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HORMONALLY MEDIATED REPROGRAMMING OF MUSCLES AND MOTONEURONES DURING THE LARVAL–PUPAL TRANSFORMATION OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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SUMMARY

The larval–pupal transformation of *Manduca sexta* results from an exposure to ecdysteroids in the absence of juvenile hormone (the commitment pulse), followed by a larger exposure to ecdysteroids (the prepupal peak) with a reappearance of juvenile hormone (JH). The prepupal ecdysteroid peak triggers the degeneration of abdominal muscles, and the dendritic regression and death of identified motoneurones. The present experiments examined the role of the commitment pulse in the larval–pupal reprogramming of these cells. The commitment pulse did not overtly affect the muscles and motoneurones, but it switched their hormonal responsiveness; before the commitment pulse, exposure to ecdysteroids in the presence of JH had no effect on the larval cells, whereas after the commitment pulse the same treatment caused regression and death. Thus, JH lost its ability to prevent pupal development. Furthermore, treatment with ecdysteroids in the absence of JH before the commitment pulse promoted pupal development much less effectively than did the same treatment given after the commitment pulse, indicating that the commitment pulse facilitates the subsequent responsiveness to ecdysteroids. Thus, the commitment pulse covertly causes both qualitative and quantitative changes in the hormonal sensitivity of the larval muscles and motoneurones.

INTRODUCTION

Many aspects of the remodelling of the nervous and muscular systems during insect metamorphosis are controlled by the blood titres of JH and ecdysteroids (reviewed in Weeks & Truman, 1986). For example, in the tobacco hornworm, *Manduca sexta*, these hormones are implicated in the control of muscle degeneration (Schwartz & Truman, 1983; Weeks & Truman, 1985), postembryonic neurogenesis (Booker & Truman, 1984, 1985), programmed neuronal death (Truman &

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Schwartz, 1984; Bennett & Truman, 1985) and the growth (Levine, Truman, Linn & Bate, 1986) and regression (Weeks & Truman, 1985) of neuronal arbors.

We have previously investigated the hormonal control of muscle and neurone fates during the larval–pupal transformation of *Manduca* (Weeks & Truman, 1985). Following the moult to the fifth (final) larval instar, the major hormonal events are: first, a drop in the blood JH titre; second, a small peak of ecdysteroids in the absence of JH (the commitment pulse), and finally, a larger release of ecdysteroids (the prepupal peak) accompanied by the reappearance of JH. The commitment pulse triggers wandering behaviour (Dominick & Truman, 1985), while more covertly, it commits the epidermal cells to produce pupal cuticle at the next moult. This change in cellular commitment has been termed 'reprogramming' (Riddiford, 1985). The subsequent prepupal peak then causes pupal cuticle synthesis, followed by the shedding of the old larval cuticle at pupal ecdysis.

The majority of the larval abdominal muscles in *Manduca* die during waves of degeneration which precede and follow pupal ecdysis (Weeks & Truman, 1985). The motoneurones which innervate the degenerating muscles either die or else survive to innervate new adult muscles which differentiate during the pupal–adult transition (Levine & Truman, 1985). A subset of these motoneurones, including some which are not fated to die, undergo substantial dendritic regression during the final days of the fifth instar (Weeks & Truman, 1984*b*, 1985). In our previous study (Weeks & Truman, 1985), we showed that the prepupal peak of ecdysteroids triggers the waves of muscle degeneration and the regression and death of motoneurones. The present experiments examine the role of the commitment pulse in reprogramming the larval muscles and motoneurones to respond in this way to the prepupal peak. Although the commitment pulse appears to have no overt effects on these cells, it has the covert action of altering their subsequent responses to JH and ecdysteroids.

MATERIALS AND METHODS

Experimental animals

Larvae of the tobacco hornworm, *Manduca sexta*, were reared individually on an artificial diet (Bell & Joachim, 1976), at 27°C on a 17L:7D (non-diapause) photoperiod. Lights-off occurred at midnight. After wandering (see Fig. 1), larvae were placed into holes bored in wooden blocks. Both males and females were used for experiments.

Hormonal manipulations

To isolate the abdomen from its normal sources of ecdysteroids and JH, CO_2-anaesthetized larvae were ligated with silk thread around the first abdominal segment, and the anterior fragment was cut off and discarded. Abdomens were isolated either on the morning after wandering began (day W; see Fig. 1), or on the late afternoon of day L2 (Fig. 1). In the latter case, larvae weighing more than 7·5 g were selected to ensure that JH had been cleared from the blood (Nijhout & Williams,

1974). Abdomens isolated on day L2 will be termed 'pre-wandering', whereas those isolated on day W will be termed 'post-wandering'.

Some groups of isolated abdomens were infused with 20-hydroxyecdysone (20-HE; Sigma Chemical Co, St Louis, MO, or Rohto Pharmaceutical Co, Ltd, Osaka, Japan), as described in Weeks & Truman (1985). 20-HE was prepared at 1 mg ml^{-1} in saline (Ephrussi & Beadle, 1936) and the concentration was adjusted spectrophotometrically (Meltzer, 1971). 20-HE was delivered into the haemolymph *via* a fine polyethylene cannula inserted into the dorsal abdominal horn, which led to a Hamilton syringe driven by a syringe pump. All abdomens were infused for 12 h starting on the morning after the day of ligation, at a rate of 5·6 μg 20-HE h^{-1}. This infusion paradigm stimulates pupal development in post-wandering abdomens (Nijhout, 1976; Weeks & Truman, 1985). Some 20-HE-infused abdomens were also treated with the juvenile hormone analogue (JHA) Zoecon ZR-515, which was dissolved at 1 mg ml^{-1} in cyclohexane and applied topically using a calibrated micropipette. JHA-treated abdomens received two, 10 μg doses of ZR-515: one in the late afternoon of the ligation day, and one the next morning at the start of the infusion.

Quantification of muscle degeneration

Abdomens were opened dorsally, pinned flat under physiological saline (Weeks & Truman, 1985), and denervated. As described in Weeks & Truman (1985), muscle degeneration was quantified by measuring the diameter of unfixed muscle fibres using an ocular micrometer in a Wild stereomicroscope. Single fibres in characteristic locations within the muscle were measured in muscles VIM (ventral internal medial) and VEO (ventral exterior oblique), whereas the diameters of the two fibres comprising PPRM (principal planta retractor muscle) were measured and summed. PPRM and VEO were measured in the third to sixth abdominal segments whereas VIM was measured in segments 4 and 5 only.

Quantification of motoneurone regression and death

Motoneurone PPR (principal planta retractor) is the only motoneurone which has an axon in the lateral branch of the ventral nerve (VNL), and which sends dendritic processes into the contra- as well as ipsilateral ganglionic neuropiles (Weeks & Truman, 1984*b*). Hence, the contralateral portion of its arbor can be visualized in isolation by cobalt-backfilling a VNL. Ganglia were dissected from proleg-bearing segments (abdominal segments 3 to 6) and each was placed in a pool of physiological saline while the cut end of a VNL was placed in a pool of 2% cobalt chloride. After 24 h at 4°C, the cobalt was precipitated and silver-intensified (Bacon & Altman, 1977), and the ganglia were dehydrated, cleared and mounted. A minority of PPRs were stained individually by intracellular iontophoresis of cobalt *via* a recording microelectrode (Weeks & Truman, 1984*a*,*b*).

A *camera lucida* drawing was made from the dorsal view of each ganglion containing a satisfactory backfill of PPR, and the drawings were analysed quantitatively using a morphometric system described in Weeks & Truman (1985). This

technique produced, for each analysed neurone, a 'dendritic density' score, reflecting the proportion of the hemineuropile area containing dendritic processes of PPR's contralateral arbor. High dendritic density scores reflected extensively branching neurones, while lower values indicated regressing neurones. Unless otherwise indicated, five neurones from five different abdomens were analysed for each experimental group.

PPR's death was also determined from cobalt backfills of VNL. The motoneurone was presumed to have died if (1) its contralateral arbor was absent, and (2) six rather than the usual seven cell bodies were stained with VNL backfills (Weeks & Truman, 1984*b*, 1985).

Statistical tests

Differences between means were tested using the Mann–Whitney U-test, with significance assumed when $P < 0{\cdot}05$.

RESULTS

Effect of prepupal juvenile hormone on muscles and motoneurones

Fig. 1 shows a diagrammatic representation of the hormonal titres during the larval–pupal transformation of *Manduca*, and indicates the developmental fates of three muscles and one motoneurone. Muscle VIM (ventral internal medial) is a persistent larval muscle which remains unchanged at pupation, whereas PPRM (principal planta retractor muscle) and VEO (ventral external oblique) represent muscles which initiate degeneration before and after pupal ecdysis, respectively (Weeks & Truman, 1985). Motoneurone PPR, which innervates PPRM, loses about 40 % of its larval dendritic arbor during the prepupal period and then dies (Weeks & Truman, 1985).

When abdomens were isolated on day W (post-wandering), to prevent the prepupal peak and reappearance of JH, pupal cuticle production was prevented and the muscles and motoneurones remained indefinitely in their larval states (Weeks & Truman, 1985). Thus the commitment pulse by itself had no overt action on the cells. Infusion of 20-HE into post-wandering abdomens either 1 or 5 days after isolation caused PPRM and VEO to degenerate, PPR to regress and die, and the epidermis to produce pupal cuticle (Weeks & Truman, 1985).

In these infused abdomens, PPRM degenerated with the same time course as in intact insects, and VEO degenerated after PPRM but approximately 48 h earlier than in intact insects. One possible cause of this abnormality was the absence of JH during the 20-HE infusion. To test this possibility, post-wandering abdomens were infused with 20-HE 1 day after isolation, with or without JHA treatment. Three days after infusion, all abdomens had produced a new pupal cuticle ($N = 9/9$, +JHA; $N = 10/10$, −JHA) and muscle degeneration was similar whether or not JHA had been present during the 20-HE infusion (Fig. 2). VIM did not degenerate under any condition (Fig. 2A); PPRM's degeneration preceded that of VEO as *in situ*, but even

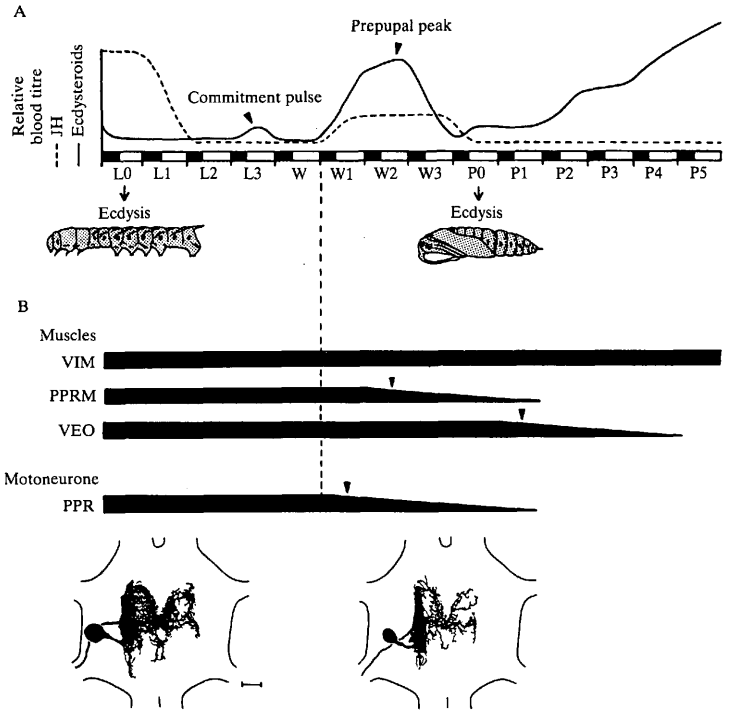

Fig. 1. Events during the larval–pupal transformation of *Manduca*. (A) Endocrine profile. Time is in days, with darkened portions indicating the scotophase. Days are numbered from ecdysis to the fifth instar (day L0), wandering (W) and ecdysis to the pupa (day P0). Ecdysteroid (solid line) and juvenile hormone (JH) (dashed line) blood titres are taken from Bollenbacher, Smith, Goodman & Gilbert (1981), B. J. Bergot (unpublished data cited in Sedlak, Marchione, Devorkin & Davino, 1983) and L. M. Riddiford (unpublished data). (B) Time line of the fates of muscles and motoneurones. Time is the same as in A, with the dashed vertical line indicating the start of the prepupal peak. The fates of abdominal muscles VIM, PPRM and VEO (described in text) are indicated by black bars; a full width bar indicates healthy contractile muscle fibres whereas the narrowing and termination of the bar signify degeneration and final histolysis of the muscles, respectively. Arrowheads indicate the days on which muscle fibre degeneration is first statistically significant (Weeks & Truman, 1985). For motoneurone PPR, the full width bar signifies a larval dendritic density (see text), whereas the narrowing and termination of the bar signify dendritic regression and neuronal death, respectively. The arrowhead indicates the day on which dendritic regression is first statistically significant (Weeks & Truman, 1985). At the bottom are shown *camera lucida* drawings of PPR in a pre-wandering larva (left) and at pupal ecdysis (right). Neurones were stained by intracellular iontophoresis of cobalt chloride. The outline of the abdominal ganglion is shown. Scale bar, 50 μm.

with JHA present, VEO degenerated earlier than in intact insects (Fig. 2B,C; Weeks & Truman, 1985).

The time course of PPR's dendritic regression and death in post-wandering abdomens was similarly unaffected by JHA during the 20-HE infusion (Fig. 3). These data indicate that the presence or absence of JH during the prepupal ecdysteroid peak does not influence the larval–pupal transformation of the muscles and motoneurones.

The role of the commitment pulse for larval–pupal reprogramming of muscles and motoneurones

To examine the role of the commitment pulse of ecdysteroids on the developmental fates of the muscles and motoneurones, we isolated abdomens from larvae on day L2 (pre-wandering), then infused 20-HE on the next day in the presence or absence of JHA. The same infusion regime was used as described above. Muscles and motoneurones were measured 3 and 7 days after infusion.

Of 26 pre-wandering abdomens which received 20-HE alone, all exposed the dorsal vessel and purged the gut on the following day, which is characteristic of the wandering stage (Nijhout, 1976). Of 10 of these abdomens dissected 3 days after infusion, seven showed apolysis (the separation of the cuticle from the epidermis in preparation for a moult) and one of these had produced some pupal cuticle. By 7 days after infusion all of the remaining abdomens had apolysed and eight of the 16 had either patchy ($N = 5$) or complete ($N = 3$) pupal cuticle.

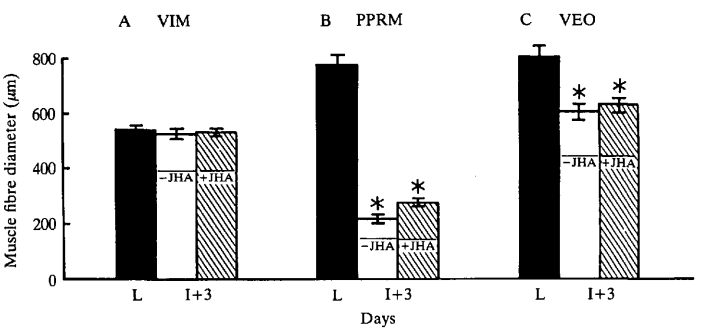

Fig. 2. Muscle responses to 20-HE infusion in post-wandering isolated abdomens, with or without JHA treatment. Each bar gives the mean (±S.E.M.) muscle fibre diameter, from at least 20 fibres in three or more abdomens. (A),(B) and (C) show data from muscles VIM, PPRM and VEO (described in the text), respectively. Control measurements were made on day W at the time of abdomen ligation (L; solid bars). 20-Hydroxyecdysone (20-HE) was infused on the day after ligation, and muscle measurements were made 3 days post-infusion (I+3). Striped bars, juvenile hormone analogue (JHA) treated; open bars, no JHA treatment; *, significantly reduced from value at ligation (Mann–Whitney U-test; $P < 0{\cdot}05$).

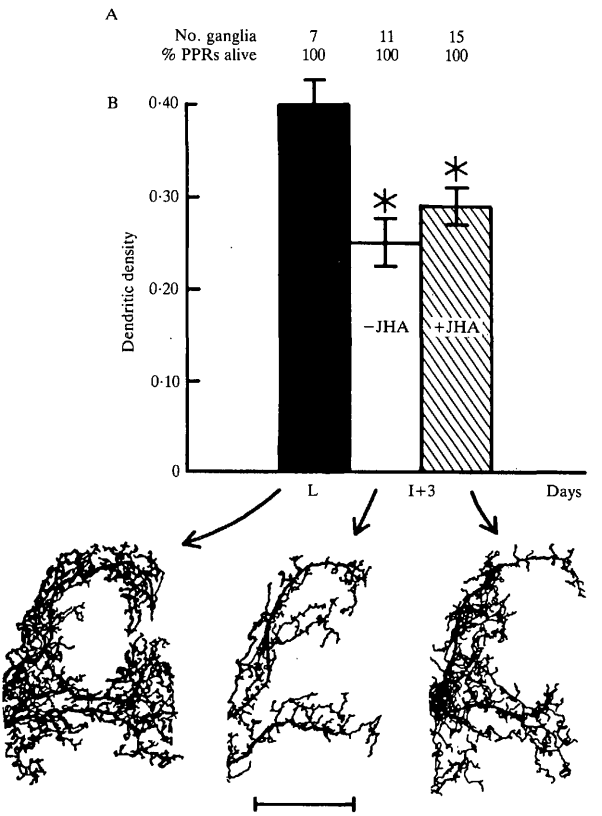

Fig. 3. Responses of motoneurone PPR to 20-hydroxyecdysone (20-HE) infusion in post-wandering abdomens, with or without juvenile hormone analogue (JHA) treatment. Experimental regime was the same as in Fig. 2. Cobalt backfills of nerve VNL were made at the time of ligation (L) on day W, and 3 days post-infusion (I+3). (A) Survival of PPR. The three sets of values correspond to the three experimental cases shown directly below in B. The upper row indicates the number of ganglia in which VNL was backfilled, and the lower row indicates the percentage of PPRs found to be alive. (B) Dendritic structure of PPR. Each bar gives the mean (±S.E.M.) dendritic density of five motoneurones from different abdomens. Solid bar, value at time of ligation; striped bar, JHA treated; open bar, no JHA treatment; *, significantly reduced from value at ligation (Mann–Whitney U-test; $P < 0{\cdot}05$). Representative *camera lucida* drawings of PPR's contralateral arbor (see Fig. 1) are shown for each of the three experimental groups. Scale bar, 50 μm.

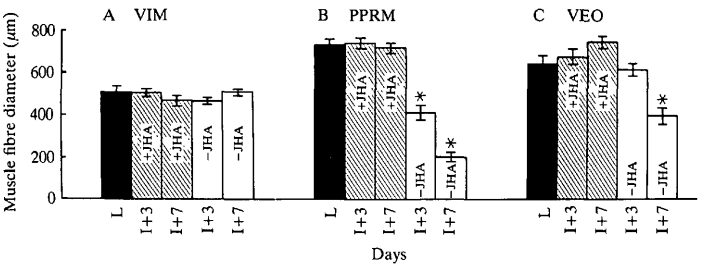

Fig. 4. Muscle responses to 20-hydroxyecdysone (20-HE) infusion in pre-wandering abdomens, with or without juvenile hormone analogue (JHA) treatment. Each bar gives the mean (±S.E.M.) muscle fibre diameter, from at least 20 fibres in three or more abdomens. (A),(B) and (C) show data from muscles VIM, PPRM and VEO (described in the text), respectively. Control measurements were made at the time of abdomen ligation (L; solid bars) on day L2. 20-HE was infused on the day after ligation, and muscle measurements were made 3 and 7 days after infusion (I+3 and I+7, respectively). Striped bars, JHA treated; open bars, no JHA treatment; *, significantly reduced from value at ligation (Mann–Whitney U-test; $P < 0{\cdot}05$).

Different responses were seen when 20-HE infusion was paired with JHA treatment. In this case, 10 of 13 abdomens dissected 3 days after infusion had undergone apolysis and produced a new larval cuticle which was complete except for the absence of crochets on the prolegs (Fain & Riddiford, 1977). The other three abdomens had not apolysed but remained fully larval in appearance. Abdomens which moulted were trapped in their cuticles and died soon after, so by 7 days after infusion, only non-apolysed abdomens were alive ($N = 7$). Control abdomens isolated on day L2 which were not treated with hormone ($N = 10$) never showed apolysis or cuticle production.

Fig. 4A indicates that VIM was indifferent to 20-HE infusion, irrespective of the presence or absence of JHA. When JHA treatment accompanied 20-HE infusion, PPRM and VEO were retained for at least 7 days after infusion (Fig. 4B,C). When infused in the absence of JHA the muscles degenerated in the proper sequence (PPRM before VEO), but more slowly than in response to the same infusion delivered to post-wandering abdomens (cf. Fig. 2). There was some correlation between muscle degeneration and epidermal responses, e.g. 3 days after infusion, PPRM fibre diameter in abdomens which showed apolysis was 44 % smaller than in non-apolysed abdomens. Yet in both groups PPRM was reduced as compared to control muscles. In isolated abdomens that were not hormone-treated, all muscles survived indefinitely in their larval forms (data not shown).

Similarly, the presence of JHA during 20-HE infusion prevented the regression (Fig. 5B) and death (Fig. 5A) of motoneurone PPR. In the pre-wandering abdomens infused without JHA, dendritic regression and neurone death were apparent by day 7 (Fig. 5). This time course was much slower than that seen after similar 20-HE infusion of post-wandering abdomens (cf. Fig. 3). Again, regression was somewhat correlated with epidermal development, with the mean dendritic

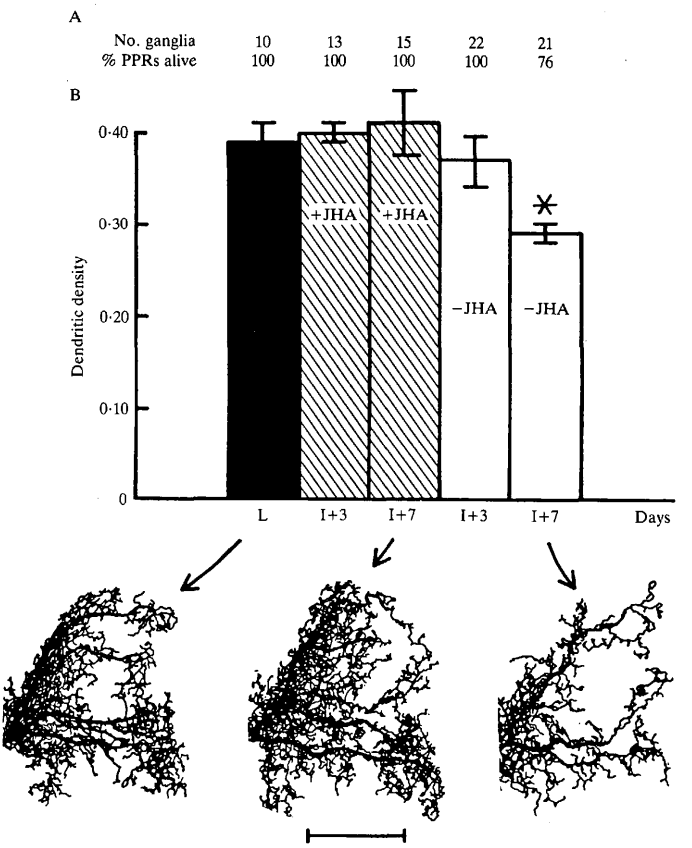

Fig. 5. Responses of motoneurone PPR to 20-hydroxyecdysone (20-HE) infusion in pre-wandering abdomens, with or without juvenile hormone analogue (JHA) treatment. Experimental regime was the same as in Fig. 4, and figure format is the same as in Fig. 3. Cobalt backfills of VNL were made at the time of ligation (L) on day L2, and 3 and 7 days post-infusion (I+3 and I+7, respectively). (A) Survival of PPR. The five sets of values correspond to the five experimental cases shown directly below in part B. The upper row indicates the number of ganglia in which VNL was backfilled, and the lower row indicates the percentage of PPRs found to be alive. (B) Dendritic structure of PPR. Each bar gives the mean (±S.E.M.) dendritic density of five motoneurones from different abdomens, with the exception of days I+3 and I+7 in the −JHA groups, in which eight and seven motoneurones from different abdomens were analysed, respectively. Solid bar, value at time of ligation; striped bars, JHA treated; open bars, no JHA treatment; *, significantly reduced from value at ligation (Mann–Whitney U-test; $P < 0{\cdot}05$). Representative *camera lucida* drawings of PPR's contralateral arbor are shown for the control and I+7 experimental groups. Scale bar, 50 μm.

density 3 days after infusion being 0·34 ($N = 5$) in abdomens with apolysis as compared to 0·41 ($N = 3$) in non-apolysed abdomens. PPR's death was not as well correlated with the state of the epidermis: of the five neurones which died by 7 days post-infusion (Fig. 5A), three were in non-apolysis abdomens, one abdomen had patchy pupal cuticle, and one had complete pupal cuticle. In control abdomens which were not hormone-treated, PPR survived in its larval form indefinitely (Weeks & Truman, 1985; data not shown).

DISCUSSION

Two components of the larval–pupal transformation of muscles and motoneurones in *Manduca* were revealed in these experiments: the commitment to pupal differentiation required ecdysteroids in the absence of JH, whereas expression of the pupal phenotype was independent of JH and depended solely on ecdysteroids. On day L2, prior to the commitment pulse, neither muscles nor motoneurones were pupally committed. They retained their larval forms indefinitely in abdomens isolated at this time, even if the abdomens were induced to undergo a supernumerary larval moult by infusion of 20-HE in the presence of JHA (Figs 4, 5; Weeks & Truman, 1985). Thus, earlier endocrine events in the fifth instar, such as the decline in JH midway through the feeding period (Fig. 1), do not trigger pupal reprogramming of the muscles and motoneurones.

In abdomens isolated after wandering and not hormone-treated, the muscles and motoneurones similarly retained their larval phenotypes indefinitely (Weeks & Truman, 1985). Thus, the commitment pulse does not produce any overt changes in these cells. However, it clearly alters their responses to subsequent hormonal cues. One such effect was to render the muscles and motoneurones insensitive to JH. For example, in post-wandering abdomens infused with 20-HE, the muscles and motoneurones showed regressive changes irrespective of the presence or absence of JHA (Figs 2, 3). This is in marked contrast to the ability of JHA to preserve the larval structure of the cells during 20-HE infusions in pre-wandering abdomens (Figs 4, 5). Muscles and motoneurones responded to the commitment pulse in the same way as do epidermal cells, which become irrevocably committed to produce pupal cuticle and refractory to JH when exposed to ecdysteroids in the absence of JH (Truman, Riddiford & Safranek, 1974; Riddiford, 1976, 1978; Mitsui & Riddiford, 1978). Not only was JHA unable to prevent muscle degeneration and motoneurone regression and death in post-wandering abdomens infused with 20-HE, but it also had no effect on the time course of these events (Figs 2, 3). In particular, JHA treatment did not reverse VEO's premature degeneration in infused ligated abdomens as compared to intact insects (Weeks & Truman, 1985): the cause of this phenomenon remains unexplained.

Although prepupal JH is seen in many Lepidopteran species (see Sparagana, Bhaskaran & Barrera, 1985), its presence appears to be required by only a subset of tissues. For instance, in *Manduca*, the epidermis from post-wandering insects produces normal pupal cuticle when exposed to ecdysteroids in the absence of JH

(Nijhout, 1976; Mitsui & Riddiford, 1976), whereas imaginal discs exposed to this environment show precocious, inappropriate adult development which is preventable by JH treatment (Kiguchi & Riddiford, 1978). In their insensitivity to prepupal JH, the muscles and motoneurones examined in this study thus resemble epidermis and not imaginal discs.

During normal development, the reprogramming of muscles and motoneurones for pupal development, and the expression of the pupal phenotype, are controlled by sequential exposures to ecdysteroids. However, in abdomens from pre-wandering insects which were infused with 20-HE in the absence of JHA, the muscles and motoneurones showed some degree of pupal development (Figs 4, 5). The muscles showed the correct pattern of degeneration, but the time course was 2–4 days slower than in post-wandering infused abdomens (Fig. 2) or in intact insects (Weeks & Truman, 1985). Similarly, 20-HE infusion was less effective in triggering motoneurone regression and death when given to pre-wandering, as compared to post-wandering, abdomens (Figs 3, 5).

The finding that the same 20-HE infusion was more effective after the wandering phase suggests that the small commitment pulse facilitates the subsequent responsiveness of the muscles and motoneurones to the larger ecdysteroid peak which follows. The mechanism for this is not known, but may involve biochemical changes such as increased synthesis of ecdysteroid receptors or other alterations in mRNA and protein synthesis (see Riddiford, 1985). Assuming that 20-HE infused into a pre-wandering abdomen in the absence of JH activates the same biochemical changes as does the commitment pulse (certainly, the overt signs of wandering are elicited by this treatment), but that the changes develop with some latency after the appearance of ecdysteroids, then the single infusion given to a pre-wandering abdomen would not be as effective as when given to a post-wandering abdomen in which the commitment pulse-mediated changes are fully developed. In order to test such hypotheses a better understanding of the biochemical consequences of ecdysteroid action in neurones and muscles is needed.

The inability of a single 20-HE exposure to promote complete pupal development has been reported in tissues such as *Manduca* epidermis (Nijhout, 1976; Mitsui & Riddiford, 1978) and *Drosophila* imaginal discs (Fristrom *et al.* 1982) and salivary glands (Richards, 1976). In the present experiments only one infusion regimen was tested: i.e. 5·6 μg 20-HE h^{-1} for 12 h. The peak titre attained at the end of this infusion in isolated, post-wandering abdomens is 2·3 μg 20-HE equivalents ml^{-1} haemolymph, which declines with a half-life of approximately 24 h (Morton & Truman, 1985). Haemolymph titres in infused, pre-wandering abdomens were not measured. The peak titres in intact insects during the commitment pulse and prepupal peak are 0·07 and 1·5 μg 20-HE equivalents ml^{-1} haemolymph, respectively (Bollenbacher, Smith, Goodman & Gilbert, 1981). Based on these estimates, the 20-HE infusion regime we used produced an ecdysteroid elevation which was approximately similar in duration and magnitude to the prepupal peak, but which was approximately three times the duration and 33 times the magnitude of the commitment pulse. The developmental effects of ecdysteroids depend on dosage and

duration (e.g. Nijhout, 1976; Riddiford, 1978; Mitsui & Riddiford, 1976, 1978), and the determination of whether a single exposure can adequately substitute for the normal two-peak sequence will require tests of the effects of other infusion regimes on the muscles and motoneurones.

In conclusion, the responses of neurones and muscles to circulating hormones during development may be strongly influenced by prior hormonal events which do not produce obvious effects on cell phenotype. The studies reported here show that the small commitment pulse has no overt effects on the muscles and motoneurones, but nevertheless it has the subtle effect of changing the state of determination of the cells. Subsequently, their responses to hormonal cues differ both qualitatively and quantitatively. The nature of these subtle changes in cellular programming remain to be determined.

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