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THE DEVELOPMENT OF ELECTRICAL PROPERTIES OF IDENTIFIED NEURONES IN GRASSHOPPER EMBRYOS

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SUMMARY

1. We have examined the development of the electrical properties of five identified neurones in grasshopper embryos between days 10 and 13 of embryogenesis (hatching occurs on day 20). DUM 3,4,5; DUM 4,5; DUM 5; the H cell; and the H cell sibling are the progeny of two different precursor cells. Electrical coupling and electrical excitability were assayed by intracellular recordings.

2. Midway through embryogenesis, on day 10, the five cells are highly electrically coupled to each other and are electrically inexcitable. The temporal sequence of the development of electrical excitability and electrical uncoupling is described for DUM 3,4,5; 4,5; and 5. The H cell and H cell sib undergo the same sequence one day later.

3. The first non-linear membrane property to appear is delayed rectification which appears on day 11 and can be blocked by tetraethylammonium (TEA). In some cells at about day 11, the addition of TEA to normal saline unmasks a Na^+-dependent action potential in the axon.

4. The first action potential in normal saline is a Na^+-dependent response that appears in the axon at day 11–11·5.

5. The next stage of excitability in normal saline is the appearance about day 11·5 of a Na^+-dependent action potential in the median neurite between the soma and the two axons. In some cells at about day 11·5, the addition of TEA unmasks an excitable response in the soma.

6. Overshooting action potentials appear in the soma about day 12; the inward current is carried by both Na^+ and Ca^{2+}; TEA causes a prolonged shoulder on the falling phase of the action potential. A short time later, TEA causes a long-duration Ca^{2+} plateau.

7. A progressive decrease in the degree of electrical coupling among the cells occurs between days 10 and 12·5. Complete uncoupling is never observed before day 11, but has always occurred by day 12·5.

8. Two methods were used to demonstrate that electrical coupling does not mask the presence of excitable inward current channels and thus make the cells appear inexcitable. First, we exposed the cells to veratridine. The cells which normally generate excitable Na^+ responses are depolarized by it; the younger inexcitable cells

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are not. Secondly, we electrically isolated the cells by killing the somata of their neighbours. The input resistance increased, yet the extent of excitability remained unchanged.

9. There is variability in the precise temporal relationship of excitability and uncoupling. Pairs of cells from different embryos of the same age can generate the same type of action potentials and yet be coupled in one embryo and uncoupled in another. Electrical excitability and uncoupling appear to be causally unrelated and independent events, occurring at about the same developmental stage.

INTRODUCTION

In the previous paper (Goodman & Spitzer, 1981) we described the mature electrical properties of five identified neurones in grasshopper embryos between days 13–20 (hatching occurs on day 20 at 35 °C). These five neurones arise from two different precursor cells: the median neuroblast, whose first three progeny are DUM 3,4,5; DUM 4,5; and DUM 5 (Goodman & Spitzer, 1979), and mid line precursor 3 (MP3), which divides once to produce the H cell and H cell sibling (Goodman, Bate & Spitzer, 1979, 1981). Between days 13 and 20, all five neurones generate action potentials and in several cases show cell-specific electrical properties; no signs of electrical coupling can be detected among the five cells.

Midway through embryogenesis, on day 10, these five cells are highly electrically coupled to each other and electrically inexcitable (Goodman & Spitzer, 1979; Spitzer, Bate & Goodman, 1979). In this paper, we describe the development of the electrical properties of these five cells between days 10–13, including the onset of electrical excitability and the cessation of electrical coupling. Brief accounts of some of these results have already appeared (Spitzer & Goodman, 1978; Goodman & Spitzer, 1979; Spitzer *et al.* 1979).

METHODS

All methods were described in the previous paper (Goodman & Spitzer, 1981).

RESULTS

Neurones are coupled and inexcitable at early stages

The first three progeny of the median neuroblast are DUM 3,4,5; DUM 4,5; and DUM 5 (Goodman & Spitzer, 1979). The two progeny of mid line precursor 3 (MP3) are the H cell and H cell sib (Goodman *et al.* 1979). Before day 10, these five cells are highly electrically coupled. Intracellular penetrations of pairs of cells that arise from the same precursor show a coupling coefficient ($\Delta V_2/\Delta V_1 = J$) greater than 0·5, and often $\sim$ 0·7 on day 10. Pairs of cells that arise from different precursors (the median neuroblast and MP3) are also found to be electrically coupled.

Before day 10, the five cells have linear current–voltage ($I-V$) relationships and appear completely inexcitable. The apparent input resistance (R_{in}) of all five cells is about 100 MΩ on day 10 and greater than 200 MΩ on and after day 11. They are inexcitable even when they are electrically isolated by removing the somata from many of the cells to which they are coupled. In such cases, the measured R_{in} increases

to over 300 MΩ, yet the cells still show no non-linearities in their membrane properties and no signs of excitability. Furthermore, the cells on day 10 show no sign of voltage dependent inward current channels in the presence of 30 mM-TEA, although this agent blocks outward current a day later.

The five identified neurones are fully excitable and totally uncoupled by day 13 (Goodman & Spitzer, 1981). The developmental timetable for the H cell and H cell sib is generally shifted one day later as compared to DUM 3,4,5; DUM 4,5; and DUM 5, which develop nearly in synchrony. All five cells, however, follow the same developmental sequence; the temporal appearance of electrical excitability proceeds from no action potential, to the axon action potential, to the neurite action potential, to the soma action potential (Fig. 1), as described below.

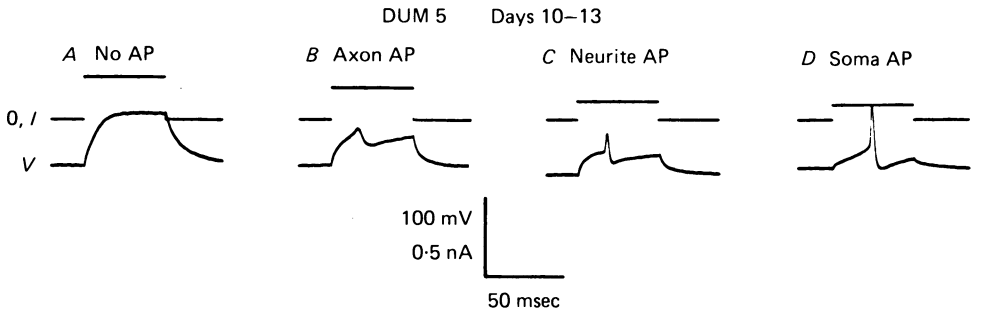

Fig. 1. Onset of excitability in DUM 5 between days 10 and 13. The development of the action potential (AP) proceeds from no AP (*A*), to axon AP (*B*), to neurite AP (*C*), to soma AP (*D*). Records from four cells.

PART I. DEVELOPMENT OF EXCITABILITY

No action potential in normal saline

By day 10, dye injections reveal that all five identified neurones already have axons beginning to grow out of the metathoracic ganglion, either in peripheral nerve 5 or the ventral nerve cord. Thus, from days 10–13 we examined the excitability of these cells by intracellular stimulation of the soma and by extracellular stimulation of the nerve bundles containing the axons. Between days 10–11, we were unable to evoke an action potential in any of these cells by either form of stimulation (e.g. Fig. 1*A*). The following description of development applies to DUM 3,4,5; 4,5; and 5. The first non-linear membrane property to appear is delayed rectification. The addition of 30 mM-TEA to block voltage-dependent outward current abolishes this membrane rectification (Fig. 2). At these early developmental stages, the addition of TEA reveals no evidence for excitable inward current. Thus, voltage-dependent outward current appears before voltage-dependent inward current.

At about day 11, a time when excitability in normal saline is sometimes first observed, TEA unmasks in some cells a small excitable response that is of short duration and is abolished by TEA solutions in which Na^+ is replaced by Tris or choline (Fig. 3). The response is likely to be a Na^+-dependent action potential

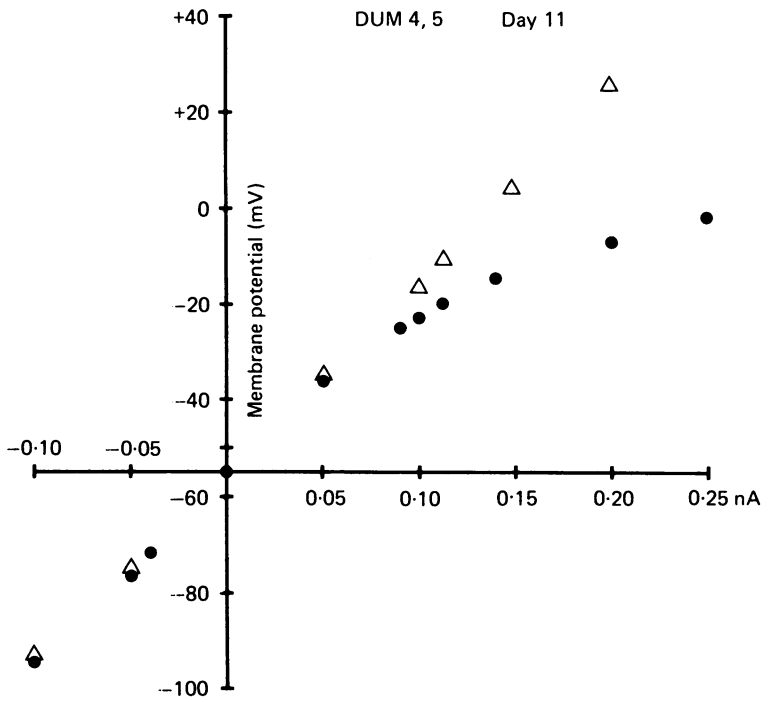

Fig. 2. Current–voltage relationship demonstrating delayed rectification in DUM 4,5 in a day 11 embryo; all data from one cell. In normal saline, depolarizing current pulses produce smaller voltage changes than hyperpolarizing current pulses of comparable amplitude. TEA abolishes the apparent conductance increase in the depolarizing direction. Circles, normal saline; open triangles, saline with 30 mM-TEA.

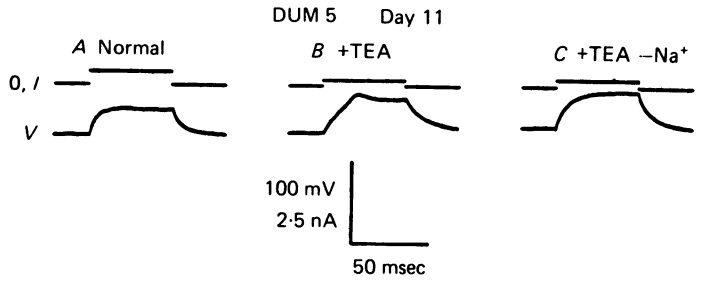

Fig. 3. TEA reveals a Na^+-dependent event not seen in normal saline. All records from DUM 5 in a day 11 embryo.

generated in the axon because it can also be evoked by extracellular stimulation of the nerves containing the axons. The suggestion that TEA unmasks Na^+ channels first appearing in the axon is supported by the next developmental stage, in which Na^+-dependent action potentials can be evoked in the axon in normal saline.

Axon action potential in normal saline

The first sign of inward current we observe in normal saline is the appearance about day 11 of an axon action potential that passively spreads into the soma (Fig. 1*B*). This axon action potential is evoked by either intracellular stimulation of the soma (Fig. 4*A*1) or by antidromic stimulation of the axon (Fig. 4*B*1). The axon action potential has the same amplitude but a different shape and duration when evoked by these two means. If the antidromic action potential is superimposed on top of a subthreshold depolarization of the soma, the action potential recorded in the soma is of similar amplitude, but of shorter duration and is followed by a more pronounced

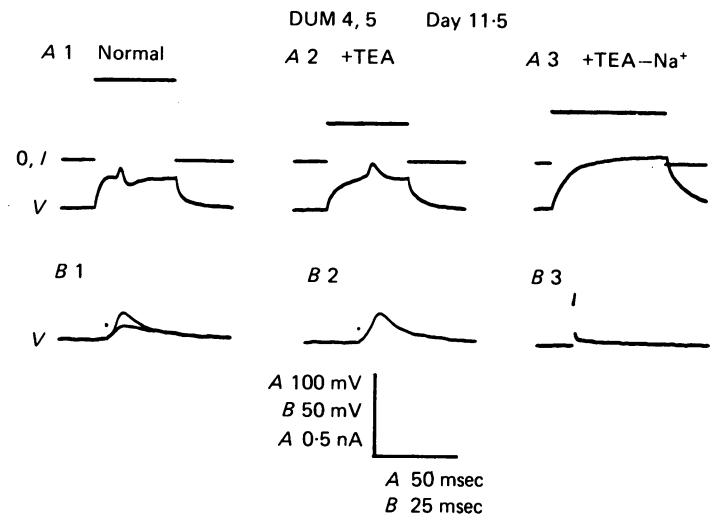

Fig. 4. Axon action potential in DUM 4,5 in a day 11·5 embryo, elicited by intracellular stimulation of the soma (*A*) and extracellular stimulation of the axon (*B*). TEA causes slight broadening of the action potential; removal of Na^+ abolishes this response. Note bimodal amplitude of the action potential (*B*1), probably due to action potentials in one or both of the peripheral axons. All records from one cell.

hyperpolarization; it appears very similar in shape and duration to the action potential evoked by direct intracellular stimulation of the soma. The shortened duration and increased hyperpolarization are likely to be due to delayed rectification in the soma in response to the additive depolarization produced by the electrotonically propagated axon action potential and the direct depolarization of the soma.

When the axon action potential first appears, it often exhibits two different amplitudes (Fig. 4*B*1). In mature embryonic neurones, the smaller response is likely to be characteristic of an action potential in only one of the two axons, while the larger response is characteristic of action potentials in both the stimulated and contralateral axons (Heitler & Goodman, 1978).

The axon action potentials are abolished by removal of Na^+ or addition of TTX 10^{-9} g/ml.; the addition of 10 mM-Co^{2+} has little effect. The addition of 30 mM-TEA abolishes much of the hyperpolarization following the action potential evoked by

intracellular stimulation of the soma, supporting the notion that this hyperpolarization is due at least in part to delayed rectification of the soma membrane (Fig. 4*A*2). However, in TEA, the action potential does not significantly increase in duration (Fig. 4*A*2, *B*2). No excitable response is observed in TEA solutions in which Na^+ is replaced by Tris or choline (Fig. 4*A*3, *B*3). Thus, there is no evidence for the presence of Ca^{2+} inward current during the developmental stage in which the Na^+-dependent axon action potential first appears.

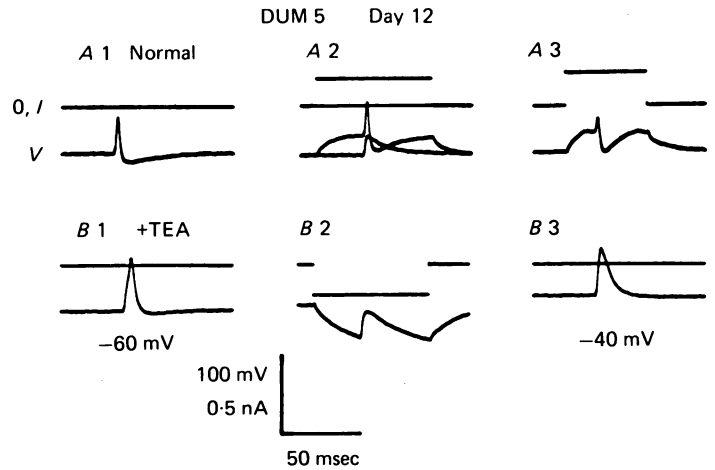

Fig. 5. Neurite action potential in DUM 5 in a day 12 embryo, elicited by intracellular (*A*3) or antidromic (*A*1, 2; *B*) stimulation. The action potential arises in the neurite (*A*1), since repetitive stimulation yields an axon action potential (the smaller, slow response) (*A*2), that sums with somatic depolarization to elicit an action potential with an amplitude similar to that in *A*1. This action potential can also be elicited by intracellular injection of current (*A*3). *B*, TEA reveals an overshooting action potential not seen in normal saline (*B*1). Hyperpolarization reveals an axon action potential (*B*2), while depolarization fails to elicit a plateau of long duration (*B*3). All records from one cell.

Neurite action potential in normal saline

After the appearance of the axon action potential, and before the appearance of the overshooting soma action potential, we often observed an intermediate developmental stage at about day 11·5 (Fig. 1*C*). This response, whether evoked by extracellular stimulation of the peripheral axon (Fig. 5*A*1) or by intracellular stimulation of the soma (Fig. 5*A*3), was not overshooting but was larger than the passively propagated axon action potential. During repetitive stimulation of the peripheral nerve (Fig. 5*A*2), this intermediate response is often not observed but rather the smaller amplitude axon action potential is recorded. By comparison with action potentials recorded in mature neurones (Heitler & Goodman, 1978), we consider this intermediate response to be a neurite action potential, where the neurite is defined as the median cell process between the soma and the T-junction leading to the two symmetrical axons. In mature embryonic neurones, the median neurite is capable of generating an action potential, but this neurite action potential is rarely seen alone in soma recordings because it usually gives rise to a soma action potential.

An alternative explanation is that this response represents a partial appearance of the soma action potential, that is, an active but not yet overshooting response of the soma membrane. This seems unlikely, however, since the depolarizing amplitude of the response is electrotonically additive with a direct soma depolarization (Fig. 5*A*2), suggesting that the response is actively generated in the neurite and passively propagated into the soma membrane. Although the depolarizing amplitude of the response is additive with direct soma depolarization, the duration of the response is shorter and the after-hyperpolarization more pronounced, probably due to the delayed rectification of the soma membrane.

In cells which generate a neurite action potential in normal saline, we record two different types of responses when outward current is blocked by TEA: (i) the neurite action potential remains relatively unchanged in amplitude, or (ii) the neurite action potential becomes overshooting, with an inflection between the neurite response and the unmasked response (Fig. 5*B*1). The first type of response in TEA may represent cases in which the neurite action potential has appeared but in which there is little development of inward current channels in the soma membrane. The second type of response probably reflects the appearance of inward current channels in the soma membrane, which though still masked by outward current, are capable of generating an overshooting action potential when outward current is blocked by TEA. During the first stages in which neurite action potentials are evoked, just like the earlier stages in which only axon action potentials are evoked, TEA (or TEA – Na^+) does not lead to long-duration Ca^{2+} action potentials. This is true even when the soma membrane potential is depolarized to −40 mV (Fig. 5*B*3), a procedure that can lead to long-duration Ca^{2+} action potentials in mature embryonic neurones (Goodman & Spitzer, 1981). The neurite action potential is abolished by removal of Na^+, as is the smaller axon action potential; the addition of 10 mM-Co^{2+} has little effect.

Soma action potential in normal saline

The overshooting soma action potential appears about day 12 (Fig. 1*D*, 6). The inward current of the soma action potential on day 12 is carried by both Na^+ and Ca^{2+}, similar to soma action potentials from days 13–20. Removal of Na^+ or addition of Co^{2+} does not abolish the action potential, but simultaneous application of both treatments does. The addition of 30 mM-TEA distinguishes two different types of responses in the development of the soma action potential, as illustrated in Fig. 6. At first, TEA causes a prolonged shoulder and gradual repolarization of the action potential at resting potential (−60 mV) (Fig. 6*A*2). If the membrane potential is depolarized to −40 mV, the cell produces a long-duration action potential, but the plateau, based on Ca^{2+} inward current (blocked by Co^{2+}), does not overshoot the zero potential. In later developmental stages, TEA causes a long-duration soma action potential at the resting potential, and the plateau is overshooting (Fig. 6*B*2, 3). These two types of responses in TEA may be the result of a developmental increase in the Ca^{2+} inward current in the soma. Alternatively, there could be an increase in the fraction of K^+ outward current blocked by TEA. This seems unlikely since we observe no change between days 12 and 13 in the brief after-hyperpolarization following the action potential in normal saline, and no change in the degree to which it is affected by TEA. The after-hyperpolarization increases markedly in amplitude and duration

after this stage (by day 16). This increment is blocked by Co^{2+} and not by TEA and probably reflects an increase in K^+ outward current. One explanation of this result is the appearance of a Ca^{2+}-activated K^+ outward current (Goodman & Spitzer, 1980).

Effects of veratridine

The five identified neurones can be electrically coupled to each other and to additional neighbours as late as day 12 (see below). It seemed possible that voltage-dependent channels might not be revealed by current injection because of the longer time constant of the coupled network of cells preventing depolarization to threshold before channel inactivation occurs. Accordingly, we exposed the cells

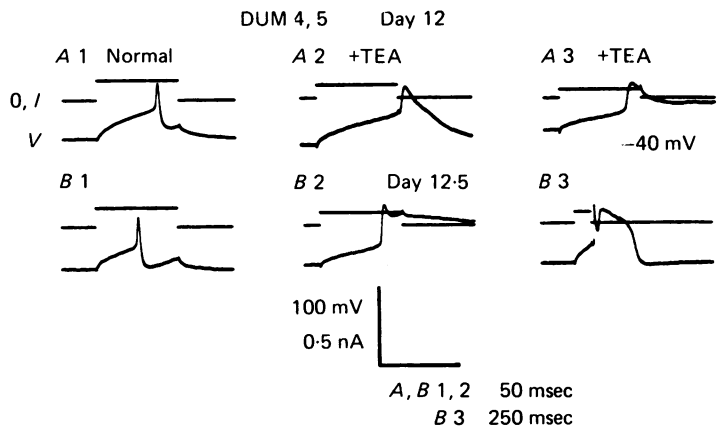

Fig. 6. Soma action potentials of DUM 4,5 in day 12 (*A*) and day 12·5 (*B*) embryos. All records from two cells. On day 12, a non-overshooting plateau is elicited in TEA only when the membrane potential is depolarized (*A*2, 3). An overshooting plateau is generated in TEA on day 12·5, at the resting potential (*B*2, 3).

to veratridine (10^{-5} g/ml.) which specifically depolarizes cells possessing voltage-dependent Na^+ channels by holding the channels open (Narahashi, 1974).

Between days 12 and 13 the median neuroblast has a mean resting potential of about −55 mV, whether exposed to normal saline or to veratridine for 15 min prior to impalement. The normally inexcitable first progeny of the median neuroblast between days 10 and 11 have a mean resting potential of about −50 mV, whether exposed to normal saline or veratridine; the largest value was −56 mV. The median neuroblast and its inexcitable progeny are usually coupled to cells capable of generating Na^+-dependent action potentials in their axons (coupling coefficient ~ 0·25, see below). Our data suggest that the depolarization produced by the action of veratridine on these excitable axons is so attenuated by the time it reaches the cell bodies of inexcitable cells that we cannot detect it. The normally excitable first progeny of the median neuroblast between days 12 and 13 are depolarized by over 30 mV by exposure to veratridine. Removal of Na^+ or addition of TTX before the addition of veratridine protects much (TTX) or all (Na^+-free) of the normal resting potential (Table 1). The cells normally hyperpolarize in Na^+-free saline, and thus it is not surprising that the largest value recorded in Na^+-free plus veratridine was −76 mV. These results are in parallel with the first set of observations on the temporal appearance of voltage-dependent Na^+ channels.

TABLE 1. Effects of veratridine on the membrane potential of identified cells at different stages of development. Values in column four are means ± S.D. (number of cells). The quality of impalements could not be evaluated on the basis of resting potential because veratridine is expected to depolarize cells possessing Na^+ channels; accordingly all values were accepted. The mean values are thus lower than those noted elsewhere

Cell type	Age (days)	Saline	Membrane potential (mV, mean ± S.D.)	Largest membrane potential (mV)
Median neuroblast	12–13	normal	−56 ± 5 (12)	−63
Median neuroblast	12–13	V	−55 ± 3 (5)	−60
Normally inexcitable neurones	10–11	normal	−52 ± 6 (12)	−60
Normally inexcitable neurones	10–11	V	−48 ± 4 (15)	−56
Normally excitable neurones	12–13	V	−11 ± 3 (32)	−16
Normally excitable neurones	12–13	V−Na^+	53 ± 14 (12)	−76
Normally excitable neurones	12–13	V+TTX	−36 ± 8 (15)	−48

V = veratridine 10^{-5} g/ml. in normal saline; $-Na^+$ = Na^+ replaced by Tris; +TTX = tetrodotoxin 10^{-9} g/ml.

TABLE 2. The coupling coefficient, input resistance, and excitability of cells at different stages of development. *A*, cells in the intact nervous system are strongly coupled and have low apparent input resistances at early stages (values are means ± S.D. (number of cells)). *B*, cells isolated from their neighbours have a larger input resistance (see text for details of isolation). The degree of electrical excitability is the same as that of cells in the intact nervous system, and depends on the stage of development

A Input resistance, excitability, and coupling of normal cells

Age (days)	Coupling coefficient (J)	Input resistance (megohms, mean ± S.D.)	Excitability
10	$0{\cdot}5 < J < 0{\cdot}8$	71 ± 10 (3)	No AP
11–11·5	$0{\cdot}15 < J < 0{\cdot}4$	246 ± 60 (13)	no AP, axon AP, or neurite AP
12	$0{\cdot}005 < J < 0{\cdot}07$	266 ± 57 (4)	Soma AP
12–13	$J < 0{\cdot}001$ (uncoupled)	374 ± 102 (7)	Soma AP

B Input resistance and excitability of electrically isolated cells

Age (days)	Input resistance (MΩ)	Excitability
10	360	No AP
11	410	No AP
11·5	380	Axon AP
11·5	450	Neurite AP

Effects of electrical isolation

Although the presence or absence of coupling appeared to bear no relationship to the stage of excitability, we were nevertheless concerned that the presence of coupling might significantly decrease the apparent R_{in} and thus mask the presence of excitability. Cells that are coupled have lower input resistances, as shown in Table 2*A*. The observed coupling coefficient between pairs of cells decreases from about 0·7

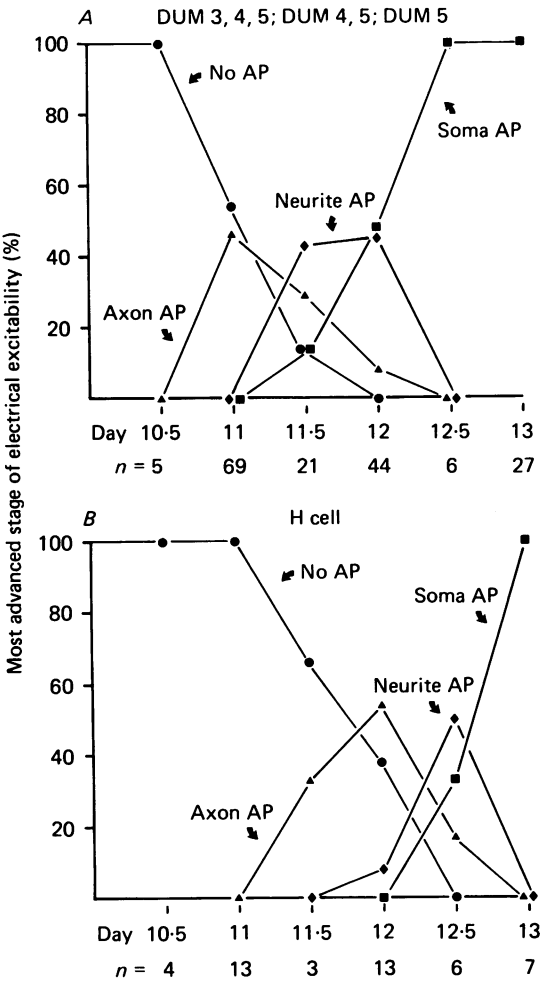

Fig. 7. Most advanced state of electrical excitability of cells, as a function of embryonic age, for DUM 3,4,5; 4,5; 5 (*A*), and for the H cell (*B*). The ordinate is the percent of cells with a particular phenotype; the abscissa indicates the age of the embryo; *n* is the total number of cells examined. The order of appearance of these properties is constant for these four identified cells, although the H cell is shifted about 1 day later. While the sequence is constant, there is some variability in the precise timing of appearance of any one phenotype.

on day 10, to 0·0 (less than 0·001) on day 12·5. The measured R_{in} increases from a mean of 71 MΩ on day 10, to 374 MΩ in uncoupled cells on days 12–13. To eliminate the possibility that the lower R_{in} was responsible for the reduced degree of excitability on days 10–12, we electrically isolated single neurones from many of their neighbours by selective destruction of the latter (achieved by impaling cells one at a time and stirring vigorously). As shown in Table 2*B*, such 'isolated' somata between days 10–12 has a much higher R_{in} than coupled cells during the same period; this R_{in} was usually within the range recorded in uncoupled cells between days 12–13. However, none of these 'isolated' somata demonstrated overshooting action potentials. In fact, all

demonstrated the typical stage of excitability for that particular embryonic age, whether it be for cells that are normally inexcitable on days 10 or 11, or for cells that normally generate axon to neurite action potentials on day 11·5.

Variability in development of electrical excitability

The first three progeny of the median neuroblast (DUM 3,4,5; 4,5; and 5) all proceed through the same temporal sequence in the development of electrical excitability: from no action potential to the axon action potential to the neurite action potential to the soma action potential (Fig. 1). However, there is variability in the precise time of appearance of the individual stages in this sequence. For a particular neurone, the stage of excitability is not identical in every embryo of the same developmental age. For example, when we examined DUM 4,5 on day 11, we found that in about 50 % of the embryos it already generated an axon action potential in normal saline while in the other 50 % of the embryos it was inexcitable in normal saline. In most of the latter cases, however, the axon could generate an action potential in the presence of TEA. The variability of DUM 3,4,5; 4,5; and 5 appears similar from days 10–13, and thus data from these three cells are combined in Fig. 7*A*. On day 10·5, none of the cells are excitable; on day 12·5, all of the cells are fully excitable. However, on days 11, 11·5, and 12, all three cells show variability in their degree of excitability.

Timetable of excitability for progeny of MP3 compared to a median neuroblast

Of the two progeny of MP3, only the H cell develops an overshooting soma action potential. The temporal sequence for the H cell appears identical to the first three progeny of the median neuroblast, and also appears to occur in a similar length of time: one to two days. However, the timetable for the H cell is shifted 0·5–1 day later (Fig. 7). For example, the first signs of excitability appear on day 11 for DUM 4,5 and on day 11·5 for the H cell. The greatest percent of cells with only an axon action potential appears on day 11 for DUM 4,5 and on day 12 for the H cell. The H cell sib develops only an axon action potential, and does so at about the same time (day 12) as does the H cell. The excitability of the H cell sib does not progress beyond this stage.

PART II. DEVELOPMENT OF UNCOUPLING

Timing of uncoupling of progeny of the median neuroblast

DUM 3,4,5; 4,5; and 5 are inexcitable and strongly electrically coupled to each other on day 10·5 (Fig. 8*A*). They can have coupling coefficients (J) as high as 0·8 on days 9·5–10, but by day 10·5 this coupling has begun to decrease, to values as low as 0·3 (0·4 in Fig. 8). The earliest we have observed the total uncoupling among the first three progeny is on day 11. In most embryos, however, these cells are still coupled on day 11 (Fig. 9). A typical example of coupling on day 11·5 is shown in Fig. 10. The two cells, DUM 4,5 and DUM 3,4,5; both generate neurite action potentials and have a high R_{in} (330 MΩ for DUM 4,5). Coupling is still present but at reduced strength; $J = 0{\cdot}27$. Values of J from 0·15–0·30 are typical for cells that are still coupled on day 11·5, but occasionally cells are sufficiently strongly coupled that action

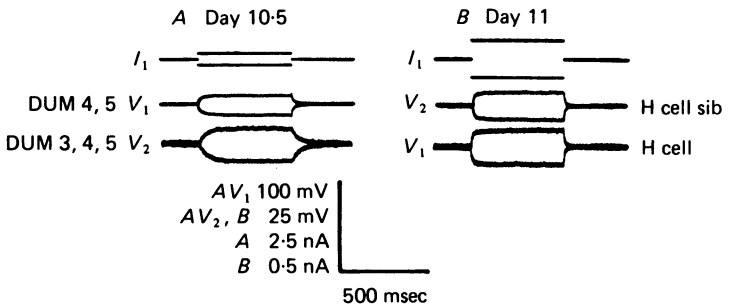

Fig. 8. Electrical coupling of identified neurones in 10·5 and 11 day embryos; the cells are not yet able to generate action potentials. *A*, V_1 is DUM 4,5; V_2 is DUM 3,4,5. $J = 0{\cdot}4$. *B*, V_1 is the H cell; V_2 is the H cell sib. $J = 0{\cdot}8$. Resting potentials are shown for V_1; values for V_2 are similar.

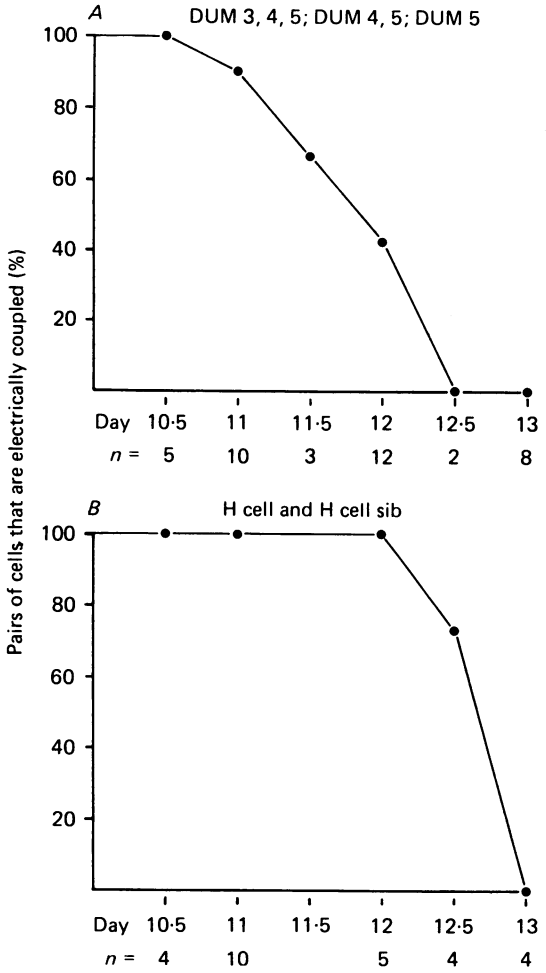

Fig. 9. Percentage of cells that are electrically coupled as a function of embryonic age, for DUM 3,4,5; 4,5; 5 (*A*) and the H cell and its sib (*B*). Cells are scored as uncoupled when the coupling coefficient is $< 0{\cdot}001$; *n* is the number of pairs of cells tested. Although there is some variability in the time of uncoupling of these cells, the H cell and its sib become uncoupled about a day after the progeny of the median neuroblast.

potentials in one cell elicit action potentials in another. In about 50% of embryos at day 12, the first three median neuroblast progeny were totally uncoupled ($J < 0{\cdot}001$ with $R_{in} \geqslant 300$ MΩ); by day 12·5, they were uncoupled in all embryos examined. In those cells still coupled on day 12, J ranged from 0·05 to 0·07 (e.g. Fig. 11). The input resistance of these cells at resting potential ranged from 300 to 450 MΩ.

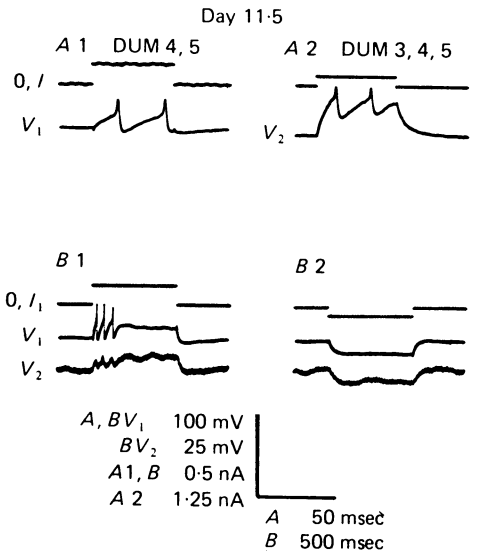

Fig. 10. Electrical coupling of DUM 4,5 and DUM 3,4,5 in a day 11·5 embryo. Both cells fire action potentials in response to depolarizing current pulses (*A*1, 2), and are coupled in both the depolarizing (*B*1) and hyperpolarizing (*B*2) directions.

Variability in development of electrical uncoupling

In a previous section we noted variability in the precise time of appearance of different stages of electrical excitability in individual neurones. A similar degree of variability was observed for the precise timing of electrical uncoupling, as shown in Fig. 9. The onset of excitability and the cessation of coupling are not consistently correlated. We were unable to predict, for example, whether two cells on day 11·5 would be coupled or not by the particular type of action potential that each was able to generate. Conversely, we were unable to predict the particular type of excitability at day 11·5 by whether the cells were coupled or not. On day 12, pairs of cells with overshooting soma action potentials of apparently identical amplitude and shape can be coupled or uncoupled (Fig. 11).

Timetable of uncoupling for progeny of MP3 as compared to the median neuroblast

We have shown that the temporal sequence of development of electrical excitability is similar for the H cell and the first three progeny of the median neuroblast, except that the comparable developmental stages in the H cell occur 0·5–1 day later than in DUM 3,4,5; 4,5; and 5. There is a similar shift of one day in the time of cessation of electrical coupling (Fig. 9). On day 11, the coupling coefficient between the H cell and the H cell sib is still 0·8, but it is only about 0·4 among the first three progeny

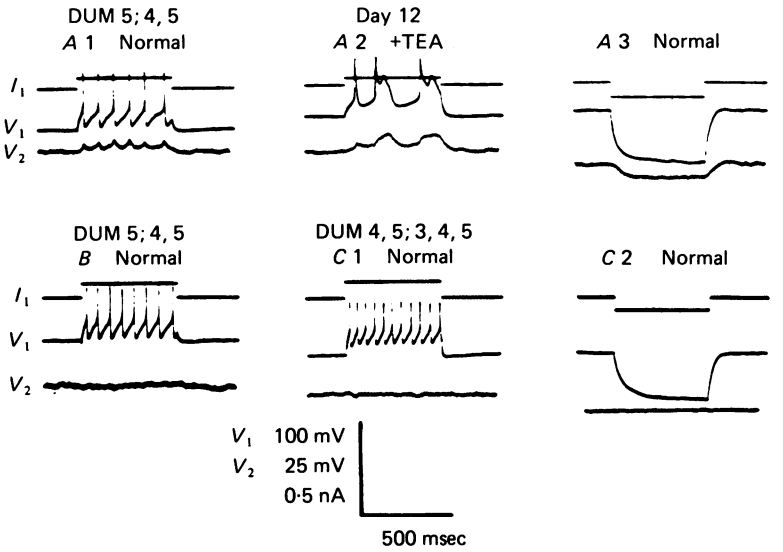

Fig. 11. Variability in the degree of coupling observed in day 12 embryos, between the progeny of the median neuroblast. Cells are often weakly coupled ($J = 0{\cdot}07$, *A*), but more commonly are not ($J < 0{\cdot}001$, *B*, *C*). *A* and *B*, V_1 is DUM 5; V_2 is DUM 4,5. *C*, V_1 is DUM 4,5; V_2 is DUM 3,4,5. Records from three preparations.

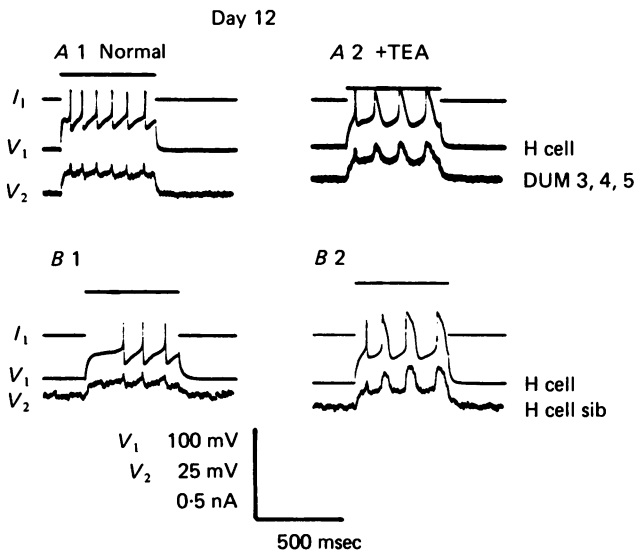

Fig. 12. Coupling between progeny of the MP3 and between MP3 and the median neuroblast progeny in day 12 embryos. The weak coupling is more evident in the presence of TEA, that prolongs the duration of the action potentials elicited in the first cell. *A*, V_1 is the H cell; V_2 is the H cell sib. $J = 0{\cdot}16$ *B*, V_1 is the H cell; V_2 is DUM 3,4,5. $J = 0{\cdot}13$. Records from two preparations.

of the median neuroblast. On day 12, the three progeny of the median neuroblast are either uncoupled or exhibit weak coupling (J = 0·005–0·07) while the H cell and H cell sib are still coupled by about 0·15 (Fig. 12*B*). On day 12·5, we were no longer able to measure coupling among DUM 3,4,5; 4,5; and 5. On day 13, we were no longer able to measure any coupling between the H cell and H cell sib. As with DUM 3,4,5; 4,5; and 5; the precise stage of electrical excitability and the extent of electrical uncoupling between the H cell and H cell sib appear to be independent of one another.

The site of electrical coupling

The undifferentiated progeny of the median neuroblast in early embryos are both electrically coupled and dye coupled with Lucifer Yellow (Goodman & Spitzer, 1979). This high degree of coupling in early embryos must be directly between cell bodies because the cells at this stage lack axons. When the axons of the first three progeny begin to grow out about day 7, these cells become dye uncoupled, although they remain highly electrically coupled. At this stage and for several more days, it is possible that the cell bodies remain directly coupled since they tightly adhere to each other. We do not know whether the cells at these stages are coupled via their processes. However, by day 11, all of the electrical coupling among the cells DUM 3,4,5; 4,5; and 5 is likely to be in their neuropil processes since the cell bodies can be physically separated from each other with micro-electrodes without affecting the coupling. When visualized with Nomarski optics, there do not appear to be direct morphological connections between the somata that have been separated.

Where, then, is the site of the electrical coupling on day 11? It is unlikely that the coupling is along the axons in the peripheral nerves, since all of the cells examined have divergent peripheral morphologies. The coupling probably occurs within their common pathways in the ganglion. One observation suggests that the coupling is from neurite to neurite in the median fibre bundle that contains processes from at least four of the five cells. During the period from days 10–12, the H cell, which arises from a different precursor cell (MP3) and whose soma is separated from those of the progeny of the median neuroblast by over 30 μm on day 11, is coupled to the first three progeny of the median neuroblast (DUM 3,4,5; 4,5; and 5). The coupling coefficient between the H cell and the progeny of the median neuroblast is about the same as those between the progeny at this stage. In the example in Fig. 12*A*, the coupling between the H cell and DUM 3,4,5 on day 12 is about 0·15, a value similar to that between the H cell and H cell sib on day 12 (Fig. 12*B*) and to that among DUM 3,4,5; 4,5; and 5 on day 11·5. The only place where the processes of the H cell and the median neuroblast progeny run together is in the median neurite bundle, between the anterior and posterior commissures (Goodman & Spitzer, 1979; Goodman *et al.* 1979).

DISCUSSION

Relationship of uncoupling to onset of electrical excitability

We have been studying the embryonic development of electrical excitability and electrical uncoupling in five identified neurones that are the progeny of two different precursor cells in the grasshopper. Early during the 20 days of embryonic development,

the two neuronal precursor cells (median neuroblast and MP3) and their undifferentiated progeny are electrically inexcitable and highly electrically coupled. During differentiation, the five identified neurones (DUM 3,4,5; DUM 4,5; DUM 5; the H cell, and the H cell sib) acquire the ability to make action potentials and become electrically uncoupled from each other. In the previous paper (Goodman & Spitzer, 1980), we showed that by day 13 the five cells have acquired their cell-specific action potentials and are fully uncoupled from each other. Although some changes in the action potential occur after day 13, particularly in the duration of the after-hyperpolarization, the Na^+ and Ca^{2+} inward currents and the depolarizing phase of the action potential appear in their mature forms in the axons, median neurite, and soma of each cell by day 13. On day 10, however, the five cells are still electrically inexcitable and electrically coupled. What is the temporal sequence in the development of these two phenotypes?

The onset of electrical excitability occurs between days 10–13; most of the developmental changes in the first three progeny of the median neuroblast occur between days 11–12. We have confirmed and extended our previous observations of electrical coupling (Goodman & Spitzer, 1979). There is a progressive decrease in the coupling coefficient (J) during this period. The earliest we have observed complete uncoupling between DUM 3,4,5; and DUM 5 is on day 11. However, these cells usually become uncoupled between days 11–12, the same period in which they are becoming excitable. Two experimental methods demonstrated that the electrical coupling is not masking excitable inward current channels, and thereby making the cells appear inexcitable. First, exposure to veratridine (Table 1) depolarized only the cells which can generate Na^+-dependent responses in either normal saline or in TEA; it did not change the resting potential of younger inexcitable cells. Secondly, electrically isolating the cells by killing the somata of their neighbours (Table 2B) caused the input resistance to increase to that of normally uncoupled cells, but did not cause a change in the extent of excitability. Thus, the temporal sequence of appearance of voltage-dependent outward current and inward Na^+ and Ca^{2+} currents appears to be accurate and not an artifact of the temporal sequence of uncoupling.

There is variability in the precise temporal relationship of excitability and uncoupling. Some pairs of cells can be fully uncoupled on day 11·5 while pairs in other embryos are still coupled on this day by a coefficient as high as 0·25. Yet, all may generate only neurite action potentials and be incapable, even in TEA, of generating overshooting soma action potentials. Conversely, some pairs of cells can have the same degree of coupling yet generate different types of action potentials. This variable relationship in the timing of uncoupling and changes in excitability further supports the notion that the presence of coupling does not mask the presence of excitability. It also leads us to suggest that these two events are not causally related, but rather that they are two independent events which occur at about the same developmental stage during the differentiation of these cells.

Temporal relationship between axon outgrowth and changes in electrical excitability

From days 10–13, excitability first appears in the axons, then in the median neurite, and finally in the soma. The axons of DUM 3,4,5; 4,5; and 5 grow out the peripheral nerves beginning on day 10. We know the most about the morphological

development of DUM 5 (Goodman & Spitzer, 1979). Its axons reach their peripheral targets, the extensor tibiae muscles, on day 12 and extend over the whole muscles by day 13. DUM 3,4,5 and 4,5 follow a similar morphological timetable. The first non-linear membrane property to appear in these three cells is delayed rectification, on day 11; it can be blocked by TEA, and is probably due to a voltage-dependent outward current. Na^+-dependent responses to voltage changes first appear in the axons about day 11–11·5, and then in the median neurites about 12 hr later. Na^+-Ca^{2+} dependent action potentials can be evoked from somata in normal saline by days 12–12·5. As soon as a particular part of the neurone (i.e. axon, neurite, soma) is capable of generating an action potential in normal saline, the ionic dependence and the depolarizing phase of that action potential appear to be in their mature form. Thus, when the axons reach and extend over their peripheral targets on day 13, the neurones are fully excitable and fully uncoupled.

The two progeny of MP3 (the H cell and H cell sib) follow the same temporal sequence in the development of excitability and uncoupling as do the first three progeny of the median neuroblast. However, the timetable for the H cell begins about 1 day later. The time of axonal outgrowth occurs in the H cell with a similar one day delay as compared to DUM 3,4,5; 4,5; and 5 (day 8 *vs.* day 7; see Goodman & Spitzer, 1979; Goodman *et al.* 1979). Thus, the temporal sequence of axonal outgrowth, the onset of electrical excitability, and the cessation of electrical coupling is similar for five identified neurones that are the progeny of two different embryonic precursor cells.

Gradual appearance of electrical excitability

Our recordings suggest that voltage-dependent channels in a particular part of a neurone appear gradually rather than all at once. For example, shortly before an overshooting soma action potential can be evoked in normal saline, the addition of TEA unmasks the inward current channels and results in an overshooting soma action potential. When the overshooting soma action potential first appears in normal saline, the addition of TEA leads to a long-duration Ca^{2+}-dependent plateau. However, this plateau is not overshooting at first and can be elicited only when the membrane potential is depolarized from the resting potential (-60 mV) to -40 mV. A short time later, the addition of TEA results in an overshooting Ca^{2+} plateau that can be elicited at resting potential. We interpret these developmental changes as indicating the sequential appearance of increasing numbers of active inward current channels in the soma membrane. We have no evidence for changes in outward current during this one day period.

Comparisons with other neurones

How general is the temporal sequence of excitability and uncoupling which we have observed in the embryonic neurones of grasshoppers? The development of excitability and uncoupling of neurones during embryogenesis has also been studied in the spinal cord of the frog, *Xenopus laevis*. Rohon–Beard neurones become electrically excitable a substantial time after their final DNA synthesis, and presumably after the outgrowth of processes (Spitzer & Spitzer, 1975; Lamborghini, 1980; Baccaglini & Spitzer, 1977). Excitability begins to appear in the Rohon–Beard cells before they are fully uncoupled; excitability and uncoupling occur during the same period of development (N. C. Spitzer, in preparation). The development of electrical excitability

of the axons of embryonic frog neurones precedes that of the soma by a small time interval (Willard, 1980). All of these observations on the development of these frog neurones are compatible with the sequence we have observed in the identified grasshopper neurones.

However, there is one striking difference between these frog and grasshopper neurones. The ionic dependence and duration of the action potential changes during development of Rohon–Beard neurones (reviewed by Spitzer, 1979). Somata of Rohon–Beard neurones generate Ca^{2+}-dependent action potentials at early times of development, Ca^{2+}-Na^{+}-dependent action potentials at later times, and predominantly Na^{+}-dependent action potentials at the latest times examined (Spitzer & Baccaglini, 1976; Baccaglini & Spitzer, 1977). A similar sequence may occur in the somata of dorsal root ganglion neurones in *Xenopus* (Baccaglini, 1978). Furthermore, both the cell bodies and the axons of frog neurones in dissociated cell cultures undergo a similar series of changes in the ionic dependence and duration of the action potential (Spitzer & Lamborghini, 1976; Willard, 1980).

In grasshopper neurones that are the progeny of the median neuroblast and MP3, we have not observed this type of qualitative change in the ionic dependence of the action potential. From the first appearance of the soma action potential about day 12 until hatching on day 20, the ionic dependence and depolarizing phase of the action potential remain relatively unchanged, although some less dramatic changes do occur. For example, on day 13, either Na^{+} or Ca^{2+} inward currents alone can generate an overshooting action potential; in contrast, by day 18, neither inward current alone can generate an overshooting response. This change may represent a decrease in the specific membrane resistance, or alternatively, a change in the density or properties of the inward current channels. After day 12, there are also changes in the duration of the after-hyperpolarization. However, these changes are not qualitatively as great as those observed in amphibian neurones.

Thus, while the temporal sequence of axon outgrowth, onset of electrical excitability, and cessation of electrical coupling appears quite similar for neuronal development in both grasshopper and frog, there is one difference. The ionic dependence of the action potential changes dramatically during development in one case, but not in the other. The different types of neurones may simply be following different intrinsic programmes leading to the same mature phenotypes: functional neurones which are fully excitable and fully uncoupled. Alternatively, the difference between grasshopper and frog neurones may be related to some special requirement for the differentiation of these cells.

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