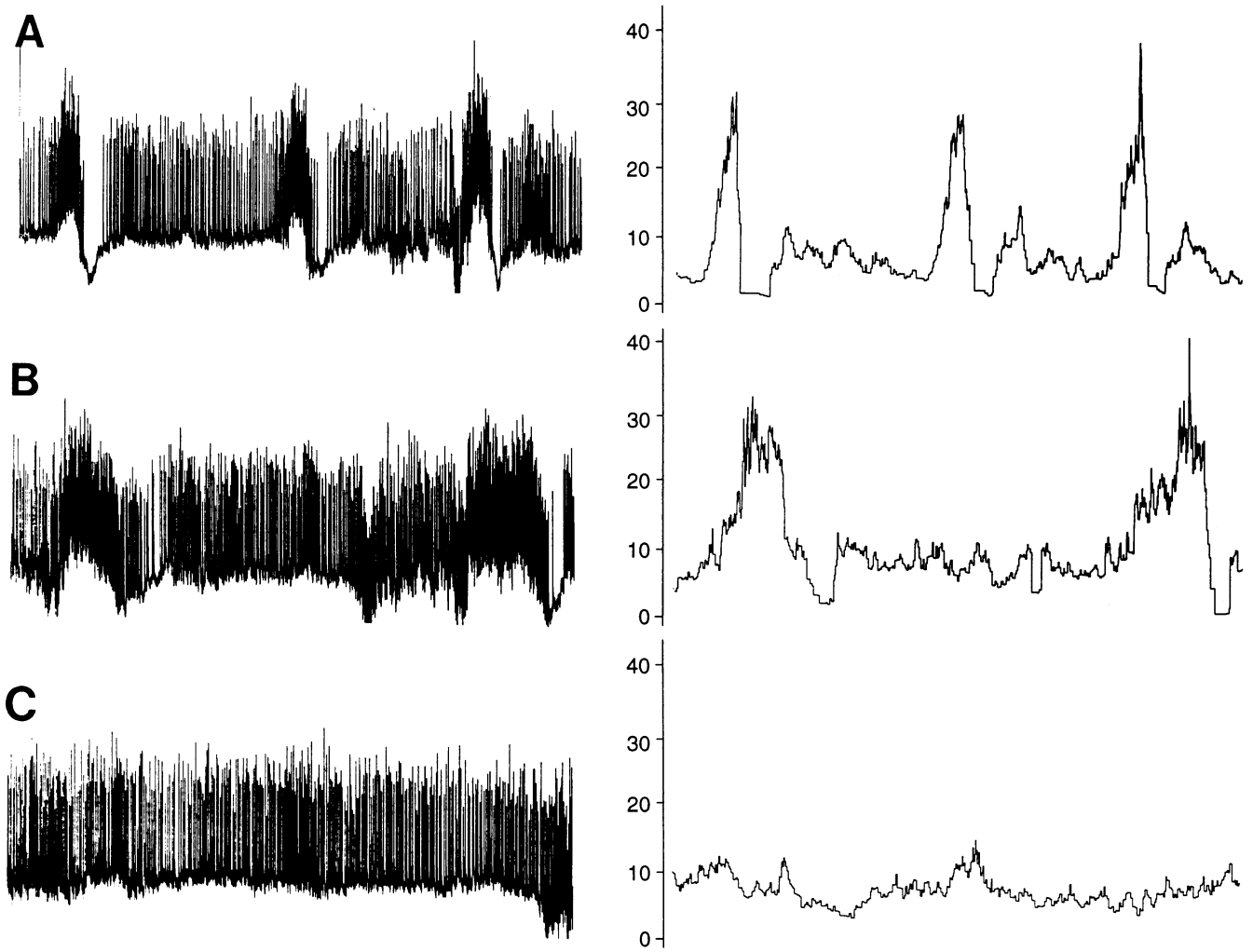

Fig. 4. The spontaneous activity of the motor neurons is altered by dopamine. The left-hand column shows the raw spiking activity while the right-hand column shows plots of the instantaneous firing frequencies (Hz) of the response to the left. (A) The spontaneous activity in the motor neurons is easily assessed by recording the firing frequency of the motor nerve root. (B) Upon addition of 10 μM dopamine a slight enhancement in the duration of the bursting phase can be seen, with the bursts less frequent. This was observed in five preparations in which the CNS was left intact to the segmental nerves. (C), After 5 min of being bathed in 10 μM dopamine, the bursts tended to disappear, and a steady firing frequency remained. Although there was temporal variation among the preparations, the same general trend was observed in that both the interburst and bursting frequencies were increased while the after burst silent period was reduced. The entire length of each trace was 1 min 20 s.

enhancement followed by reduction of the EJCs varied among the preparations, possibly due to access to the nerve terminal engulfed in the subsynaptic reticulum. When the preparations were exposed to low levels of dopamine, the reductions in the EJCs were reversible upon exchange of the medium back to saline only. The same washout procedures did not remove the strong EJC reductions when the preparations were exposed to the higher concentration of dopamine (1 mM). To determine whether the terminals were still able to release transmitter, the bathing medium was exchanged from a 1 mM Ca^{2+} containing solution to a 5 mM Ca^{2+} saline solution (Fig. 6A). The EJCs were enhanced substantially, and the muscle began to twitch rapidly.

The measure of evoked 'charge' or the evoked peak 'current' amplitudes can be used in determining quantal content. The charge measure is more reliable when events do not appear in synchrony. When release occurs with an evoked latency jitter, the result produces a broader and smaller evoked current which would underestimate mean quantal content when the peak amplitude measure is utilized. These differences in estimating mean quantal content have been examined in an earlier study of this preparation and of crustacean preparations [13].

Graphs of frequency histograms represent the distribution of evoked and spontaneous events and allow leftward shifts to be observed that occur due to the application of dopamine (Fig. 7). The mEJCs indicate

the unitary nature of the evoked events. There were no discernible differences in the distribution of the area or of the peak amplitudes of the mEJCs from the saline bath and the dopamine treated preparations. This indicates that fewer vesicles were being released during evoked release; this is not an alteration in the receptivity to dopamine by the postsynaptic receptors, since the spontaneous events did not show any differences. Fig. 7 shows the responses graphed in histograms from an Ib terminal in which the EJCs were plotted for both the peak amplitude (A1, B1) and charge (A2, B2) (or area under the current trace) while exposed to saline (A) or 10 μM dopamine (B).

The focal, macro-patch electrodes used in this study had approximately 10 μm inside diameters. This electrode diameter corresponds to the length of terminal that was recorded. The length of terminal recorded was similar for each recording session, so that the variation in response measured was intrinsic to the location along a terminal and among the Ib and Is terminals. Several studies have reported differences in the synaptic efficacy among the Ib and Is terminals, and the influence of different ionic compositions of the recording medium

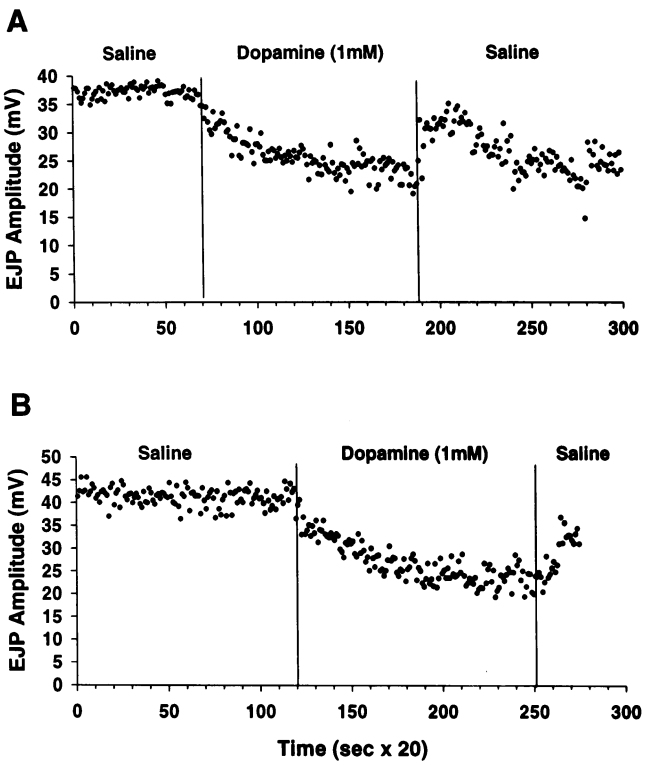

Fig. 5. The effects of dopamine on the Excitatory Junctional Potentials (EJPs). Segmental nerves 3 or 4 were stimulated while the EJPs were recorded with an intracellular electrode in muscle 6 of the stimulated segment. Isolated responses from stimulation of both the Is and the Ib motor neurons are shown while the preparations were bathed in saline and during the exposure to 1 mM dopamine (A, and B). The stimulation frequency was 0.5 Hz, and 10 EJPs were averaged for each point shown. Note the effects of dopamine are not due to run-down of the preparation since in B the dopamine was not added until 40 min of recording, compared to 23 min of recording in A.

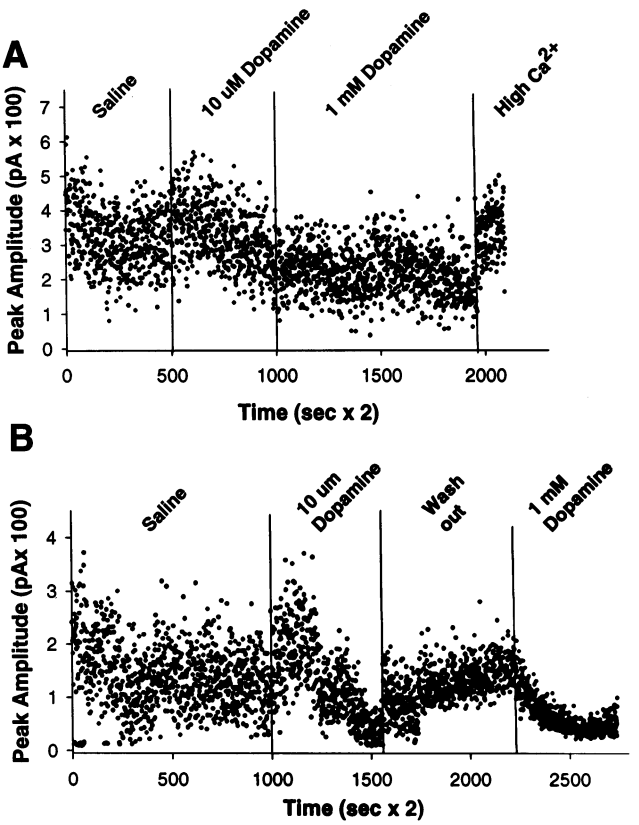

Fig. 6. EJC amplitudes from Is and Ib neuromuscular junctions over time in saline and exposure to dopamine. All the preparations show a small transient increase in the synaptic currents followed by a substantial decrease over time when exposed to a low concentration (10 μM) of dopamine. This decrease can be recovered with a saline wash, but a high concentration (1 mM) of dopamine results in a rapid decrease of the evoked currents. In all cases, exchanging a low (1 mM) to high calcium (5 mM) saline resulted in a substantial increase in the currents, indicating that the terminals still have the ability to increase synaptic transmission after being strongly depressed. A is a response from an Is terminal, whereas B is from an Ib terminal. Variation in the onset time and the time of decay varied among the preparations which may well be differences in the ability of dopamine to access the terminals from which the macropatch electrode is recording.

on transmission [1,5,28,47]. Independent of the measurement procedure, the mean quantal response is decreased in both the Ib and Is terminals (Table 1). The percent decrease is greater for the high-output Is terminals than for the low-output Ib terminals. Estimates of the mean quantal content from measures of current area or charge (m_{charge}) were calculated under the curve for both evoked and spontaneous measurements [13]. By dividing the averaged evoked response by the average of the spontaneous events, the mean quantal content was estimated. As mentioned above, this method is preferred when latency jitter in release occurs [7,13]. The same calculations were used to obtain the mean quantal content by measures of the peak current amplitude (m_{peak}). Some preparations were first examined during exposure to 1 mM dopamine; others were ex-

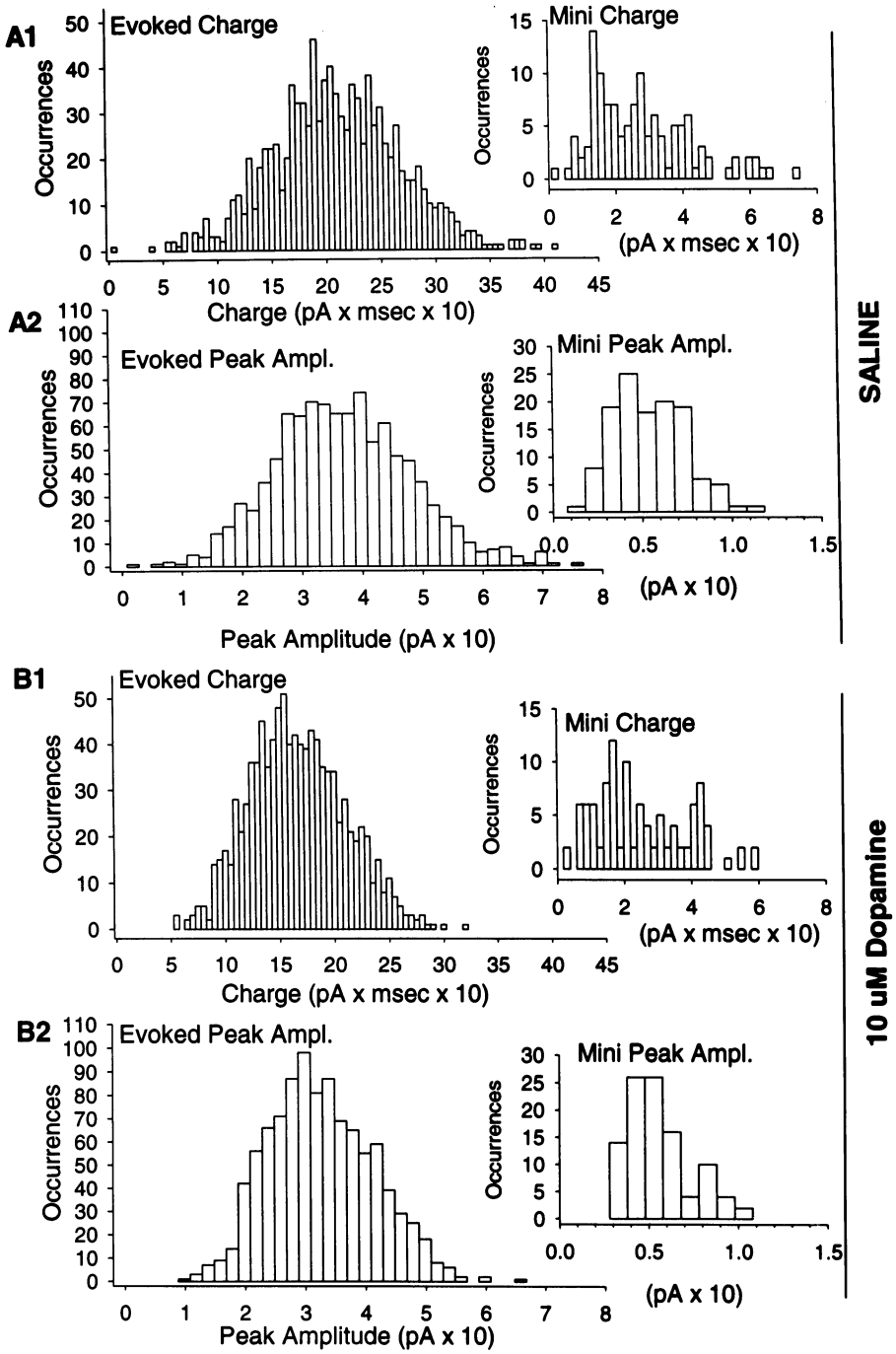

Fig. 7. Changes in the quantal content induced by dopamine. Peak evoked and spontaneous EJCs are plotted in histograms to show overall shifts in the evoked response. No change in the amplitudes of spontaneous events occurred in the presence of dopamine. The responses were recorded during a 0.1-Hz stimulation frequency for 1000 trials in saline and for the last 1000 trials in the presence of dopamine (10 μM). Note the size of the spontaneous events did not shift in size while the preparation was exposed to dopamine. This indicates a presynaptic site of action for dopamine. The general trend is consistent for Is and Ib terminals. The evoked and spontaneous events are displayed by the two standard methods of measure: peak amplitude (left column) and charge or area under the curve (right column).

posed to 10 μM followed by 1 mM dopamine. Since the reduction in synaptic currents could not be readily reversed back to baseline conditions after exposure to dopamine, the second addition of dopamine was not calculated for a percent change (the reason for blanks shown in Table 1).

4. Discussion

In the fruit fly, dopamine is known to have an effect on behavior as well as in development. If dopamine reserves are depleted in the larva, they will become aphagic and succumb to death [35]. In adults, depletion

Table 1
Mean quantal content determined by charge measures (m_{charge} and m_{peak})[a]

Terminal	m	Saline	10 μM dopamine	%Δ	Wash	1 mM dopamine	%Δ
Ib							
Prep. 1	m_{charge}	4.17	3.77	↓10	4.28	3.7	–
	m_{peak}	3.59	3.31	↓7	3.69	3.4	–
Prep. 2	m_{charge}	4.65	3.9	↓16	–	–	–
	m_{peak}	4.77	3.81	↓20	–	–	–
Prep. 3	m_{charge}	3.22	3.04	↓6	–	–	–
	m_{peak}	3.53	3.35	↓5	–	–	–
Prep. 4	m_{charge}	4.35	4.0	↓9	–	–	–
	m_{peak}	3.95	3.5	↓11	–	–	–
Is							
Prep. 5	m_{charge}	7.0	4.33	↓38	6.6	1.9	–
	m_{peak}	8.4	5.06	↓40	7.6	4.7	–
Prep. 6	m_{charge}	7.31	4.57	↓37	–	2.99	–
	m_{peak}	7.96	4.25	↓47	–	3.36	–
Prep. 7	m_{charge}	7.1	–	–	–	3.35	↓53
	m_{peak}	8.54	–	–	–	3.83	↓55
Prep. 8	m_{charge}	7.28	–	–	–	4.46	↓39
	m_{peak}	8.83	–	–	–	4.97	↓44

[a] The mean quantal content was determined by measuring evoked charge (m_{charge} and m_{peak}) in the saline groups as well as in the dopamine groups. The % difference was calculated by the following method: $\{[(\text{saline})-(\text{dopamine})]/(\text{saline})\} \times 100\%$. There is a trend for a greater decrease in the m_{charge} for the Is terminals than that of the Ib terminals in the presence of dopamine.

of dopamine alters female sexual receptivity and a learning behavior [36,37]. If the depleted dopamine levels are restored by L-DOPA, the previously altered behaviors are rescued. HPLC quantification of dopamine and 5-HT in whole larvae revealed distinct levels of expression which could reflect the differing requirements for these molecules. Dopamine, although present in the larval CNS, has not yet been shown to act as a transmitter at this developmental stage, excluding the modulation of exploratory behavior [35]. Depletion of dopamine during the second instar results in developmental abnormalities and lethality [35], suggesting that the primary role of dopamine in this stage may be as a developmental signal. However, catecholaminergic fluorescence is clearly detected in cell bodies within the larval body wall. These results suggest that the immunoreactive cells may have a role in releasing dopamine and thus have a modulator role in altering neuronal function. Similar sized cells within the *Drosophila* larval body wall are recognized by antibodies raised against the amino acid transmitter γ-aminobutyric acid (GABA) and the rat GABA transporter GAT-1; this result is apparently specific as antibodies against the GAT-2 and GAT-3 transporters, although capable of detecting other neurons in both central and peripheral tissues, do not recognize cells within the body wall [38]. This also suggests that GABAnergic cells in the body wall may have a functional role.

Levels of 5-HT are approximately 10-fold lower in concentration than dopamine but increase with increasing larval age. It is not clear whether 5-HT is required for normal non-neuronal development of the adult fly, and its expression appears largely limited to neuronal tissues. It was therefore surprising that application of 5-HT had little effect on transmission at the neuromuscular junction. Conversely, application of dopamine resulted in distinct changes in transmission, suggesting that the systemic decrease of dopamine from second to third instar larvae might reflect a greater regulation of transmitter levels.

The observations we report here support the notion that dopamine plays a role in altering the physiological responses of the CNS and the periphery at the neuromuscular junctions. The altered activity in the segmental roots indicates a general reduction in the bursting rhythmicity of the motor command. In addition, the application of dopamine to neuromuscular preparations, isolated from the CNS shows a depressive influence of the evoked quantal response without any measurable effects directly on the postsynaptic muscle fibers. The rate of reduction in evoked transmission is concentration dependent.

Estimates of mean quantal content of discrete regions of the Is and Ib terminals generally show that the Is has higher output varicosities than the Ib terminals. It has also been reported [46,48] that there is variation along the length of the terminal which probably accounts for the variations that we have observed (Table 1). The synaptic structural differences among the Ib and Is terminals and the variation along a given terminal have previously been examined [1,2,5,32]. The synaptic structural differences parallel those of crustacean high- and low-output terminals [4,8,12,14–16]. The quantal anal-

ysis indicates that the reduced synaptic efficacy in both Is and Ib terminals is due to a presynaptic effect of dopamine. The differences in transmission among Ib and Is terminals is correlated to the degree of calcium entry, which was shown with calcium-sensitive indicators [27] and was likely due to synaptic structural differences [5]. These intrinsic differences in the two types of terminals may be why there is a greater decrease in synaptic efficacy among the Is terminals when exposed to dopamine as compared to the Ib terminals, but at this time we do not know how dopamine reduces vesicular release. Other neuromodulators, such as the active form of ecdysone (20-hydroxyecdysone), have been first shown in lobsters [18,19], and later in tonic crayfish neuromuscular junctions [17], as well as in *Drosophila* neuromuscular junctions [44], to have a rapid non-genomic influence at the presynaptic motor nerve terminals in decreasing synaptic efficacy. Because of the multi-quantal evoked responses of the Ib and Is terminals measured with the focal macro-patch electrodes, the quantal parameters of n (the number of release sites) and p (the probability of release) are not easily obtainable as in lower output motor nerve terminals [13].

Comparisons of motor neurons among arthropods, particularly insects and crustaceans, have shown many of the same morphological and physiological phenotypes [1,3], although the influences of some neuromodulators on synaptic efficacy are strikingly different. For example, application of 5-HT results in a substantial enhancement in the number of vesicles released from the presynaptic nerve terminals in both high- and low-output motor neurons of crustaceans. We applied serotonin to the preparations used in this *Drosophila* study at concentrations of 100 nM, 1 μM, and 10 μM without observing any alterations in the EJP amplitudes or shapes. It is possible serotonin acts as a neuromodulator at other peripheral sites, or directly on the CNS, since it is found within the hemolymph of third instar *Drosophila* larvae. Pilot studies examining the effect of 10 μM and 1 mM dopamine on the tonic opener neuromuscular junctions in crayfish did not show any significant alterations in the evoked postsynaptic potentials (unpublished personal observations). In other neural circuits involved in the stomatogastric ganglion of lobsters, dopamine has been shown to have differential effects on spike evoked and graded transmission [6]. Dopamine was shown to reduce the responsiveness of the postsynaptic cell to glutamate in some neurons, while in some presynaptic neurons it alters the input resistance causing an enhancement of release [6].

We examined the wandering third instar larval stage because of ease in dissection; there may be quite a difference in earlier stages in the responsiveness to dopamine in altering CNS activity as well as the evoked neuromuscular transmission. Given that the HPLC results indicate that dopamine concentrations decrease from the second to the mid third instar stage in the whole animal, there may also be an alteration in the receptivity to the neuromodulator.

In this report we have shown a specific presynaptic response to dopamine. We are pursuing this question in *Drosophila* mutants with defined presynaptic deficits in dopamine machinery, and are examining other possible insect modulators such as octopamine and tyramine.

Acknowledgements

Illustrations were provided by the courtesy of Hye Won Cooper. We thank Joe Wegrzyn (University of Kentucky) for editorial assistance. Funding was provided by University of Kentucky Research and Graduate Studies Office (R.L.C.) and NSF grants IBN-9808631 (R. Cooper) and IBN-9423616 (W. Neckameyer).

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