DEGENERATIVE CHANGES IN THE FUNCTION OF NEUROMUSCULAR JUNCTIONS OF *MANDUCA SEXTA* DURING METAMORPHOSIS

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Summary

In *Manduca sexta* the decline in neuromuscular function during metamorphic degeneration was compared in two muscles which differed characteristically with regard to pre- and postsynaptic physiological properties. In both muscles, morphological evidence indicated that a significant number of the active zones within the population of neuromuscular junctions on a given fiber were non-functional. Nevertheless, the degenerating nerve terminals were able to produce an above-threshold excitatory junction potential (EJP) which was facilitated in a manner characteristic of the muscle being observed. Abnormal findings during the early stages of degeneration included a *larger* than normal EJP, a decline in EJP amplitude over a 20 min period even with low frequencies of stimulation, an increase in EJP duration, a decline in muscle fiber resting potential amplitude with age, a decrease or disappearance of post-tetanic potentiation and long-term facilitation, and an increased likelihood that the motor nerve would fail to conduct a stimulus. The two muscles were qualitatively similiar but quantitatively different with regard to these degenerative changes. It is suggested that this combination of relatively normal function with abnormal properties might be associated with the withdrawal of glial processes from the neuromuscular junctions, changes in the cable properties associated with shrivelling of the muscle fibers, and a decline in the metabolic functions supporting both muscle fiber resting potentials and those underlying transmitter synthesis, mobilization and release.

Introduction

Many studies of the change in function associated with degeneration of nerve terminals have been made following axotomy (Birks *et al.* 1960; Nüesch and Stocker, 1975; Purves, 1975; Hodgkiss and Usherwood, 1978; Ko, 1981; Washio, 1989) or chemical destruction of neuronal cell bodies (Deshpande *et al.* 1978). However, artificially induced degeneration may not evoke precisely the same

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responses as degeneration during development, metamorphosis, disease or aging. For instance, in the moth *Antheraea polyphemus*, axotomy led to the appearance of abnormal vesicles and mitochondria in the affected nerve terminals, while these changes were not seen during metamorphic degeneration (Nüesch and Stocker, 1975). Thus, the study of neuromuscular function during metamorphic degeneration might provide insights into the correlation between synaptic structure and function and into processes unique to dying or diseased neurons and their associated glia.

In additional papers in this series (Rheuben, 1992*a,b*), we examined the ultrastructure of normal and degenerating larval mesothoracic dorsal longitudinal (AB and C) muscles and nerves of *Manduca sexta* (Lepidoptera) during the larval and prepupal period of the fifth instar and the first day after ecdysis to the pupal stage. This set of muscles is used by the larva for locomotion and ecdysis; they begin to degenerate at the end of the fifth instar during the prepupal period. Some (but probably not all) fibers are conserved to form a scaffold for subsequent fusion of myoblasts and redevelopment of the adult flight muscles (Heinertz, 1976; M. B. Rheuben, unpublished observations). Some of the motor neurons are also conserved, in particular the five that innervate the mesothoracic dorsal longitudinal flight muscle (Casaday and Camhi, 1976). During the period of premetamorphic degeneration studied here and by Rheuben (1992*a,b*), we observed a variety of morphological changes which might well influence the performance of the motor nerves, neuromuscular junctions and muscle fibers.

In peripheral nerve trunks, glial cells surrounding the axons swelled and withdrew their processes from around the axons, leaving the axons in direct contact with each other. Muscle fibers shrivelled, with a decrease in cross-sectional area and an increase in circumference relative to their larval shapes, thus affecting their cable properties and their subsequent ability to propagate nerve-evoked electrical signals within the muscle. Degeneration of muscle fiber mitochondria and withdrawal of their oxygen-supplying tracheoles may have been associated with decreased capabilities for oxidative metabolism (Rheuben, 1992*a*). The set of glial cells associated with the neuromuscular junction also withdrew their interdigitating processes, leading to greater direct contact between the nerve terminal and the muscle fiber. Some presynaptic active zones appeared normal and some exhibited disorganized particle arrays in freeze-fractured material (Rheuben, 1992*b*), a finding that has previously been noted for other degenerating (Ko, 1985; Ko and Propst, 1986) or diseased neuromuscular junctions (Engel *et al.* 1987; Fukuoka *et al.* 1987).

Many of the structural changes would be expected to modify evoked neurotransmitter release directly and immediately and therefore to be detectable as an altered response to a single stimulus. Other changes might have more subtle results with an unpredictable time course. If there were a decline in overall metabolic function, as suggested by the degeneration of mitochondria and the withdrawal of tracheoles from the muscle, then synthesis and mobilization of stores of neurotransmitter might be decreased within the nerve terminal.

Impairment of these functions might only be detected with more rigorous stimulation regimes. Synaptic depression might be more marked, reflecting depletion of the immediately releasable pool of neurotransmitter or of the pools 'upstream' to it (Betz, 1970; Furukawa and Matsuura, 1978; Glavinovic, 1987). The complicated processes leading to post-tetanic potentiation and long-term facilitation following tetanic stimulation might also be disturbed.

We therefore investigated the responses of normal fifth-instar larval and degenerating day 1 pupal neuromuscular junctions to single stimuli and to short and prolonged trains of stimuli at various frequencies to determine whether physiological changes occurred that might be correlated with the ultrastructural changes. We found that, during degeneration, simple phenomena such as the production of an above-threshold excitatory junction potential (EJP) by the population of release sites that were still functioning were not greatly affected by degeneration during the first 12 h following ecdysis, although the structural changes in glial and muscle cells appeared to influence the postsynaptic response considerably. However, tetanic and post-tetanic potentiation and long-term facilitation, all processes that are presumed to have complex metabolic components, were impaired during metamorphosis.

Materials and methods
Experimental animals

Tobacco hornworms [*Manduca sexta* (Linnaeus)] were reared on an artificial diet (after the methods of Yamamoto, 1969) with a 16 h:8 h L:D photoperiod at 26°C from eggs provided by the Insect Hormone Laboratory, Department of Agriculture, Beltsville, MD. Fifth-instar larvae were studied between the second and sixth day after ecdysis. Pupae were studied between 1 and 13 h after pupal ecdysis. When pupation was not directly observed, pupal age, defined as the age in hours after pupal ecdysis at the time of anesthesia, was determined from the characteristic appearance of the pupal cuticle (Riddiford and Ajami, 1973).

Dissection

After anesthesia by chilling, the abdomen was removed. A longitudinal incision was made dorsal to the left row of spiracles and the right thoracic segments were pinned out on a sloping block of wax. The gut was reflected rostrally and the chain of central ganglia removed. The right prothoracic spiracle was positioned above a well in the wax block to keep it dry. Oxygen (95 % O_2, 5 % CO_2) was piped into the well in most of the experiments. The degree of ventilation could not be evaluated visually; if fluid filled the tracheoles the experiment was terminated.

The fat body was trimmed to allow a full view of the right mesothoracic A, B and C muscles (nomenclature of Lyonet, 1762). The boundary between muscle A and muscle B is indistinct in *Manduca sexta*; the fibers from both regions had similar responses and are referred to collectively as the AB muscle.

Electrophysiology

The experimental saline contained $20 \, \text{mmol} \, l^{-1}$ KCl, $15 \, \text{mmol} \, l^{-1}$ NaCl, $33 \, \text{mmol} \, l^{-1}$ MgCl$_2$, $10 \, \text{mmol} \, l^{-1}$ NaHCO$_3$, $5 \, \text{mmol} \, l^{-1}$ KH$_2$PO$_4$, $4 \, \text{mmol} \, l^{-1}$ CaCl$_2$, $35 \, \text{mmol} \, l^{-1}$ Tris methanesulfonate (TrisMSO$_3$), $22.2 \, \text{mmol} \, l^{-1}$ glucose, $5.3 \, \text{mmol} \, l^{-1}$ trehalose, $26.6 \, \text{mmol} \, l^{-1}$ sucrose and $8.2 \, \text{mmol} \, l^{-1}$ L-glutamine. In low-calcium solution, CaCl$_2$ was replaced by TrisMSO$_3$ to maintain osmolarity. Oxygen (95 % O$_2$, 5 % CO$_2$) was bubbled through the experimental solution, which flowed continuously across the surface of the specimen and was wicked away at its lower edge to avoid wetting the spiracles.

The motor nerve to the mesothoracic AB and C muscles was stimulated *via* a glass suction electrode filled with saline. Conventional glass microelectrodes filled with $3 \, \text{mol} \, l^{-1}$ KCl (5–15 MΩ in saline) were used to record intracellular EJPs from these muscle fibers. The resulting signals were displayed on an oscilloscope screen, and simultaneously recorded on a two-channel Brush 220 chart recorder.

After ascertaining that there was a response to stimulation of the motor nerve, the solution was changed to an appropriate low-calcium saline. Any change in external calcium concentration was always followed by at least 15 min of equilibration.

Data acquisition and analysis

Resting potentials were measured directly on the oscilloscope screen or from the deflection on the chart record. EJP amplitudes and durations were measured from the chart records with Helios calipers or with a digitizing pad and a Bioquant II morphometry program.

Stability of resting potentials was evaluated from the chart recordings, and, if possible, by comparing the resting potential at the beginning and end of an experiment. Data from fibers whose resting potentials declined by more than 20 % during the course of long-term experiments were excluded from studies in which EJP amplitudes were being examined over time. The effects of age on muscle fiber resting potentials were determined from electrode penetrations made at the onset of experiments.

To determine whether there was a significant change in amplitude of the EJP over the duration of an experiment, only fibers which had not received prolonged high-frequency stimulation were studied. Since pupal EJP amplitudes tended to decline during the course of experiments involving short trains of stimuli, we first verified whether the presence or absence of previous short trains or the total duration of an experiment might have altered the rate of decline. We used a mixed-design analysis of variance (ANOVA) to establish that there was no difference in the stability of EJP amplitudes of eight pupal C fibers which had had a variable number of previous trains of 10 stimuli and variable equilibration periods (over 15 min) and nine pupal C fibers which had had no such previous trains and had equilibrated for 43–66 min. The data from pupal C fibers were therefore pooled, as were those from pupal AB fibers.

The statistical test used on data sets was Student's one-tailed *t*-test if not otherwise stated.

Results

Even though the larval mesothoracic AB and C muscles and neuromuscular junctions in *Manduca sexta* are structurally similar and the electrical responses recorded in normal saline are identical (Rheuben and Kammer, 1980), we found that their physiological responses differed consistently in the reduced-calcium salines used in the present study. Consequently, the data have been segregated and the differences defined. In each section, properties of fifth-instar larval AB and C muscles are described first, followed by a comparison of the same features in the degenerating pupal fibers. The muscles and their innervation are shown in Fig. 1 and further illustrated in Rheuben (1992*a*). Their specific uses during larval locomotion have never been described, but their motor patterns consist of very long bursts concurrent with contractions of the entire segment (Kammer and Rheuben, 1976).
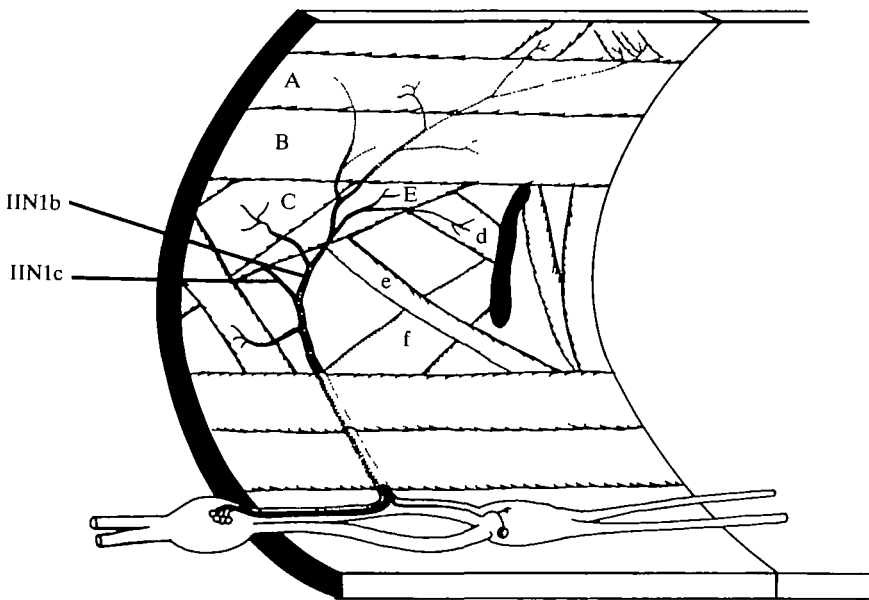

Fig. 1. Internal view of the muscles and nerves of the larval right mesothoracic segment illustrating the dorsal longitudinal muscles, including muscles A, B and C. The locations of the five motor neurons innervating the adult dorsal longitudinal muscle are also shown. Nerve IIN1 has two main branches in this region of the larval mesothorax. IIN1c contains sensory axons from the tubercles of the skin and from stretch receptors. IIN1b contains the motor axons which innervate muscles A, B, C, D, E, F, d and several others which, like D*a*F, lie underneath AB. Reprinted from Rheuben and Kammer (1980).
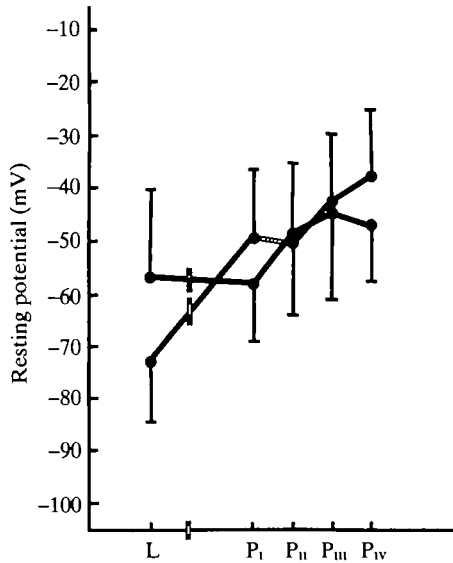
Fig. 2. Correlation between muscle fiber resting potential and developmental stage of the animal. Time not to scale; approximately 4 days intervened between the fifth-instar and pupal samples. Sample size 15–41 fibers for each point; mean±s.D. L, fifth-instar larvae; P, pupae; P_i, pupae between 0 and 2.5 h old; P_{ii}, pupae between 2.5 and 5 h old; P_{iii}, pupae between 5 and 7.5 h old; P_{iv}, pupae at least 7.5 h old. AB fibers, single line; C fibers, double line.

Resting potentials of the muscle fibers

Unexpectedly, C muscle fibers had consistently more negative resting potentials than the immediately adjacent AB fibers (-72.5 ± 12.7 mV versus -57.1 ± 17.7 mV, $P<0.05$, mean±s.D.). The range of values was similar to (Runion and Pipa, 1970; Rheuben and Kammer, 1980), or slightly more negative than (Dawson and Djamgoz, 1984; Djamgoz and Dawson, 1989), those previously reported for other larval lepidopteran muscle fibers.

Over the prepupal and the early pupal period, the amplitudes of the resting potentials of the two muscles declined (Fig. 2). This decline was comparable to that reported for other insects during metamorphosis (Runion and Pipa, 1970) and for both insect and vertebrate muscles after experimental denervation (Clark et al. 1979; Deshpande et al. 1978). The resting potentials of C fibers declined more rapidly than those of AB fibers during the prepupal days; by the pupal ecdysis their resting potentials (-49 ± 13 mV, $N=32$) were less negative than those from AB fibers (-58 ± 11 mV, $N=41$). During the hours after ecdysis the fall continued, with a significant difference being found for both AB and C fibers when comparing day 1 pupae 0–2.5 h after ecdysis to day 1 pupae more than 7.5 h after ecdysis ($P<0.05$ for both muscles). At the end of the period studied, 7.5 h post ecdysis, the average resting potential for AB fibers was -47 ± 11 mV and that for C fibers -41 ± 11 mV.
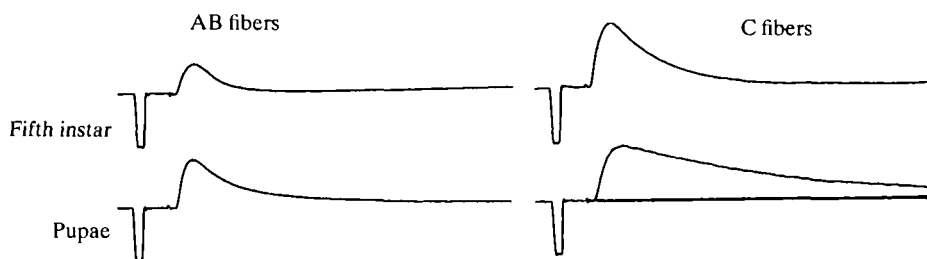
Fig. 3. Excitatory junction potentials from normal fifth-instar and degenerating pupal muscle fibers. The top row illustrates the typically larger amplitudes and slower time courses of EJPs from larval C fibers compared to the EJPs from AB fibers in reduced-Ca^{2+} saline ($1\,mmol\,l^{-1}$). In comparison, the EJPs in the bottom row show the often strikingly prolonged time courses of EJPs from pupal fibers. Pupal fibers were in $0.57\,mmol\,l^{-1}$ (AB) and $0.8\,mmol\,l^{-1}$ (C) calcium. (Baseline is indicated by solid line.) Calibration pulse on each tracing is 10 mV, 10 ms.

Properties of the excitatory junction potential

Larval AB and C excitatory junction potentials

In fifth-instar larvae in normal-calcium ($4\,mmol\,l^{-1}$) saline, a large 20–30 mV EJP typically initiated a spike-like active membrane response in AB and C muscles as was seen in previous studies (Rheuben and Kammer, 1980). In reduced-calcium salines, characteristic differences between the subthreshold EJPs of larval AB and C neuromuscular junctions became apparent (Fig. 3). In $1\,mmol\,l^{-1}$ calcium, the amplitudes of EJPs recorded from fifth-instar AB fibers were significantly smaller (by $10.5\pm3.7\,mV$ for 12 paired samples, $P<0.0005$, Student's one-tailed paired *t*-test) than those simultaneously recorded from C fibers. The average EJP amplitude of fibers with similar equilibration and stimulation histories in $1\,mmol\,l^{-1}$ calcium was $5.41\pm3.34\,mV$, $N=17$, for AB fibers and $16.35\pm5.06\,mV$, $N=22$, for C fibers.

The times to peak and to half-fall of EJPs from AB fibers were significantly shorter than those from C fibers ($P<0.05$, Fig. 3 and Table 1).

Excitatory junction potentials from degenerating pupal AB and C fibers

The EJPs from degenerating prepupal and early pupal muscle fibers were often unexpectedly larger than those from their larval counterparts and required lower calcium concentrations to reduce them below the threshold for an active membrane response and to prevent muscle contraction. The precise calcium concentration required to reduce the EJP to 15 mV or less varied from pupa to pupa, ranging from 0.4 to $1.33\,mmol\,l^{-1}$, typically 0.44–$0.57\,mmol\,l^{-1}$ for pupae 1–6 h old, whereas $1\,mmol\,l^{-1}$ Ca^{2+} reliably reduced EJPs to this level in fifth-instar larvae. In pupae older than 7 h post ecdysis, the EJPs tended to be smaller than in younger day 1 pupae, although calcium concentrations less than $1\,mmol\,l^{-1}$ were usually still required to block action potentials and contraction.

Table 1. *Comparison of the times to peak and to half-fall of fifth-instar and pupal EJPs*

	Fifth instars	Pupae
AB fibers		
Time to peak (ms)	20.2±5.3 (22)	31.0±10.5 (41)
Time to half-fall (ms)	29.8±10.2 (22)	59.4±34.0 (41)
Average EJP amplitude (mV)	6.0±2.8 (22)	9.0±5.5 (41)
C fibers		
Time to peak (ms)	31.3±5.8 (18)	42.5±19.0 (52)
Time to half-fall (ms)	61.6±13.0 (18)	110.0±52.0 (52)
Average EJP amplitude (mV)	17.2±4.8 (18)	7.6±5.6 (52)

EJPs were recorded from fifth-instar fibers in saline containing $1 \, \text{mmol} \, l^{-1}$ calcium and from pupal fibers in saline containing $0.4–1.33 \, \text{mmol} \, l^{-1}$ calcium, and the average EJP amplitudes obtained at these concentrations are shown.
Mean±S.D.; number of fibers sampled are shown in parentheses.

The characteristic difference between the amplitudes of EJPs from AB and C fibers, though still significant, was reduced in the degenerating pupal fibers. In a series of paired recordings from AB and C muscles of pupae, obtained in calcium concentrations ranging from 0.4 to $0.8 \, \text{mmol} \, l^{-1}$, the amplitudes of EJPs from pupal C fibers averaged only $2.7±4.7 \, \text{mV}$ ($N=9$) larger than those simultaneously recorded from AB fibers ($P<0.05$, Student's one-tailed paired t-test).

On average, the time courses of the EJPs from pupal fibers of both muscles were strikingly longer than those from their larval counterparts, with times to half-fall nearly doubled ($P<0.05$, Table 1, Fig. 3). Pupal C fibers continued to have EJPs with longer time courses on average than EJPs from AB fibers ($P<0.05$). Overall, the durations of pupal EJPs were much more variable than those from fifth-instar larvae. The data were grouped without regard for pupal age or external calcium concentration because no obvious correlation emerged when the times to peak or half-fall were plotted against either calcium concentration ($0.4–0.8 \, \text{mmol} \, l^{-1}$) or pupal age.

Amplitude of the EJP during the course of an experiment

Experiments often lasted 2–4 h, with those involving pupae occurring during a period when degenerative changes in the animal were rapid. As described previously (Fig. 2), the resting potentials of fibers sampled from pupae dissected at intervals during the first 12 h after ecdysis were successively lower and lower, with the average of each sample being smaller by about 1 mV per hour post ecdysis. Sustainable neurotransmission could not be obtained reliably 8–12 h after ecdysis.

In order to assess the likelihood that a decline in EJP amplitude observed during an experiment reflected an actual decrease in synaptic function in addition to either the effects of the experiment itself or those of a concomitant decline in muscle fiber resting potential, we examined the stability over time of the EJP
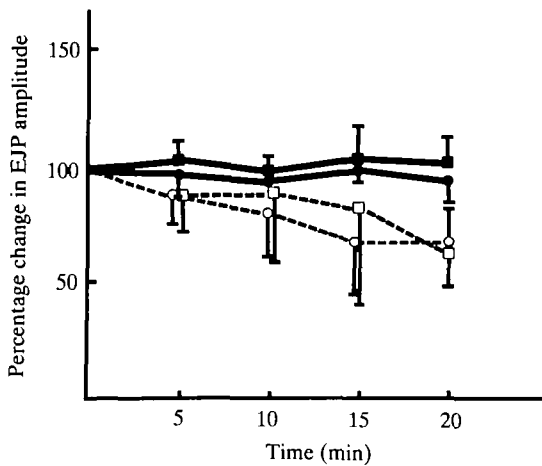
Fig. 4. Decline in EJP amplitude during an experiment. The amplitude of the first EJP of a train of 10 was compared to that of the first EJP of subsequent trains at 5 min intervals with rest in between. Sample size 4–18 for each point; mean±s.d. Solid lines and filled symbols, fifth-instar neuromuscular junctions; interrupted lines and open symbols, pupal neuromuscular junctions. Squares, AB fibers; circles, C fibers.

amplitudes of a small subset of fibers. We selected fibers which had equilibrated for at least 15 min in the low-calcium saline, had resting potentials which changed by less than 20 % over the entire experimental period (1–3 h) and which had not been subjected to prior prolonged high-frequency stimulation. The first EJP in a train of ten was compared with the first one in subsequent trains of ten EJPs at 5 min intervals over a 20 min period. Under these circumstances, larval EJPs had stable amplitudes, but pupal EJPs exhibited a statistically significant decline ($P<0.05$, Friedman's χ^2) of about 1.2–2.6 mV per 15 min (Fig. 4).

In some pupae, the EJPs decreased sharply in amplitude over a few minutes or continued to decline even when the external Ca^{2+} concentration was raised (the rate of decline was not determined for the latter, nor were they included in the above averages or Fig. 4). When data from individual experiments were examined, neither specific pupal age nor calcium concentration within the range utilized appeared to influence the rate of decline.

Although other explanations are possible, particularly given the fragile nature of both pupal nerves and muscle fibers, it seems likely that the decline in EJP amplitudes at least partly represented degenerative changes in the processes of neurotransmission occurring during the period of the experiment.

In summary, the effects associated with degeneration were qualitatively (but not quantitatively) similar for both AB and C fibers and included a decline in resting potential, an increase in apparent amplitude of a single EJP during the early stages and, frequently, a lengthening of EJP time course. There was some suggestion that the pupal neuromuscular junctions were less capable of consistent transmitter release (over 20 min or more) than were the larval ones.
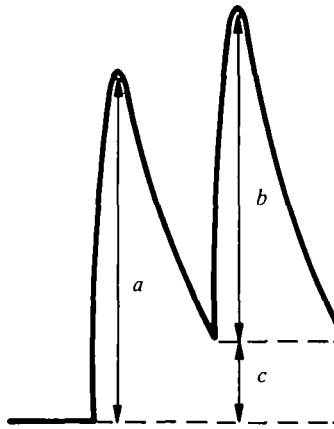
Fig. 5. Method used to measure EJP amplitudes for the calculation of facilitation and depression. a, amplitude of first EJP; b, amplitude of subsequent EJPs; c, amplitude of summation. Facilitation$=(b/a)\times100$.

Short-term facilitation and depression

To assess facilitation and depression, we investigated the responses of normal and degenerating neuromuscular junctions to trains of ten stimuli delivered to the motor nerve, each train preceded by a 5 min rest. The frequencies of stimulation within the trains were 0.4 Hz, 6 Hz and 20 Hz.

Facilitation or depression were reported as the ratio of the size of the second EJP to that of the first multiplied by 100. The population of junctions on a fiber was considered to have shown facilitation if the ratio exceeded 100 % at either the second or the tenth EJP. In cases in which summation was present, the amplitudes of second and subsequent EJPs were measured as indicated in Fig. 5. Because of inaccuracies that would be introduced in measuring some records (cf. Figs 6 and 8), we did not attempt to extrapolate the time course assuming an exponential decay, nor did we have enough information to correct for nonlinear summation (McLachlan and Martin, 1981). The method chosen thus underestimated the amount of facilitation when summation was extensive.

Short-term facilitation and depression at larval AB and C neuromuscular junctions

The normal neuromuscular junctions of larval AB and C fibers responded to trains of stimuli in characteristically different ways, with the AB junctions exhibiting a greater tendency to facilitate (Fig. 6). In 1 mmol l^{-1} Ca^{2+}, the EJPs of normal larval AB fibers gradually increased in amplitude at 6 and 20 Hz, usually throughout the train of ten responses; C fibers usually showed either no detectable increase or a depression of EJP amplitudes. Facilitation was not great in magnitude in either muscle, the maximum increase in response to the second stimulus being about 133 % for AB fibers at 6 Hz. At 0.4 Hz, the amplitudes of
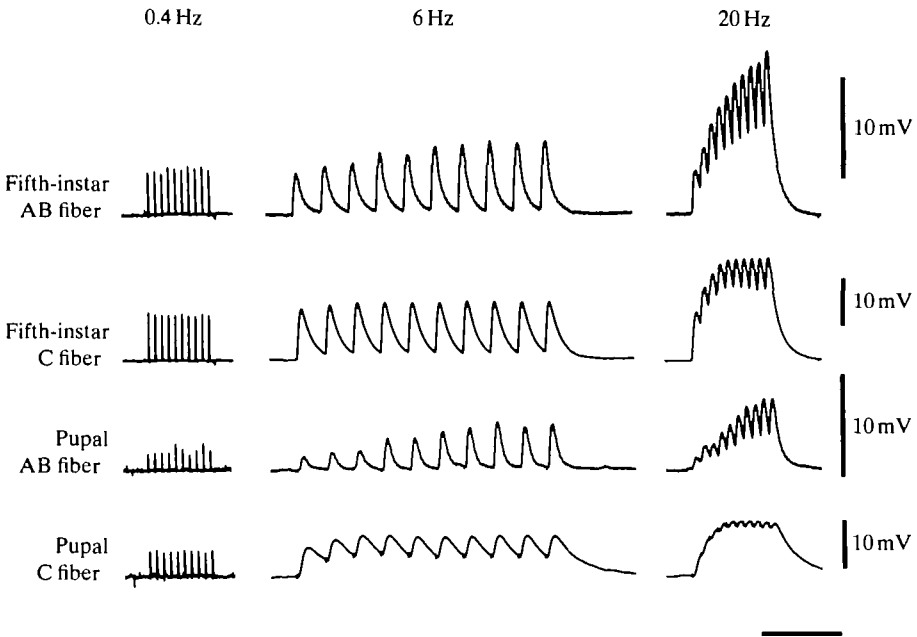
Fig. 6. Comparison of fifth-instar and pupal responses to trains of 10 stimuli at 0.4, 6 and 20 Hz. Trains were recorded simultaneously from the AB and C muscle fibers of a fifth-instar larva in 1 mmol l^{-1} calcium and similarly in another experiment from a 4-h-old pupa in 0.57 mmol l^{-1} calcium. Note that facilitation could often be detected in spite of extensive summation at 20 Hz. Horizontal bar, 30 s for trains at 0.4 Hz, 0.5 s for trains at 6 and 20 Hz.

Table 2. *Proportion of fibers which facilitated and their average EJP amplitudes*

	6 Hz		20 Hz	
Fiber type	No. of fibers	Average EJP of facilitators/ non-facilitators (mV)	No. of fibers	Average EJP of facilitators/ non-facilitators (mV)
Larval AB	9/9	4.1/−	9/9	3.9/−
Larval C	3/10	12.3/15.1	0/10	−/14.5
Pupal AB	9/10	8.3/8.6	4/9	4.0/11.4
Pupal C	7/22	3.0/9.7	7/19	2.4/7.4

EJPs recorded from both AB and C fibers were stable, showing neither marked facilitation nor depression. The results are summarized in Table 2 and Fig. 9.

To control for any variations in degree of equilibration between preparations in the low-calcium saline, we considered a subset of six preparations in which recordings were obtained simultaneously from AB and C fibers. At 6 Hz all AB neuromuscular junctions facilitated while only one out of six of the C fibers

exhibited facilitation, the other five being depressed. At 20 Hz, all the AB fibers and none of the C fibers showed facilitation. In these six experiments the mean EJP amplitude of AB fibers was 4.8 mV and that of C fibers was 13.7 mV. Overall, the results of this subset are similar to that from the entire group.

Short-term facilitation and depression at pupal AB and C neuromuscular junctions

Many pupal AB and C fibers responded to short trains of stimuli with either facilitation or depression of the EJP amplitudes comparable to that seen in the corresponding normal larval fibers (Table 2, Fig. 9). The EJPs recorded from pupal AB and C neuromuscular junctions still had relatively stable amplitudes at 0.4 Hz. At 6 Hz the proportions of AB and C pupal junctions facilitating were approximately the same as for larval junctions.

However, individual pupal fibers displayed more variable responses than those previously seen in fifth-instar larvae, possibly reflecting the wider range of EJP amplitudes, muscle fiber resting potentials and calcium concentrations used (0.4–0.8 mmol l^{-1}), or possibly reflecting variability of the degenerative processes (Figs 6, 7 and 8). In some cases, adjacent fibers in the same muscle exhibited opposite responses, with one facilitating and the other not (Fig. 7). At 20 Hz (but not 6 Hz) fewer pupal AB fibers and more pupal C fibers exhibited facilitation than was typical of the populations of larval fibers studied. The mechanisms underlying this relatively small difference are not clear.

The mean EJP amplitude of the 'anomalous' pupal C fibers facilitating at 20 Hz was smaller than typical, and it was generally true that facilitating fibers tended to have smaller average EJP amplitudes than fibers whose EJPs did not facilitate (Table 2). A significant negative correlation between EJP amplitude and the amount of facilitation at 6 Hz was apparent when data from fifth-instar AB and C fibers were plotted separately as well as for combined pupal AB and C fibers ($P<0.05$, Spearman's two-tailed ranked correlation test). This correlation was also significant at 20 Hz for fifth-instar AB fibers, but fifth-instar C fibers showed such extensive summation that quantitative estimates of changes in release were not realistically possible for most fibers.

Conversely, many of the pupal AB fibers which showed facilitation at 6 Hz did not also do so at 20 Hz, unlike their larval counterparts (Fig. 9), suggesting a greater susceptibility to depression. However, this subpopulation of pupal AB fibers had much larger EJPs than either the group of pupal AB fibers which did facilitate or the larval AB fibers, so that we cannot immediately separate the direct effects of degeneration from those related to the unusually large EJPs seen in some degenerating fibers.

In summary, the two muscles differed characteristically in their likelihood of exhibiting facilitation, but degeneration did not have a very big impact on their respective capabilities to respond to the particular protocol described here. There appeared to be a negative correlation between the amount of facilitation and the amplitude of the EJP. The differences between larval and pupal likelihood of
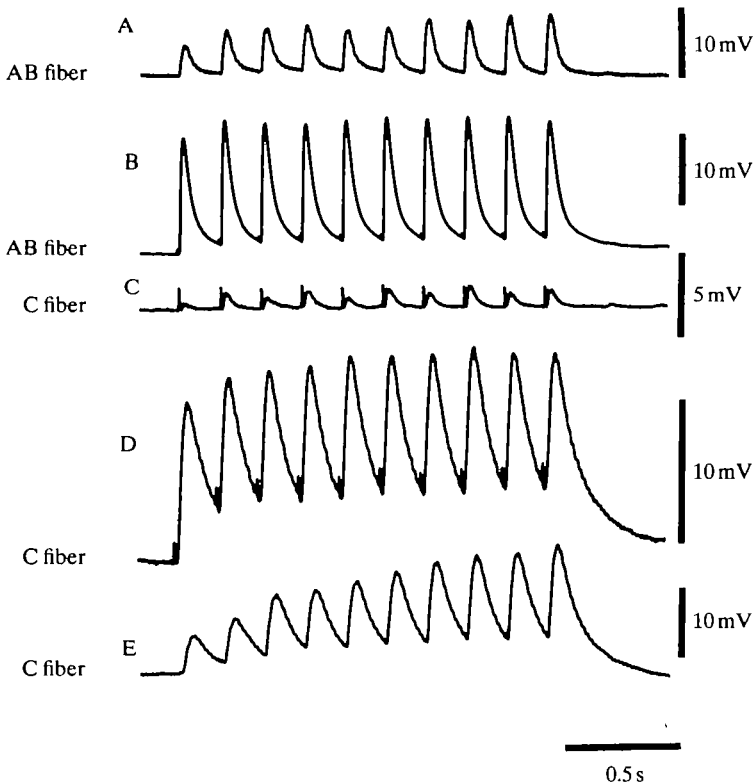
Fig. 7. Variability of pupal responses to trains of stimuli at 6 Hz. (A,B) Simultaneously recorded trains from two fibers in muscle AB. Note the difference in EJP amplitudes and the relative lack of facilitation in B. Pupa no. 36, 1.5 h old, in 0.5 mmol l^{-1} calcium. (C,D) Simultaneously recorded trains from two fibers in muscle C. Pupa no. 28, 3 h old, 0.44 mmol l^{-1} calcium. (E) C fiber which facilitated strongly, unlike the majority of pupal C fibers. Pupa no. 17, 4 h old, 0.57 mmol l^{-1} calcium.

facilitation may therefore have been a consequence of the wider range of EJP amplitudes in pupal fibers.

Responses to prolonged stimulation at high frequency

To investigate whether degeneration affected the ability of nerve terminals to maintain neurotransmitter release during prolonged intense stimulation, we studied the responses of both normal and degenerating neuromuscular junctions to protracted stimulation at 20 Hz. Continuous stimulation at this frequency usually resulted in summation of EJPs so that the muscle membrane was persistently depolarized, although the peaks of individual EJPs could still be distinguished and measured. In addition, the amplitude of a single EJP was recorded after a brief interruption (0.7±0.2 s) of the high-frequency stimulation (Fig. 10A). The interruption was just long enough to avoid any summation with preceding EJPs. The amplitude of this EJP was compared to that of a control EJP measured before the
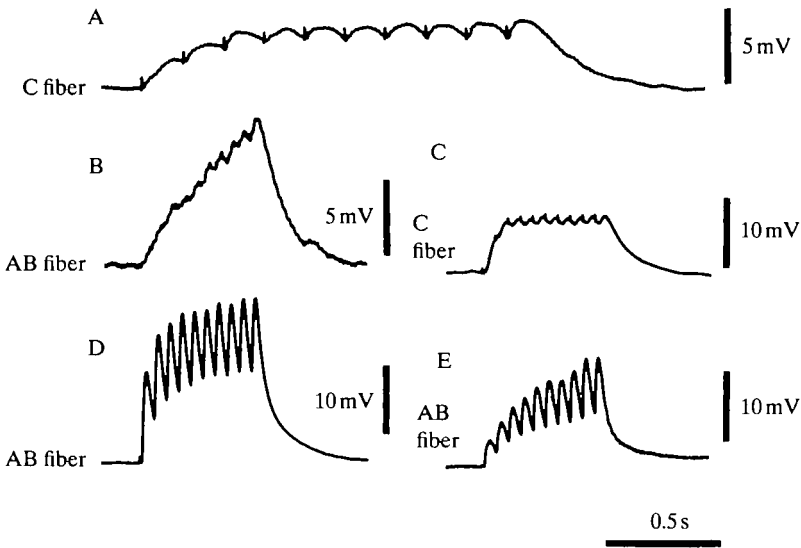
Fig. 8. Summation and facilitation at pupal neuromuscular junctions. (A) Train of EJPs recorded from a C fiber where the very long duration of EJPs resulted in extensive summation even when stimulated at 6 Hz. Pupa no. 22, 2 h old, in 0.44 mmol l^{-1} calcium. (B,C) Simultaneously recorded trains at 20 Hz from an AB (B) and a C (C) fiber. Note that facilitation can still be observed in B, despite summation, but cannot be accurately quantified. Pupa no. 25, 3 h old, in 0.44 mmol l^{-1} calcium. (D) AB fiber showing summation without obvious facilitation at 20 Hz, recorded simultaneously with E. (E) AB fiber showing facilitation at 20 Hz. Pupa no. 36, 1.5 h old, in 0.5 mmol l^{-1} calcium.

start of prolonged high-frequency stimulation. The ratio thus obtained indicated whether EJP amplitudes were increased or decreased during the course of high-frequency stimulation.

Responses of larval AB and C neuromuscular junctions to tetanic stimulation

At normal larval AB neuromuscular junctions, the amplitude of EJPs recorded during 20 Hz stimulation, excluding the steady-state summation, declined somewhat during 20 min of tetanic stimulation (from 6.2±1.5 to 5.2±1.8 mV, $N=9$, $P<0.05$, paired t-test, one-tailed). Depression of EJPs measured in this way was not seen at normal C neuromuscular junctions during tetanic stimulation: many C muscle fibers hyperpolarized by about 5 mV and the amplitude of summated EJPs tended to increase slightly (from 8.2±1.8 to 8.8±1.9 mV, $N=7$, $P<0.05$, paired t-test, one-tailed).

At both AB and C normal larval neuromuscular junctions, the single EJP recorded during each brief interruption of high-frequency stimulation was always significantly larger than the control EJP (Fig. 10B). The increase persisted even after more than 20 min of nearly continuous stimulation. This potentiated response was more marked at AB neuromuscular junctions than at C neuromuscular junctions.
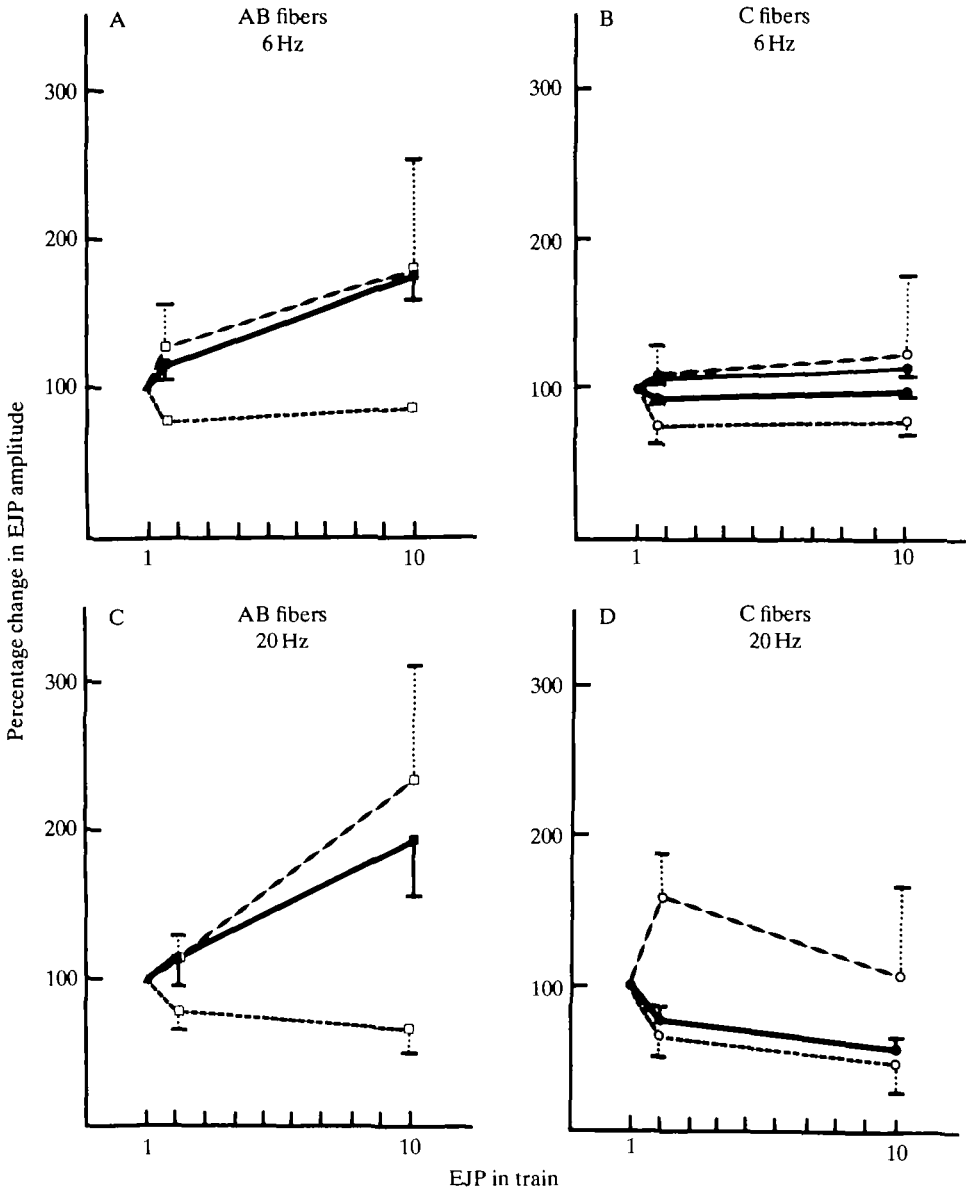
Fig. 9. Facilitation and depression during brief trains of stimuli at 6 and 20 Hz. The change in amplitude was calculated as described in Fig. 5, for the second and tenth EJP in a train of 10 (mean±s.D.). Solid lines, fifth-instar fibers; interrupted lines, pupal fibers. When the response of a class of fibers, such as the pupal fibers, was not uniform, the data from those fibers that exhibited facilitation were averaged and plotted together (long dashes), and the data from those that exhibited depression were averaged and plotted as a second group (short dashes). Only one line (e.g. larval AB fibers) was plotted if all fibers responded alike. At 20 Hz, summation led to an underestimation of the amount of facilitation (particularly in C fibers) and sometimes to generation of an active membrane response so that the amplitude of the tenth EJP could not be measured, thus reducing the number of data points.
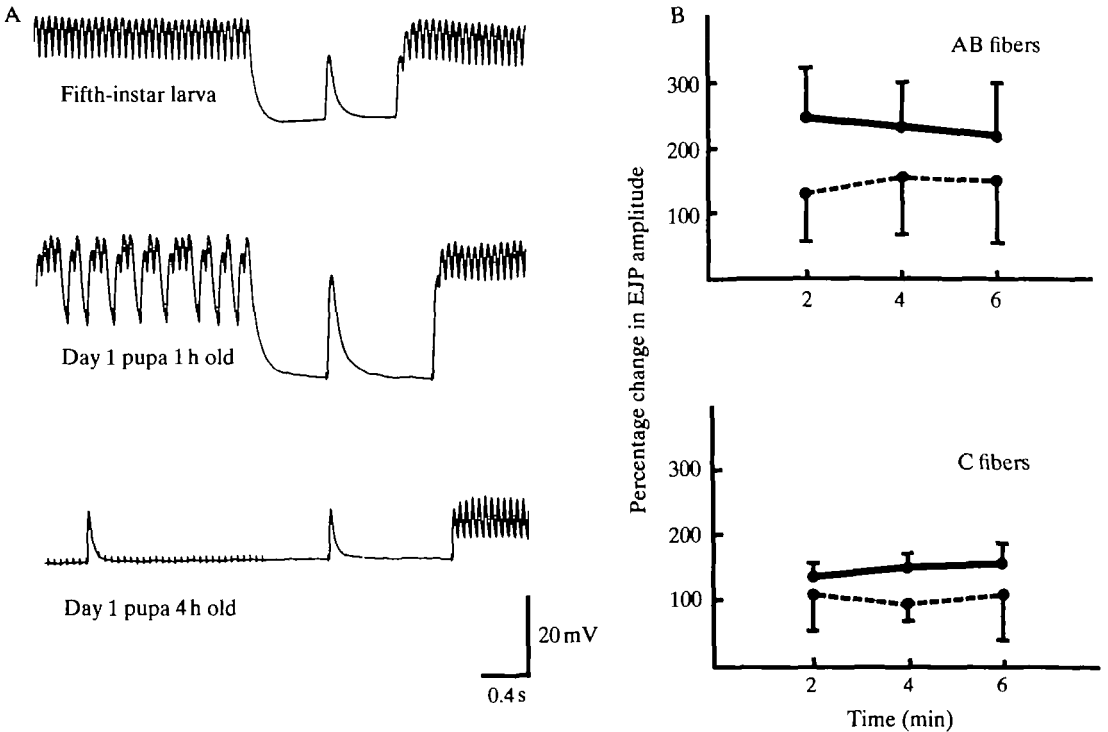
Fig. 10. Responses of normal and degenerating neuromuscular junctions to high-frequency stimulation. The motor nerve was stimulated at 20 Hz. Stimulation was briefly interrupted every 2 min for 0.7±0.2 s, a single stimulus was given, and 20 Hz stimulation then resumed. (A) Intracellular recordings of responses of normal and degenerating neuromuscular junctions from AB fibers to 20 Hz stimulation. Note the conduction blocks shown by the presence of stimulus artifacts with no subsequent EJP and the temporary relief of the conduction block after a rest. The fifth-instar recording is in 1 mmol l^{-1} calcium saline; pupal recordings are in $0.4-0.6 \text{ mmol l}^{-1}$ calcium salines. (B) Mean amplitudes of a single EJP recorded from AB and C fibers during a brief interruption of continuous stimulation at 20 Hz, compared to a control value obtained before the onset of high-frequency stimulation. Sample size 7–17 for each point; mean±s.D. Fifth instars, solid lines; pupae, interrupted lines.

Responses of pupal AB and C neuromuscular junctions to tetanic stimulation

Because of the fragile nature of pupal nerves and muscle fibers, it was rarely possible to record for more than 6 min from a given muscle fiber during prolonged high-frequency stimulation. Intermittent, often cyclical, failure of action potential conduction was common (Fig. 10A), particularly in older pupae, occurring in 78 % of pupae examined.

When the amplitudes of intratetanic EJPs from pupal fibers whose nerves had no intermittent failure of action potential conduction during 20 Hz stimulation were examined, depression seemed to predominate. The summated EJPs of the single pupal AB muscle fiber with no evidence of failure of action potential conduction during prolonged 20 Hz stimulation decreased in amplitude during

tetanus. Only one of the six pupal C muscle fibers with no evidence of action potential conduction failure and from which summated EJPs were measurable showed an increase in EJP amplitude similar to that observed in the corresponding fifth-instar fibers. Pupal C fibers did not hyperpolarize during tetanic stimulation. Results were similar when those fibers with interruptions in conduction during the train were included.

Single EJPs recorded during a brief interruption of high-frequency stimulation of pupal AB fibers still potentiated on average, but not to the same degree as did AB neuromuscular junctions of fifth-instar larvae (Fig. 10B). Pupal C neuromuscular junctions usually facilitated only slightly or were depressed, facilitating less on average than the junctions of the corresponding fifth instars ($P<0.05$, Wilcoxon–Mann–Whitney two-sample test, Fig. 10B). Pupal age, calcium concentration of the experimental solution ($0.4–1.33\,mmol\,l^{-1}$) and the presence of intermittent failure of action potential conduction were not observed to affect the amplitudes of these pupal EJPs when data from each fiber were plotted individually.

Post-tetanic phenomena

To investigate whether the mechanisms responsible for tetanic and post-tetanic phenomena, such as potentiation and long-term facilitation, might also be affected by degeneration, we measured the amplitude of EJPs following 6 or 20 min of stimulation at 20 Hz. Immediately after the tetanus, the motor nerve to the AB and C mesothoracic muscles was stimulated continuously at 0.4 Hz for at least 30 min. This frequency was chosen because EJP amplitudes were typically stable at 0.4 Hz prior to prolonged tetanic stimulation. The amplitudes of the EJPs recorded after tetanic stimulation were compared to the control value obtained before tetanic stimulation.

Events after 20 min of tetanic stimulation at larval AB and C neuromuscular junctions

The first 1–2 EJPs immediately after the end of high-frequency stimulation were strongly facilitated (data not shown), with the amplitudes of the subsequent ones rapidly declining to near or below control values (Fig. 11). After about 200 s, EJP amplitudes gradually increased again and remained elevated by 161 % (AB fibers) or 131 % (C fibers) above the control value for at least 30 min (AB and C fibers, tested separately: $P<0.01$, two-way, one-tailed ANOVA and Dunnett's test for multiple comparisons to a control value). The amplitude of the EJPs was noticeably potentiated for up to 2.5 h after the end of prolonged tetanic stimulation; the maximum duration of this long-term response was not investigated.

Although overall the responses of AB and C normal neuromuscular junctions had the same time course and direction, they differed in degree: at AB neuromuscular junctions EJP amplitudes were proportionately much more
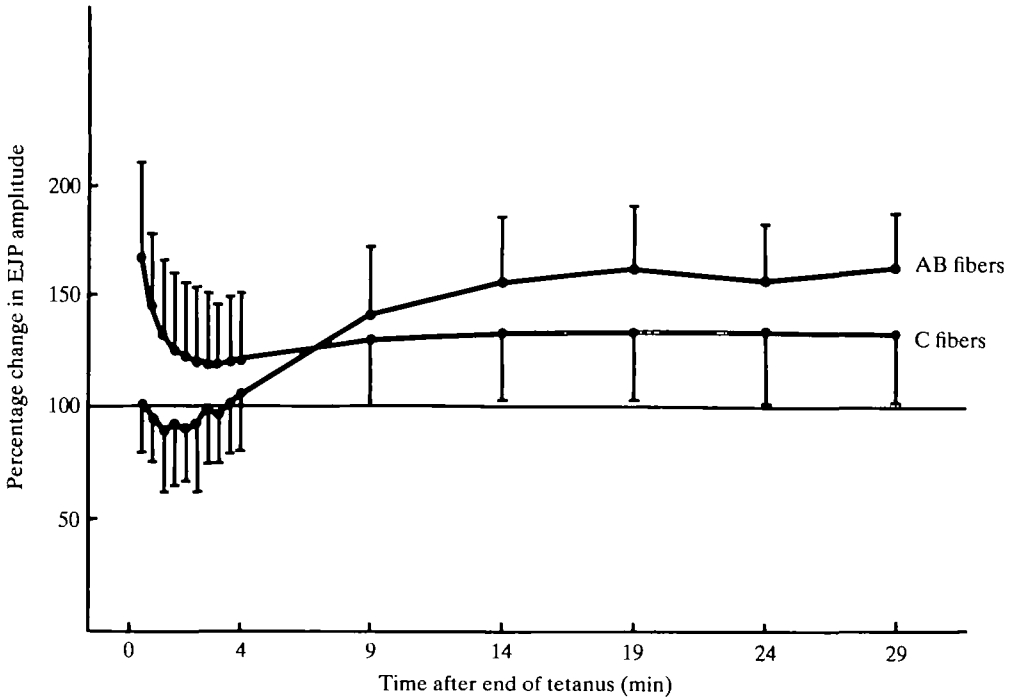
Fig. 11. Long-term changes in EJP amplitude of normal fifth-instar AB and C neuromuscular junctions after 20 min of 20 Hz stimulation. After the end of 20 Hz stimulation (time=0 min), the motor nerve was stimulated at 0.4 Hz for at least 30 min. EJP amplitudes were compared to a control EJP obtained just prior to 20 Hz stimulation (mean±s.D.). AB fibers, single solid line, N=7–9; C fibers; double line, N=9.

depressed during the first few minutes following the end of tetanic stimulation and more elevated thereafter than at C neuromuscular junctions.

Events after 6 min of tetanic stimulation at larval AB and C neuromuscular junctions

For comparison with the degenerating pupal fibers, we also examined the responses of fifth-instar larvae to 6 min of tetanic stimulation, which was the average time we could successfully stimulate and record from the fragile pupal fibers at 20 Hz. The time course of the changes in amplitude of the test EJPs of fifth-instar AB neuromuscular junctions after 6 min of tetanic stimulation was similar to the one observed after 20 min of 20 Hz stimulation. At normal fifth-instar AB neuromuscular junctions a significant increase in EJP amplitudes compared to control values was present from 4 to 29 min after the end of high-frequency stimulation ($P<0.05$, two-way one-tailed ANOVA and Dunnett's test for multiple comparisons to a control value; Fig. 12A) and was of the same magnitude as that seen after 20 min of 20 Hz stimulation ($P<0.05$, mixed-design
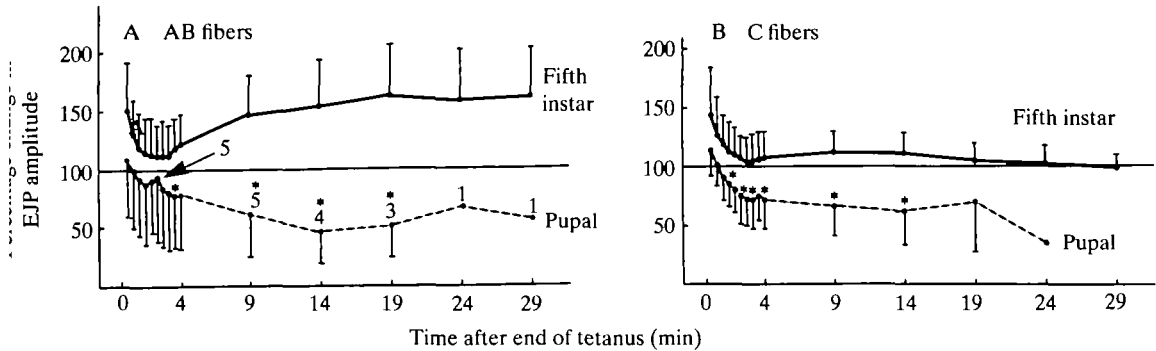
Fig. 12. Long-term changes in EJP amplitudes from normal and degenerating neuro-muscular junctions after 6 min of 20 Hz stimulation. After the end of 20 Hz stimulation (time=0 min), the motor nerve was stimulated at 0.4 Hz for at least 30 min. EJP amplitudes were compared to a control EJP obtained just prior to 20 Hz stimulation (mean±S.D.). Sample size 6–8 fibers unless otherwise indicated. * indicates a statistically significant difference between the degrees of change observed at pupal and fifth-instar neuromuscular junctions ($P<0.05$, Fisher's rank sum test). Solid lines, fifth-instar fibers; interrupted lines, pupal fibers. (A) AB fibers; (B) C fibers.

ANOVA). Depression immediately after the end of 6 min of high-frequency stimulation was much less marked than after 20 min of 20 Hz stimulation.

At normal fifth-instar C neuromuscular junctions, the enhancement of EJP amplitudes after 6 min of tetanic stimulation was much less marked and less durable than after 20 min of 20 Hz stimulation. The amplitudes of EJPs were only significantly greater than control values 9 and 14 min after the end of high-frequency stimulation ($P<0.05$, Fig. 12B).

Events at pupal AB and C neuromuscular junctions after 6 min of tetanic stimulation

In contrast to larval neuromuscular junctions, after 6 min of 20 Hz stimulation pupal neuromuscular junctions exhibited on average no long-term increase in EJP amplitudes (Fig. 12). The ratio of test EJP amplitude to control EJP amplitude was instead much smaller in pupae than in corresponding fifth instars during the 30 min after tetanic stimulation, although statistical analysis was not always possible owing to the small number of pupal fibers successfully examined (AB fibers: $P<0.05$ for 9, 14 and 19 min; C fibers, $P<0.05$ for 4, 9 and 14 min after the end of tetanic stimulation, Fisher's rank sum test).

The presence of intermittent failures of action potential conduction, quite common during tetanic stimulation in pupae, would be expected to reduce the long-term increase of EJP amplitudes if the phenomenon observed at *Manduca* neuromuscular junctions resembled long-term facilitation in other invertebrates in its dependence on a minimum number of stimuli for its expression (Atwood and

Wojtowicz, 1986). However, no long-term increment of EJP amplitudes was present in the four pupal C muscle fibers with no interruption of action potential conduction during tetanic stimulation; one of these had received 20 min of high-frequency stimulation, which in fifth-instar C muscle fibers usually led to the development of long-term facilitation. We observed no difference between the post-tetanic responses of neuromuscular junctions without intermittent failures of action potential conduction and those with some or many such interruptions during 20 Hz stimulation and therefore grouped the data for presentation in Fig. 12.

Discussion
Characteristic differences between AB and C muscles

In the course of this study, characteristic differences between AB and C muscle fibers and neuromuscular junctions emerged. These differences were most marked in normal fifth-instar fibers, and persisted, often in an attenuated form, in the degenerating muscles of the pupae. In fifth-instar larvae, C muscle fibers had more negative resting potentials by about 15 mV, and their subthreshold EJPs were larger in amplitude and longer in time course than those from AB fibers. When short trains of stimuli were used, the smaller EJPs from AB fibers tended to facilitate in $1 \, \text{mmol} \, \text{l}^{-1} \, Ca^{2+}$, while those from C fibers did not. After prolonged tetani, the neuromuscular junctions of both AB and C muscle fibers exhibited a complicated combination of potentiation and depression, followed by long-term facilitation lasting many minutes; the C neuromuscular junctions showed less depression and less long-term facilitation.

The specific mechanisms underlying these characteristic differences between the fibers of the two muscles and the populations of neuromuscular junctions formed by the motor neurons innervating them are largely undefined. The differences in muscle fiber resting potentials may well relate to the metabolic processes which are thought to underlie up to 50 % of muscle membrane potentials in Lepidoptera (Rheuben, 1972; Djamgoz, 1986). Indirect evidence to support this possibility is provided both by the observation that the difference disappears in pupal fibers, as will be discussed later, and because the difference is not observed in a saline lacking nutrients and HCO_3^- (I. M. Sonea, M. B. Rheuben and C. Young, in preparation). The negative correlation between EJP amplitude and the amount of facilitation and the differing responses to tetanic stimulation suggest that there are intrinsic differences in release properties between the motor neurons innervating the AB and C muscles. We have at this point no insight into any contributions that may arise from postsynaptic receptor properties, nor can we comment upon the functional implications of these differences, which are observable at subthreshold levels of calcium. Most of the characteristic features associated with the AB and C muscles continue to be observable into pupal degeneration but, strikingly, in some cases there are significant quantitative differences.

Degenerative changes

Resting potentials

We observed a decrease in amplitude (depolarization) of muscle fiber resting potentials starting before ecdysis to the pupal stage and accelerating thereafter. Similar changes have been reported in muscles destined to disappear after pupal ecdysis in *Galleria mellonella* (Runion and Pipa, 1970) or after adult eclosion in *Manduca sexta* (Lockshin, 1973). The decline in resting potential may have been partly due to the withdrawal of tracheoles from their association with the muscle fibers (Rheuben, 1992*b*). A major fraction of the muscle fiber resting potential in Lepidoptera is dependent on oxidative metabolism (Huddart and Wood, 1966; Rheuben, 1972; Djamgoz, 1986), and good oxygenation *via* the tracheoles is now known to be essential for the maintenance of normal resting potentials in some larval (Yamaoka and Ikeda, 1988) and adult insects (Rheuben, 1972; Djamgoz, 1986). In addition to suffering from decreased availability of oxygen for cellular processes, metamorphosing muscles have been shown to exhibit several changes suggestive of impaired metabolic functions, including changes in calcium handling and a decline in the activity of lactic dehydrogenase respiratory enzymes (Lockshin, 1985; Bidlack and Lockshin, 1976; Beaulaton, 1986). The combination of these factors might easily lead to the decline in the amplitude of membrane potentials dependent upon active processes.

In addition to the direct effects of metamorphosis on muscle cell metabolism, it is also possible that the gradual loss of functional neuromuscular junctions may have helped to trigger the decline in muscle fiber resting potentials, since the resting potentials of normal locust muscle fibers decrease after denervation (Clark *et al.* 1979).

Amplitude and duration of the EJP

Early pupal EJPs initially recorded from a preparation were both larger and longer on average than those recorded from normal fifth-instar fibers. The degenerative structural changes in the nerve terminal occurring at this time (Rheuben, 1992*b*) had led us to expect, if anything, deficits in transmission at the neuromuscular junction. Several factors may have contributed to this anomalous finding.

The passive electrical properties of degenerating muscle fibers probably differed from those of normal larval fibers, given their decreased cross-sectional areas and increased surface membrane. In the ventral abdominal intersegmental muscles of *Manduca* and *Antheraea* during degeneration after adult eclosion, Lockshin (1973) found an increase in input resistance that began about 3 h after eclosion and tripled over the next 17 h. He attributed this, in part, to muscle fiber shrivelling. Calculations using measurements of the dimensions of muscles AB and C in larvae and in pupae, and assuming membrane properties to remain constant, predicted an increase in input resistance from 1.25 to 2 times the larval values (Rheuben, 1992*a*). Such an increase in input resistance would give rise to a larger

intracellularly recorded EJP if no other factors changed at the same time. This effect could help to offset the fact that significant parts of each of the junctions formed on any give fiber appeared to be non-functional, and synaptic currents were probably decreasing.

The EJPs recorded from degenerating pupal muscle fibers had, in many cases, strikingly prolonged time courses. Several factors might reasonably have been involved, including the membrane properties and morphological features of the muscle that are involved in determining its cable properties. In addition, however, the morphological results suggested that the time course of the synaptic current itself might also be prolonged by the withdrawal of glial cells, resulting in decreased uptake of glutamate from around the synaptic cleft, and by the increased areas occupied by postsynaptic receptors, giving more opportunity for repetitive binding of transmitter. The possible impacts of the structural changes in both the muscle fiber and the neuromuscular junction as a whole on synaptic transmission and the electrical properties of the junction are more fully explored elsewhere (Rheuben, 1992b; M. B. Rheuben and S. M. Baer, in preparation).

Decline in EJP amplitudes during the course of an experiment

Even in pupal fibers selected for stable resting potentials, EJPs declined on average to approximately 65 % of their initial amplitudes over a 20 min period even with a modest stimulation regime (Fig. 4). If the decline of metabolic functions thought to accompany degeneration of the muscle fiber, as cited above (Lockshin, 1985; Bidlack and Lockshin, 1976), extends to nerve terminals, even the regions of the pupal nerve terminals which appeared to have essentially normal ultrastructural components (Rheuben, 1992b) may have had deficiencies of synthesis, storage and uptake of neurotransmitter and smaller reserves of neurotransmitter than normal nerve terminals. Such deficiencies may have led to diminished release capabilities during repetitive stimulation.

In addition, normal nerve terminal function may have been further impaired by the withdrawal of glia from the neuromuscular junction, which probably adversely affected the transfer of nutrients, neurotransmitter precursors and other trophic substances from glia to nerve terminals, a glial function well documented elsewhere in the insect nervous system (Treherne, 1960; Wigglesworth, 1960; Lane and Treherne, 1980; Tsacopoulos *et al.* 1987). The inhibition of glial metabolism or glutamine synthesis in rat brains led to a decrease (though slower than that observed here) in the amount of glutamate released during K^+-induced depolariz-ation (Paulsen and Fonnum, 1989). Although data from insect tissue are not available, it is likely that impairment of glial function at degenerating *Manduca* neuromuscular junctions would have a similar deleterious effect on neurotransmit-ter storage and release.

Responses to short trains of stimuli

Degeneration did not significantly modify the characteristic responses of functional pupal neuromuscular junctions to short trains of stimuli. On average,

pupal neuromuscular junctions tended to facilitate (ABs) or depress (Cs) in the same manner that the corresponding fifth-instar neuromuscular junctions had, although the responses were more variable than those of fifth-instar fibers. Short-term presynaptic facilitation would be expected to be less affected by a decline of metabolic functions associated with metamorphosis than long-term facilitation since it is thought to depend on the transient accumulation of Ca^{2+} near individual active zones following a nerve impulse (Katz and Miledi, 1968; Simon and Llinás, 1985).

In contrast, facilitation might be expected to be decreased if the precise geometry of the Ca^{2+} channels within the active zone was disarrayed. In degenerating junctions, the structure of some active zones was disrupted or disorganized, as indicated by the particle arrays (Rheuben, 1992b), and this might well accompany or reflect a concurrent disruption of the calcium channels. The correlation between EJP amplitude and the amount of facilitation as well as a degeneration-associated variability of EJP amplitudes precluded our investigating this in the populations of junctions included in the study of intracellularly recorded EJPs. Furthermore, we cannot at this time indicate what fraction of the disrupted active zones was participating in transmitter release, and therefore 'visible' to the electrical recording.

Responses during prolonged tetanic stimulation

Summated EJPs recorded during tetanic stimulation from normal fifth-instar AB fibers gradually declined in amplitude, although only slightly compared to those of C fibers. Since this is the opposite of the characteristic response of AB and C junctions to short-term facilitation, it suggests the presence of a separate and distinct depressive process. EJPs recorded during brief interruptions of tetanic stimulation were always strongly facilitated at normal fifth-instar neuromuscular junctions of both muscles, but more so for AB than for C fibers. This increase in EJP amplitude which predominated during tetanic stimulation may have been due to increased intraterminal free Ca^{2+} and Na^+ concentrations, as reported for other preparations under similar circumstances (Rahamimoff *et al.* 1980; Parnas *et al.* 1982; Wojtowicz and Atwood, 1985; Misler *et al.* 1987).

On average, the amplitudes of EJPs from degenerating pupal fibers of both muscles exhibited less potentiation and appeared more affected by depression during tetanic stimulation or during brief interruptions of it than those from normal neuromuscular junctions. Several causes can be suggested. It is possible that less total Ca^{2+} and Na^+ entered the degenerating nerve terminals. Structurally normal active zones were found side by side on the same terminal with ones whose particle arrays were dispersed (Rheuben, 1992b). If the abnormal active zones admitted less Ca^{2+}, the overall Ca^{2+} concentration rise in the terminal during a long tetanus would be lower, leading one to expect less potentiation (Delaney *et al.* 1989) of release from the remaining functional active zones. The slightly lower calcium concentrations necessary to record from pupal fibers in

comparison with that needed for fifth-instar larvae would further add to that effect.

As in the case of shorter stimulus protocols, neurotransmitter stores may have been depleted during long tetani by impaired uptake, synthesis and storage of neurotransmitter, or reduced by the decreased ability of glial cells to furnish neurotransmitter precursors and other substrates to nerve terminals in degenerating neuromuscular junctions. All of these factors would lead to a greater emphasis of depressive processes in degenerating terminals. Conduction failures in distal motor nerve branches may also have contributed to reduce EJP amplitudes during high-frequency stimulation, as they do at some crayfish neuromuscular junctions (Hatt and Smith, 1976).

Responses after tetanic stimulation

The nomenclature used to describe the events occurring after the end of a prolonged tetanus is at times confusing. The 10- to 20-min period immediately after the end of tetanus, during which phenomena such as augmentation and then potentiation may predominate (Magleby, 1973; Magleby and Zengel, 1975), has been called the 'tetanic phase' of long-term facilitation in crustaceans (Atwood and Wojtowicz, 1986). After this period, some neuromuscular junctions exhibit a prolonged increase in EJP amplitude, termed long-term facilitation, lasting tens to hundreds of minutes (Sherman and Atwood, 1971). In crustaceans, facilitation during this latter period has also been called the 'long-lasting phase' of long-term facilitation (Atwood and Wojtowicz, 1986). In our preparation, we have arbitrarily designated the period up to 14 min following prolonged tetanus as the 'tetanic' period, and considered phenomena observed at 14 min and later as pertaining to the 'long-lasting' period.

The mechanisms underlying the long-lasting phase of long-term facilitation have not been elucidated, but are thought to involve the generation of a second messenger and phosphorylation of certain proteins, leading to a larger quantal content through an increased probability of release and/or recruitment of previously inactive release sites (Wojtowicz and Atwood, 1986, 1988; Atwood *et al.* 1989; Wojtowicz *et al.* 1989; also see Atwood and Wojtowicz, 1986, for a review).

Normal larval neuromuscular junctions were capable of long-term facilitation after 6 min or more of tetanic stimulation. The 'tetanic' period presumably included post-tetanic potentiation partially or completely overlapped by concomitant depression, the latter accounting for the rapid but transient decline in EJP amplitudes from strongly potentiated levels immediately after the tetanus to near control levels 2-4 min later. The phenomena underlying the long-lasting phase may have begun during the tetanus itself; their initiation is presumably obscured by the preceding two processes of potentiation and depression. The time course of the long-lasting facilitation could extend beyond 30 min, depending upon the duration of the tetanus. Its amplitude was greater in AB than in C fibers.

In degenerating neuromuscular junctions, the EJP amplitudes immediately

following the tetanus were potentiated, but much less so than those from normal muscle. Furthermore, degenerating neuromuscular junctions showed little or no evidence of long-term facilitation, with depression appearing to predominate, as it had during tetanic stimulation.

Several mechanisms could explain the absence of the long-lasting phase of facilitation of the EJPs generated by degenerating pupal muscle fibers. Their nerve terminals may have lacked the metabolic pathways or substrates needed to establish the long-lasting phase of long-term facilitation because of the probable decline of metabolic function associated with metamorphosis. Recruitment of inactive release sites may have been reduced or absent at degenerating pupal neuromuscular junctions, either because all functional release sites were already activated, or because of the lack of second messengers and substrates essential for recruitment. These mechanisms are thought to contribute to the production of long-term facilitation in other preparations (for a review, see Atwood and Wojtowicz, 1986).

It is also possible that pupal neuromuscular junctions were still capable of long-term facilitation but that partial or complete failures of action potential conduction reduced the number of stimuli reaching each release site to below the threshold required for the development of the long-lasting phase of this phenomenon (in crayfish, between 10 and 30 min of 5–20 Hz stimulation are required; Atwood and Wojtowicz, 1986). However, the four pupal C fibers with no obvious failures of action potential conduction during tetanic stimulation did not exhibit a greater post-tetanic increase in EJP amplitude than those with many such failures, so that it is most likely that pupal neuromuscular junctions lacked the ability to produce long-term facilitation.

Implications for neuromuscular function during metamorphosis

The functional changes accompanying degeneration probably had important implications for the motor function of larvae during the prepupal period and the larval to pupal transformation. The normal larval motor pattern consists of single EJPs or long (1 s) bursts of impulses, leading to prolonged, slow contractions (Kammer and Rheuben, 1976). The postsynaptic responses would therefore resemble those recorded during short or long trains of stimuli.

During the early prepupal period, when muscles are already beginning to show signs of degeneration, larvae become very mobile and burrow prior to pupation. After burrowing, prepupae remain quiescent until ecdysis, when the whole body contracts rhythmically to shed the larval cuticle (Weeks and Truman, 1984) at a period when degenerative changes are already advanced (Rheuben, 1992a,b). Some of the physiological changes in neuromuscular function which accompany metamorphic degeneration, particularly the larger and longer EJPs, may serve to enhance neurotransmission during the critical period of ecdysis when muscle fiber resting potentials are declining and fewer functional neuromuscular junctions remain. As we have shown, the degenerating post-ecdysial pupal neuromuscular junctions are still capable of responding to trains of stimuli similar to the ones that

would occur during shedding of the larval cuticle. Since this event is relatively brief and is followed by relative quiescence in the mesothoracic muscles, the increased tendencies of the pupal neuromuscular junctions to be susceptible to depression after prolonged stimulation might have little effect at the behavioral level.

In conclusion, the structural and physiological changes accompanying metamorphosis are complex, so that determining the contribution of individual structures to neuromuscular function is not easy. However, this study of normal and degenerating neuromuscular junctions allowed us to gain some interesting insights into neuromuscular function.

The mechanisms responsible for simple neurotransmitter release seemed unimpaired or may even have been enhanced in the functional population of release sites. Single EJPs were produced normally or may even have had a greater than normal safety factor since lower calcium concentrations were required to reduce EJPs below threshold. This was surprising in view of the morphological evidence that nearly half of the active zones appeared on structural grounds to be incapable of functioning (Rheuben, 1992b). Presumably, increases in the input resistance of the muscle fibers contributed to this effect, but might not account for it entirely. Short-term facilitation was only slightly affected during the first 12 h after ecdysis. It therefore seems likely that simple neurotransmitter release at those active zones that remained functional was relatively independent of the more generalized decline in the metabolic processes presumed to occur in nerve terminals.

In contrast, physiological processes thought to be very dependent on metabolic functions, such as neurotransmitter synthesis and storage, and on the unknown mechanisms responsible for long-term facilitation appeared to be more impaired during metamorphosis even in those sites that continued to release transmitter. Degenerating nerve terminals could not sustain a stable level of neurotransmitter release during long periods of low-frequency stimulation, nor did they exhibit potentiation and facilitation during and after tetanic stimulation to the degree that normal nerve terminals did.

During degeneration there were significant changes in the non-neuronal cells associated with the neuromuscular junction. Metamorphosis led to structural and functional changes in the glia and muscle cells which, in turn, probably affected impulse conduction, neurotransmitter reserves in nerve terminals and the durations and amplitudes of intracellularly recorded EJPs through diminished uptake of transmitter. Further studies of neuromuscular function during degeneration should include an examination of the contributions of the glial components of the neuromuscular junction to neurotransmission as well as the structural and physiological characteristics of the nerve terminals themselves.

References

ATWOOD, H. L., DIXON, D. AND WOJTOWICZ, J. M. (1989). Rapid introduction of long-lasting synaptic changes at crustacean neuromuscular junctions. *J. Neurobiol.* **20**, 373–385.

ATWOOD, H. L. AND WOJTOWICZ, J. M. (1986). Short-term and long-term plasticity and physiological differentiation of crustacean motor synapses. *Int. Rev. Neurobiol.* **28**, 275–362.

BEAULATON, J. (1986). Programmed cell death. Cytochemical evidence for accumulation of calcium in mitochondria and its translocation into lysosomes: X-ray microanalysis in metamorphosing insect muscles. *Histochem. J.* **18**, 527–536.

BETZ, W. J. (1970). Depression of transmitter release at the neuromuscular junction of the frog. *J. Physiol., Lond.* **206**, 629–644.

BIDLACK, J. M. AND LOCKSHIN, R. A. (1976). Evolution of LDH isozymes during programmed cell death. *Comp. Biochem. Physiol.* **55B**, 161–166.

BIRKS, R., KATZ, B. AND MILEDI, R. (1960). Physiological and structural changes at the amphibian myoneural junction, in the course of nerve degeneration. *J. Physiol., Lond.* **150**, 145–168.

CASADAY, G. B. AND CAMHI, J. M. (1976). Metamorphosis of flight motor neurons in the moth *Manduca sexta. J. comp. Physiol.* **112**, 143–158.

CLARK, R. B., GRATION, K. A. F. AND USHERWOOD, P. N. R. (1979). Desensitization of glutamate receptors in innervated and denervated locust muscle fibers. *J. Physiol., Lond.* **290**, 551–568.

DAWSON, C. J. AND DJAMGOZ, M. B. A. (1984). Intracellular potassium activities of the muscle cells of a Lepidopteran larva. *J. Physiol., Lond.* **351**, 36P.

DELANEY, K. R., ZUCKER, R. S. AND TANK, D. W. (1989). Calcium in motor nerve terminals associated with post-tetanic potentiation. *J. Neurosci.* **9**, 3558–3567.

DESHPANDE, S. S., ALBUQUERQUE, E. X., KAUFFMAN, F. C. AND GUTH, L. (1978). Physiological, biochemical and histological changes in skeletal muscle, neuromuscular junction and spinal cord of rats rendered paraplegic by subarachnoidal administration of 6-aminonicotinamide. *Brain Res.* **140**, 89–109.

DJAMGOZ, M. B. A. (1986). Electrophysiological aspects of metabolic pumping in insect muscle. *Comp. Biochem. Physiol.* **84A**, 207–215.

DJAMGOZ, M. B. A. AND DAWSON, J. (1989). Ion-sensitive micro-electrode measurements of intracellular K^+, Na^+ and Cl^- activities in lepidopteran skeletal muscle. *J. Insect Physiol.* **35**, 165–173.

ENGEL, A. G., FUKUOKA, T., LANG, B., NEWSON-DAVIS, J., VINCENT, A. AND WRAY, D. (1987). Lambert–Eaton myasthenic syndrome IgG: Early morphologic effects and immunolocalization at the motor endplate. *Ann. N. Y. Acad. Sci.* **505**, 333–345.

FUKUOKA, T., ENGEL, A. G., LANG, B., NEWSOM-DAVIS, J., PRIOR, C. AND WRAY, D. W. (1987). Lambert–Eaton myasthenic syndrome: I. Early morphological effects of IgG on the presynaptic membrane active zones. *Ann. Neurol.* **22**, 193–199.

FURUKAWA, T. AND MATSUURA, S. (1978). Adaptive rundown of excitatory post-synaptic potentials at synapses between haircells and eighth nerve fibers in the goldfish. *J. Physiol., Lond.* **276**, 193–209.

GLAVINOVIC, M. I. (1987). Synaptic depression in frog neuromuscular junction. *J. Neurophysiol.* **58**, 230–246.

HATT, H. AND SMITH, D. O. (1976). Synaptic depression related to presynaptic axon conduction block. *J. Physiol., Lond.* **259**, 367–393.

HEINERTZ, R. (1976). Untersuchungen am thorakalen Nervensystem von *Antheraea polyphemus* Cr. (Lepidoptera) unter besonderer Berucksichtigung der Metamorphose. *Rev. suisse Zool.* **83**, 215–242.

HODGKISS, J. P. AND USHERWOOD, P. N. R. (1978). Transmitter release from normal and degenerating locust motor nerve terminals. *J. Physiol., Lond.* **285**, 113–128.

HUDDART, H. AND WOOD, D. W. (1966). The effect of DNP on the resting potential and ionic content of some insect skeletal muscle fibres. *Comp. Biochem Physiol.* **18**, 681–688.

KAMMER, A. E. AND RHEUBEN, M. B. (1976). Metamorphosis of the motor system during the development of moths. In *Neural Control of Locomotion* (ed. R. M. Herman, S. Grillner, P. S. G. Stein and D. G. Stuart), pp. 775–779. New York: Plenum Pub. Corp.

KATZ, B. AND MILEDI, R. (1968). The role of calcium in neuromuscular facilitation. *J. Physiol., Lond.* **195**, 481–492.

KO, C.-P. (1981). Electrophysiological and freeze-fracture studies of changes following denervation at frog neuromuscular junctions. *J. Physiol., Lond.* **321**, 627–639.

KO, C.-P. (1985). Formation of the active zone at developing neuromuscular junctions in larval and adult bullfrogs. *J. Neurocytol.* **14**, 487–512.

KO, C.-P. AND PROPST, J. W. (1986). Absence of sterol-specific complexes at active zones at degenerating and regenerating frog neuromuscular junctions. *J. Neurocytol.* **15**, 231–240.

LANE, N. J. AND TREHERNE, J. (1980). Functional organization of arthropod neuroglia. In *Insect Biology in the Future 'VBW 80'* (ed. M. Locke and D. S. Smith), pp. 765–795. New York: Academic Press.

LOCKSHIN, R. A. (1973). Degeneration of insect intersegmental muscles: electrophysiological studies of populations of fibres. *J. Insect Physiol.* **19**, 2359–2372.

LOCKSHIN, R. A. (1985). Programmed cell death. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 2 (ed. G. A. Kerkut and L. I. Gilbert), pp. 301–317. Oxford: Pergamon Press.

LYONET, P. (1762). *Traité Anatomique de la Chenille qui Ronge le Bois de Saule.* Amsterdam: LaHaye.

MAGLEBY, K. L. (1973). The effect of tetanic and post-tetanic potentiation on facilitation of transmitter release at the frog neuromuscular junction. *J. Physiol., Lond.* **234**, 353–371.

MAGLEBY, K. L. AND ZENGEL, J. E. (1975). A dual effect of repetitive stimulation on post-tetanic potentiation of transmitter release at the frog neuromuscular junction. *J. Physiol., Lond.* **245**, 163–182.

McLACHLAN, E. M. AND MARTIN, A. R. (1981). Non-linear summation of end-plate potentials in the frog and mouse. *J. Physiol., Lond.* **311**, 307–324.

MISLER, S., FALKE, L. AND MARTIN, S. (1987). Cationic dependence of post-tetanic potentiation of neuromuscular transmission. *Am. J. Physiol.* **252**, C55-C62.

NÜESCH, H. AND STOCKER, R. F. (1975). Ultrastructural studies on neuromuscular contacts and the formation of junctions in the flight muscle of *Antheraea polyphemus* (Lep.). II. Changes after motor nerve section. *Cell Tissue Res.* **164**, 331–355.

PARNAS, I., PARNAS, H. AND DUDEL, J. (1982). Neurotransmitter release and its facilitation in crayfish. II. Duration of facilitation and removal processes of calcium from the terminal. *Pflügers Arch.* **393**, 232–236.

PAULSEN, R. E. AND FONNUM, F. (1989). Role of glial cells for the basal and Ca^{2+}-dependent K^+-evoked release of transmitter amino acids investigated by microdialysis. *J. Neurochem.* **52**, 1823–1829.

PURVES, D. (1975). Functional and structural changes in mammalian sympathetic neurons following interruption of their axons. *J. Physiol., Lond.* **252**, 429–463.

RAHAMIMOFF, R., LEV-TOV, A. AND MEIRI, H. (1980). Primary and secondary regulation of quantal transmitter release: calcium and sodium. *J. exp. Biol.* **89**, 5–18.

RHEUBEN, M. B. (1972). The resting potential of moth muscle fiber. *J. Physiol., Lond.* **225**, 529–554.

RHEUBEN, M. B. (1992a). Degenerative changes in the muscle fibers of *Manduca sexta* during metamorphosis. *J. exp. Biol.* **167**, 91–117.

RHEUBEN, M. B. (1992b). Degenerative changes in the structure of neuromuscular junctions of *Manduca sexta* during metamorphosis. *J. exp. Biol.* **167**, 119–154.

RHEUBEN, M. B. AND KAMMER, A. E. (1980). Comparison of slow larval and fast adult muscle innervated by the same motor neurone. *J. exp. Biol.* **84**, 103–118.

RIDDIFORD, L. M. AND AJAMI, A. M. (1973). Juvenile hormone: its assay and effects on pupae of *Manduca sexta*. *J. Insect Physiol.* **19**, 749–762.

RUNION, H. I. AND PIPA, R. L. (1970). Electrophysiological and endocrinological correlates during the metamorphic degeneration of a muscle fibre in *Galleria mellonella* (L.) (Lepidoptera). *J. exp. Biol.* **53**, 9–24.

SHERMAN, R. G. AND ATWOOD, H. L. (1971). Synaptic facilitation: Long-term neuromuscular facilitation in crustaceans. *Science* **171**, 1248–1250.

SIMON, S. M. AND LLINÁS, R. R. (1985). Compartmentalization of the submembrane calcium

activity during calcium influx and its significance in transmitter release. *Biophys. J.* **48**, 485–498.

TREHERNE, J. E. (1960). The nutrition of the central nervous system in the cockroach, *Periplaneta americana* L. *J. exp. Biol.* **37**, 513–533.

TSACOPOULOS, M., COLES, J. A. AND VAN DE WERVE, G. (1987). The supply of metabolic substrate from glia to photoreceptors in the retina of the honeybee drone. *J. Physiol., Lond.* **82**, 279–287.

WASHIO, H. (1989). Time course of failure and resumption of excitatory and inhibitory transmission in the denervated muscle fibers of the cockroach. *J. exp. Biol.* **142**, 257–266.

WEEKS, J. C. AND TRUMAN, J. W. (1984). Neural organization of peptide-activated ecdysis behaviors during the metamorphosis of *Manduca sexta. J. comp. Physiol.* A **155**, 407–422.

WIGGLESWORTH, V. B. (1960). The nutrition of the central nervous system in the cockroach *Periplaneta americana* L. The role of perineurium and glial cells in the mobilization of reserves. *J. exp. Biol.* **37**, 500–513.

WOJTOWICZ, J. M. AND ATWOOD, H. L. (1985). Correlation of presynaptic and postsynaptic events during establishment of long-term facilitation at crayfish neuromuscular junction. *J. Neurophysiol.* **54**, 220–230.

WOJTOWICZ, J. M. AND ATWOOD, H. L. (1986). Long-term facilitation alters transmitter releasing properties at the crayfish neuromuscular junction. *J. Neurophysiol.* **55**, 484–498.

WOJTOWICZ, J. M. AND ATWOOD, H. L. (1988). Presynaptic long-term facilitation at the crayfish neuromuscular junction: Voltage-dependent and ion-dependent phases. *J. Neurosci.* **8**, 4667–4674.

WOJTOWICZ, J. M., MARIN, L. AND ATWOOD, H. L. (1989). Synaptic restructuring during long-term facilitation at the crayfish neuromuscular junction. *Can. J. Physiol. Pharmac.* **67**, 167–171.

YAMAMOTO, R. T. (1969). Mass rearing of the tobacco hornworm. II. Larval rearing and pupation. *J. econ. Entomol.* **62**, 1127–1131.

YAMAOKA, K. AND IKEDA, K. (1988). Electrogenic responses elicited by transmembrane depolarizing current in aerated body wall muscles of *Drosophila melanogaster* larvae. *J. comp. Physiol.* A **163**, 705–714.