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THE APPEARANCE AND DEVELOPMENT OF NEUROTRANSMITTER SENSITIVITY IN *XENOPUS* EMBRYONIC SPINAL NEURONES *IN VITRO*

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SUMMARY

1. We have determined the time of onset and examined some of the properties of neurotransmitter sensitivity in *Xenopus* spinal neurones developing in dissociated cell culture. These cells are initially insensitive, but acquire responses to several agonists over a period of 6 h.

2. Nearly one-third of the neurones were depolarized by γ-aminobutyric acid (GABA) or by both GABA and glycine; these cells were not affected by glutamate. The reversal potential of the ionophoretic GABA response is -35 mV. These neurones are likely to be Rohon–Beard neurones. Roughly two-thirds of the neurones were depolarized by glutamate and hyperpolarized by GABA and by glycine. The reversal potential of the ionophoretic GABA response is -58 mV. These neurones are likely to include motoneurones.

3. A quantitative measure of the sensitivity to a given GABA dose was obtained at early and intermediate stages of development. The mean 'sensitivity index' (ionophoretic sensitivity/input resistance) for both classes of neurones *in vitro* was initially the same as that seen in Rohon–Beard neurones *in vivo*. This sensitivity index did not increase with time in culture to attain the value at intermediate stages *in vivo*.

4. The development of chemosensitivity in Rohon–Beard-like neurones in these cultures resembles that of Rohon–Beard neurones in the spinal cord with respect to the time of onset of responses to GABA, the reversal potential, pharmacology and desensitization of these responses, and the spectrum of agonists to which they are sensitive. It differs in the absence of a developmental increase in sensitivity to GABA. The development of chemosensitivity in motoneurone-like neurones in these cultures parallels that of Rohon–Beard-like neurones, with respect to the time of onset and level of sensitivity, as well as susceptibility to pharmacological blockers.

5. Several features of normal neurotransmitter sensitivity, like features of the action potential, differentiate in culture in the absence of normal cellular interactions.

INTRODUCTION

Neurones, like other cell types, undergo characteristic sequences of developmental changes during differentiation *in vivo*. How the expression of these developmental

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'timetables' is controlled is a central issue in developmental biology. Descriptions of the sequence of neuronal differentiation have been obtained to varying extents for the dorsal unpaired median interneurones of the grasshopper thoracic ganglia (Goodman & Spitzer, 1979, 1981; Goodman, O'Shea, McCaman & Spitzer, 1979), the primary sensory antennal neurones of the moth (Sanes & Hildebrand, 1976*a*, *b*, *c*; Schweitzer, Sanes & Hildebrand, 1976), and the Rohon–Beard neurones of the frog spinal cord (Baccaglini & Spitzer, 1977; Spitzer, 1982; Bixby & Spitzer, 1982). These descriptions are a necessary preliminary to investigations of developmental mechanisms.

The ability of cells to differentiate in culture is useful, especially when this development parallels that seen in the animal. In culture one can manipulate the environment in ways difficult to achieve *in vivo*. The differentiation of electrical excitability, electrical uncoupling and neurotransmitter sensitivity are the most fully characterized aspects of the early development of Rohon–Beard cells. The development of electrical excitability apparently proceeds in the same way and with the same time course when spinal neurones differentiate *in vitro*, as demonstrated by an examination in dissociated cell culture of populations of cells which seem to include Rohon–Beard neurones (Spitzer & Lamborghini, 1976; Willard, 1980). This culture system can be used in asking whether the development of neurotransmitter sensitivity is similarly undisturbed by growth in a controlled environment.

We have recently shown that Rohon–Beard cells become responsive to γ-aminobutyric acid (GABA) at the early tail-bud stage of development, and have described the properties of this response through the first week of life (Bixby & Spitzer, 1982). Some of the Rohon–Beard neurones acquire glycine sensitivity at the same stage, but lose this sensitivity by 3·5 days, so that of the compounds tested 'mature' Rohon–Beard cells respond only to GABA. In this paper we describe the extent to which spinal cord neurones in dissociated cell culture (apparently including Rohon–Beard cells) mimic this differentiation *in vivo*. An abstract of some of these findings has appeared (Spitzer & Bixby, 1982). The fact that *in vitro* development of neurotransmitter sensitivity in spinal neurones closely approximates that seen *in vivo* suggests a considerable degree of autonomy in the early developmental timetable of these cells. In addition, the similarity in the differentiation of these neurones in culture to that seen *in vivo* makes attractive further experiments in which normal development might be perturbed by an altered environment.

METHODS

Cell cultures

Embryos of *Xenopus laevis* were obtained by standard breeding procedures and staged according to Nieuwkoop & Faber (1956). Dissociated cell cultures were prepared by methods adapted from those of Spitzer & Lamborghini (1976). Briefly, stage 14 embryos were incubated for *ca.* 1 h in dissecting solution containing antibiotics (mycostatin, 200 u. (GIBCO); gentamycin sulphate, 200 μg/ml (Sigma)). At stage 15 they were removed from their jelly coats and the posterior neural plates and underlying mesoderm were dissected with jewellers' forceps. This tissue was rinsed in disaggregating solution and incubated in this solution until dissociated (10–15 min). Cells from a single embryo were plated on the bottom of a 60 mm Petri dish (Falcon tissue culture plastic) in 3–4 ml culture medium. Dissecting solution, disaggregating solution, and culture medium were those described previously (Willard, 1980), except that culture medium contained 10 mM-$CaCl_2$ and

no added serum albumin (Blair, 1983). Cultures were incubated at 22 °C. The age at which a neurone was examined is given as the hours after plating unless otherwise specified. In many cases when early events (6–9 h *in vitro*) were to be examined, intact sibling embryos of the same stage as those dissected were incubated at the same time as the cultures. This was necessary as development did not always proceed according to the schedule of Nieuwkoop & Faber (1956). Where a discrepancy existed between real time *in vitro* and developmental age as assessed by examination of sibling embryos, results were considered to be from the age corresponding to the stage of these embryos. Neurones were identified as cells with phase-bright cell bodies which extended neurites with growth cones. Experiments were performed from May through October, 1982.

Recording conditions

Cultures were viewed with a compound microscope with phase-contrast optics and a 40× water-immersion lens. Recordings were made in normal saline, of the following composition (mM): NaCl, 125; $CaCl_2$, 10; KCl, 3; HEPES, 5 (pH 7·4), which was perfused continuously. Blocking agents (tetrodotoxin (TTX), 10^{-6} g/ml; $CoCl_2$, 10 mM; $LaCl_3$, 0·5 mM; and TEACl, 20 mM) were added to normal saline without compensation for changes in osmolarity. Cells were impaled with glass micro-electrodes filled with 1 M-potassium acetate (100–250 MΩ). Measurements of currents, voltages and conductances, determinations of reversal potentials, and criteria for acceptance of data were as described in Bixby & Spitzer (1982). 'Na^+/Ca^{2+}' spikes were distinguished from 'Ca^{2+}' spikes by the presence of a prominent peak preceding the plateau (see Baccaglini & Spitzer, 1977).

Drug application

Neurotransmitter candidates and antagonists (Sigma) were applied to the cells by bath application or ionophoresis (Bixby & Spitzer, 1982). After the time of onset of sensitivity to GABA the absence of sensitivity to various agonists was accepted only when a cell responded to bath application of this neurotransmitter. In a few experiments, negative braking current was passed through the ionophoretic electrode to test the assumption that the high resistance (1000–2000 MΩ) of the pipettes prevented leakage. Only small increases in drug sensitivity were effected by this procedure (mean of 10%), and it was not routinely used.

RESULTS

The cultures contain a variety of cell types, including nerve, muscle, skin and fibroblast-like cells, as previously described (Spitzer & Lamborghini, 1976; Willard, 1980). Neurones first extended neurites at 5·5–6 h *in vitro*, and essentially all cultures of this age or older contained recognizable nerve cells. Most cultures retain some neurones for $\geqslant$ 3 days, but few neurones survive for longer periods.

Onset of GABA sensitivity

Neurones examined at the earliest times *in vitro* (6–7·5 h) exhibit no response to bath application of 100 μM-GABA (Fig. 1). GABA sensitivity (both depolarizing and hyperpolarizing responses) appears gradually during the next several hours in culture, so that more than half the cells respond at about 10 h, and virtually all neurones are sensitive to GABA by 12 h (e.g. Fig. 2*A*). This schedule for the acquisition of transmitter sensitivity is indistinguishable from that found earlier for the Rohon–Beard neurones *in vivo* (χ^2 test, Bixby & Spitzer, 1982; see Fig. 1*B*). On the basis of our results *in vivo*, we suggested the possibility of a causal relationship between the acquisition of GABA sensitivity and the acquisition of a prominent Na^+ component in the action potential; these events are temporally closely related for developing Rohon–Beard cells. The differentiation of the action potential in these cultured neurones parallels that of the Rohon–Beard cells (Spitzer & Lamborghini, 1976; Willard, 1980), leading to a similar correlation between the acquisition of GABA

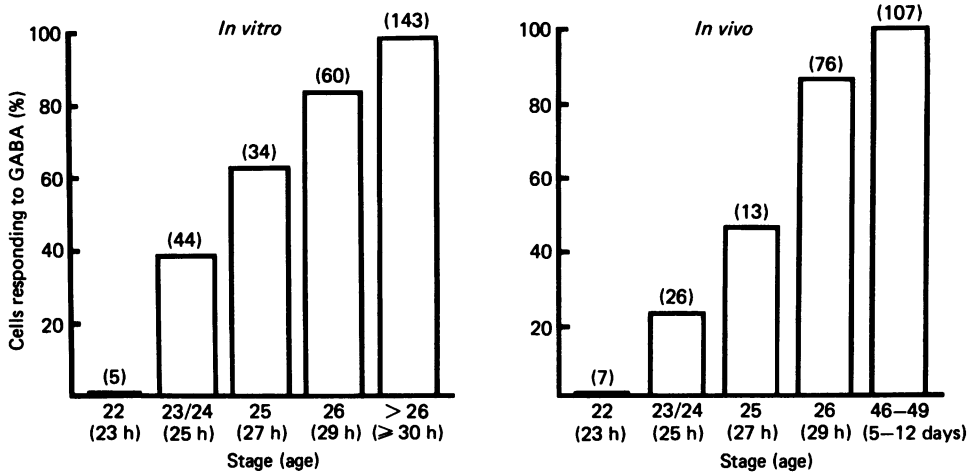

Fig. 1. The time course of acquisition of sensitivity to GABA in spinal neurones *in vitro* and in Rohon–Beard neurones *in vivo*. The percentage of cells responding to bath application of 100 μM-GABA is indicated as a function of the Nieuwkoop & Faber stage and equivalent age (hours or days since fertilization). The number of cells tested at each time is indicated in parentheses.

TABLE 1. Relationship of the presence of a GABA response to the ionic dependence of the action potential (a.p.) The data were obtained from Rohon–Beard neurones *in vivo* (Bixby & Spitzer, 1982), and from a more diverse population of spinal neurones *in vitro*, around the time of onset of GABA sensitivity

	No GABA response, Ca^{2+} a.p.	GABA response, Ca^{2+} a.p.	No GABA response, Na^+ component in a.p.	GABA response, Na^+ component in a.p.
In vivo	29	2	2	10
In vitro	26	1	9	40

responses and the Na^+ component of the impulse *in vitro*. A causal relationship now seems unlikely, however, for the correlation is not strict *in vivo*, and even less so in the present experiments (Table 1).

Responses to other transmitters

Some neurones in these cultures become sensitive not only to GABA, but also to two other amino acid neurotransmitters, glycine and glutamate. There are three categories of cells. The first is depolarized by GABA, with no response to glycine or glutamate (all at 100 μM; Fig. 2*A*). The second group of cells is similar to the first, except that the cells are depolarized both by GABA and by glycine (not illustrated). The third category is hyperpolarized by GABA and by glycine, and depolarized by glutamate (Fig. 2*B*). These results indicate that some of the neurones in the cultures exhibit a spectrum of sensitivity like that of Rohon–Beard cells (depolarized by GABA, sometimes by glycine; Bixby & Spitzer, 1982), while the others are like motoneurones (hyperpolarized by GABA and glycine, depolarized by glutamate; Nicoll, Padjen & Barker, 1976). Of the 135 cells for which satisfactory tests were made

(either by bath application or ionophoresis of the amino acids), thirty-nine, or 29%, were Rohon–Beard-like, and ninety-six, or 71%, were motoneurone-like. Identification of motoneurone-like cells was further substantiated in that action potentials elicited from them frequently caused impulse-locked twitching of striated muscle cells contacted by neuronal processes. Of those classified as Rohon–Beard-like, five

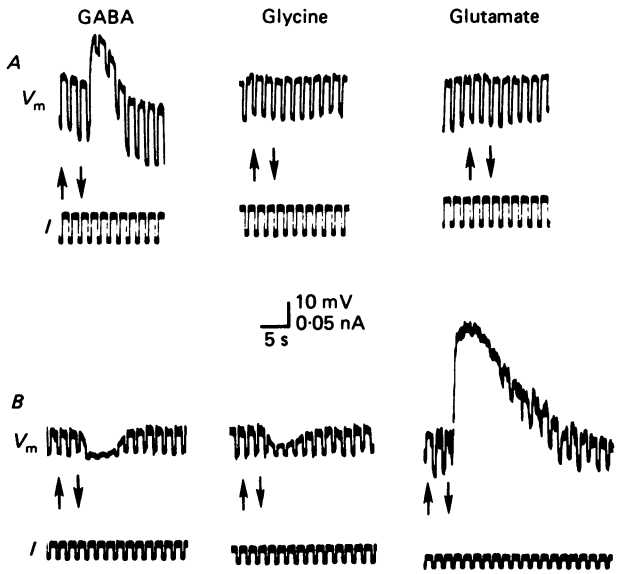

Fig. 2. Responses of cultured spinal neurones to bath application of neurotransmitters (100 μM; arrows). Records from two cells; upper trace is membrane potential, V_m; lower trace is injected current. The latency of the responses is due to the lag time of the perfusion system. *A*, a cell depolarized by GABA with an associated conductance increase is unaffected by glycine and glutamate. Resting potential −54 mV; 12 h. *B*, a cell is hyperpolarized by GABA and glycine and depolarized by glutamate; all responses involve increases in conductance. Resting potential −46 mV (V_m was held at −76 mV during application of glutamate); 12 h.

out of twelve (42%) responded to glycine, and seven out of twelve (58%) only to GABA. This distribution is similar to that seen for Rohon–Beard neurones at early times *in vivo* (45% respond to glycine at stage 26; Bixby & Spitzer, 1982). The disappearance of the glycine sensitivity of Rohon–Beard-like cells *in vitro* was not studied.

The histogram in Fig. 1 includes data from both motoneurone-like and Rohon–Beard-like cultured neurones, as we saw no difference between these two populations in the time of onset of GABA sensitivity. The two populations of spinal neurones become sensitive to GABA at about the same developmental stage. The initial acquisition of sensitivity to several transmitters occurs around the same time but does not proceed with complete synchrony. We saw examples, at 6–12 h *in vitro*, of neurones which were depolarized by glutamate but unresponsive to GABA or glycine or both (nine out of thirty-eight cells). No neurone of these categories was seen in older cultures (twenty-seven cells). We cannot define a generalizable order for the acquisition of sensitivity to the three amino acids.

For the most part, the cultured neurones do not respond to bath application of other neurotransmitters tested. No change in membrane potential or conductance was elicited by 5-hydroxytryptamine, dopamine, octopamine, histamine or noradrenaline (100 μM). This was true both at the time the response to GABA is first acquired (10–12 h; $n \geqslant 9$ for each), and at late times *in vitro* (65–72 h; $n \geqslant 5$ for each). However, a few neurones responded to acetylcholine (ACh). Although no response was seen to bath-applied ACh at 10–12 h ($n = 9$), or at 35–45 h ($n = 4$), three of eight neurones tested in five cultures at 65–72 h responded with a depolarization and conductance increase to ACh (data not shown). These three neurones were all in the same culture, and all had overlapping neurites. In addition to their ACh sensitivity, all were hyperpolarized by GABA and glycine, and depolarized by glutamate. They could represent either a late-developing population, or a group of neurones which change their sensitivity. Neurones were also tested for responses to a variety of neuroactive peptides. No change in membrane potential or conductance occurred with bath application of met-enkephalin, substance P, oxytocin, bombesin, lys-vasopressin, arg-vasopressin or vasotocin at 1 day *in vitro* ($\geqslant 20$ μM, $n = 5$). Bath application of met-enkephalin did consistently shorten Ca^{2+}-dependent action potentials, however (20 μM; $n = 4$), as was found for Rohon–Beard neurones *in vivo* (Bixby & Spitzer, 1983).

Reversal potentials of the GABA responses

To provide information about the ionic basis of the GABA responses in these spinal neurones, we measured the reversal potential of the ionophoretically elicited GABA response at 1 and 2 days *in vitro*. This was accomplished by bathing the cells in saline containing TTX, TEA^+ and Co^{2+} to block voltage-dependent currents, changing the membrane potential in either direction with current pulses, and observing the amplitude of the GABA response to brief ionophoretic currents of constant magnitude. Plots were made of the response amplitude as a function of membrane potential, using data only from the linear region of the neurone's current–voltage relation. The reversal potentials determined in this way for all the neurones examined are plotted in a histogram in Fig. 3*A*, and two examples of the reversal potential graphs are shown in Fig. 3*B* and *C*. Fig. 3*A* shows that the distribution of reversal potentials from the neurones in these cultures is bi- or trimodal (data from 1 and 2 day cultures are presented together, as no difference could be seen between them). On the hypothesis that the first group of reversal potentials represents data from Rohon–Beard neurones, and the second and third represent data from motoneurones, we calculated the mean for each of these two groups. The mean of the first group (e.g. Fig. 3*B*) is $-35 \pm 0{\cdot}7$ mV (mean $\pm$ S.E. of mean; $n = 5$), which is not significantly different from that found for mature Rohon–Beard neurones *in vivo* ($-30 \pm 1{\cdot}6$ mV; $P > 0{\cdot}07$, *t* test; Bixby & Spitzer, 1982). This suggests that these cells are Rohon–Beard neurones, and further, that the GABA response is Na^+ and K^+ dependent in these cultured neurones, as is the case *in vivo*. The mean of the remaining reversal potentials (e.g. Fig. 3*C*) is $-58 \pm 9{\cdot}8$ mV ($n = 20$), which is in the range of those found for motoneurones *in vivo* (Curtis, Hösli, Johnston & Johnston, 1968; Krnjević, Puil & Werman, 1977), where the response seems to involve an increase in Cl^- conductance (Nicoll *et al.* 1976). The justification for including the apparent third mode in the

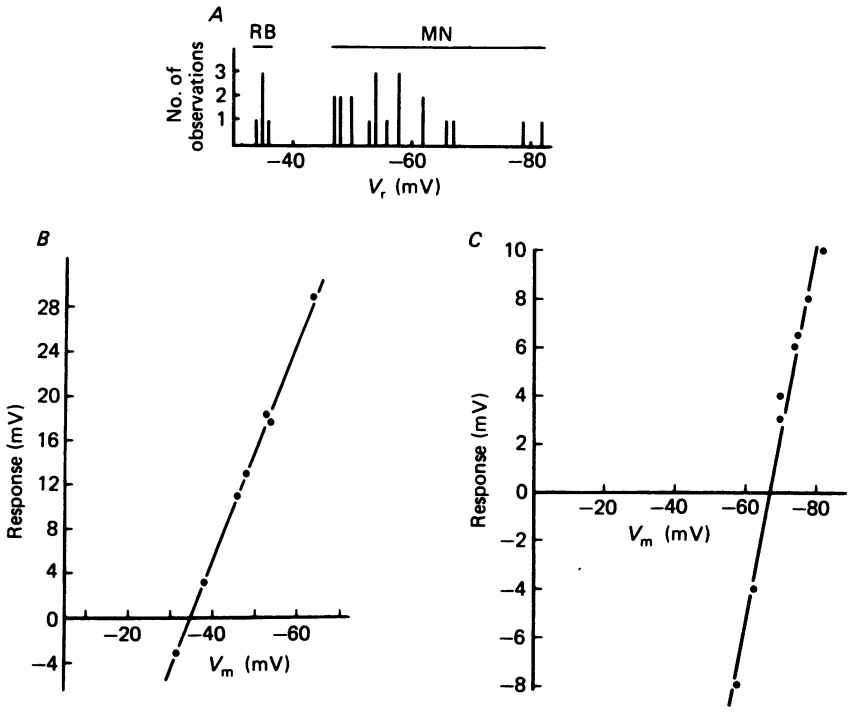

Fig. 3. The reversal potential for GABA in cultured spinal neurones. *A*, the histogram shows the distribution of reversal potentials (V_r) and seems to reveal three modes. The first is designated RB since it is not different from that reported for Rohon–Beard neurones *in vivo*. The second and third are designated MN since the values are close to those reported for motoneurones *in vivo*. *B* and *C*, reversal potentials were determined by measuring the amplitude of the ionophoretic response to GABA at different steady levels of the membrane potential (V_m); voltage-dependent currents were pharmacologically suppressed. Each line is the least-squares fit to the points, and the intersection with the abscissa is the reversal potential. *B* is an example of a Rohon–Beard-like neurone. Resting potential −42 mV; 19 h. *C* is an example of a motoneurone-like neurone. Resting potential −44 mV; 16 h.

second group is its small size and the known dependence of the reversal potential for GABA on the resting potential of motoneurones (Krnjević *et al.* 1977). However, it is possible that these represent a third population of GABA-responsive neurones.

Pharmacology of the GABA and glycine responses

The pharmacology of the amino acid responses in these neurones examined at 1 day *in vitro* is illustrated in Fig. 4. Picrotoxin (5–10 μM; $n = 10$) and curare (25 μM; $n = 5$) blocked the response to ionophoretic application of GABA virtually completely. Strychnine, a glycine antagonist, had no effect on the GABA response (3–4 μM, $n = 5$). In contrast, much lower concentrations of strychnine quickly abolished the response to ionophoretic application of glycine (0·5 μM; $n = 7$), while curare had no effect at high concentrations (50 μM; $n = 8$), and picrotoxin reduced the response only slightly (mean 25%, 10 μM; $n = 7$). Muscimol, a GABA agonist, produced changes in membrane potential and conductance increases similar to those elicited by GABA,

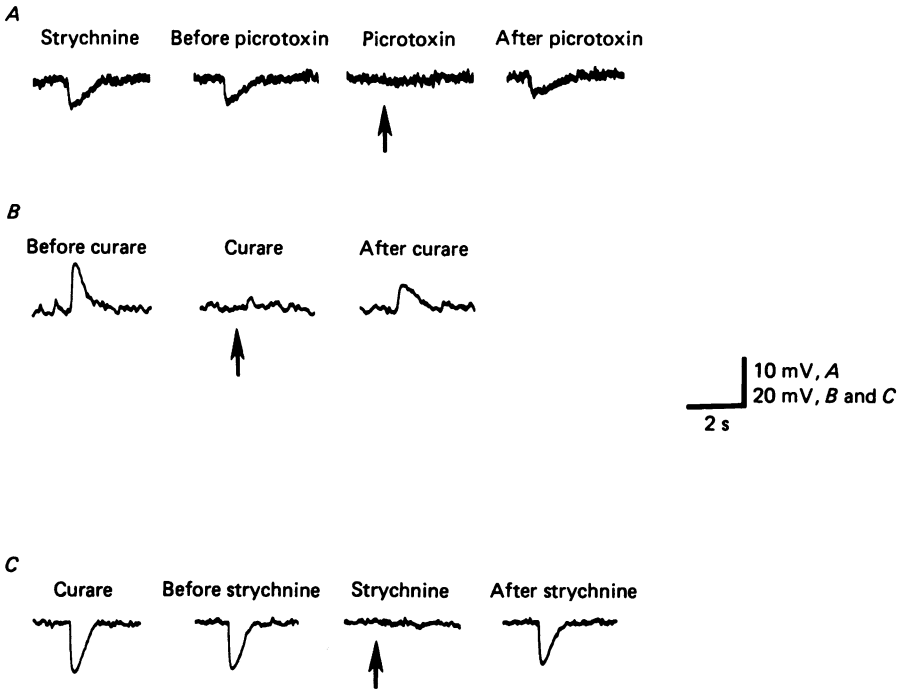

Fig. 4. Pharmacology of responses of cultured spinal neurones to GABA and glycine. Records from three cells. *A*, hyperpolarization by ionophoretic application of GABA is unaffected by bath application of 3 μM-strychnine but blocked reversibly by 5 μM-picrotoxin. Resting potential −50 mV; 20 h. *B*, depolarization by GABA is blocked reversibly by 25 μM-curare. Resting potential −70 mV; 21 h. *C*, hyperpolarization by glycine is unaffected by 50 μM-curare but blocked by 0·5 μM-strychnine. Resting potential −46 mV; 22 h. Arrows indicate time of ionophoretic application when the response is blocked.

in concentrations as low as 1 μM (data not shown). These pharmacological characteristics are similar to those found for Rohon–Beard neurones *in vivo*, and suggest that the GABA and glycine responses are mediated through different receptors or ion channels (Bixby & Spitzer, 1982). Rohon–Beard-like and motoneurone-like cells exhibited indistinguishable pharmacological sensitivities. As expected, we found that compounds affecting voltage-dependent channels (TTX, Co^{2+}, La^{3+}, TEA^{+}) had no effect on the GABA responses of these neurones at 1 day in culture ($n \geqslant 4$ for each).

Desensitization

Prolonged bath application of agonists to these neurones results in a depression of the response (Fig. 5). This desensitization was seen with bath application of GABA to both Rohon–Beard-like and motoneurone-like cells ($n = 5$ for each). The amount of desensitization seen was not the same for different amino acids: GABA responses generally exhibited faster and more complete desensitization than glycine responses, while those to glutamate scarcely desensitized at all during similar periods of application (Fig. 5). Similar observations have been made on adult motoneurones *in vivo* (Barker & Nicoll, 1973; Engberg, Flatman & Lambert, 1979). Desensitization of

the GABA response could also be observed consistently with repeated ionophoretic pulses (40 ms; data not shown), as is the case for Rohon–Beard neurones *in vivo* (Bixby & Spitzer, 1982).

Quantitation of GABA sensitivity during development

A measure of the quantitative level of sensitivity to an agonist can be obtained from the ionophoretic dose–response relation. We initially determined ionophoretic

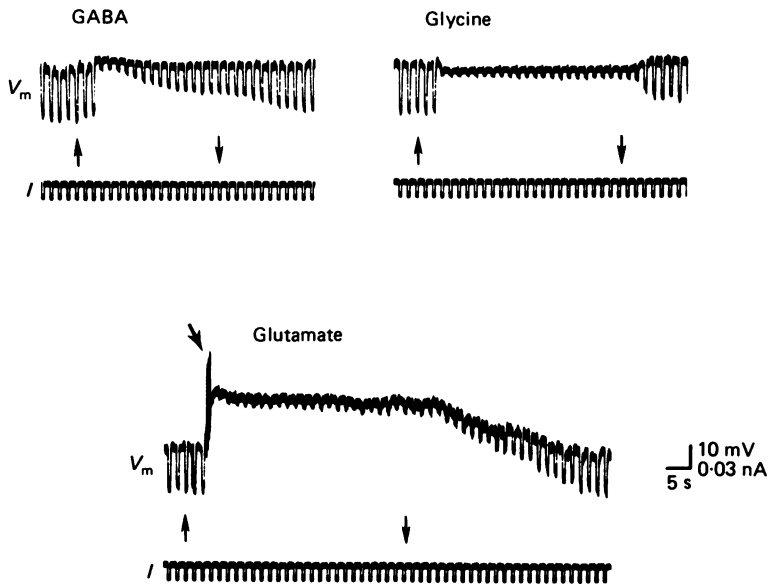

Fig. 5. Desensitization of cultured spinal neurones to bath application of GABA, glycine and glutamate (each at 100 μM). All records from one cell; upper trace is membrane potential, V_m; lower trace is injected current. Time of application of neurotransmitter is indicated by arrows. Glutamate elicits an initial burst of action potentials (arrow). Resting potential −56 mV; 48 h.

sensitivities to GABA for neurones at 10–12 h *in vitro*, the approximate time of onset of GABA sensitivity. The sensitivities (in mV/nC) were taken from the slopes of dose–response graphs, with the membrane potential held at −80 mV. For each cell, this sensitivity was divided by the input resistance to yield the sensitivity index (s.i.; Bixby & Spitzer, 1982). In many cases we made a rough estimate of the reversal potential for GABA, in order to classify neurones as Rohon–Beard-like or motoneurone-like. We saw no systematic difference in s.i. between the two populations, in spite of the larger driving force for GABA responses in Rohon–Beard neurones, and data from all neurones were considered together. The mean s.i. for cultured neurones at this stage is shown in Fig. 6, together with a similar determination of s.i. for Rohon–Beard neurones *in vivo* at the same age (Bixby & Spitzer, 1982). The two are not significantly different ($P > 0{\cdot}1$; t test). Similarly, we obtained a mean s.i. for the neurones grown for 20–65 h in culture, a time period which corresponds to stages 31–42 *in vivo* (Nieuwkoop & Faber, 1956). The s.i. values for spinal neurones *in vitro* and Rohon–Beard cells *in vivo* at this time *are* significantly different ($P < 0{\cdot}01$). There

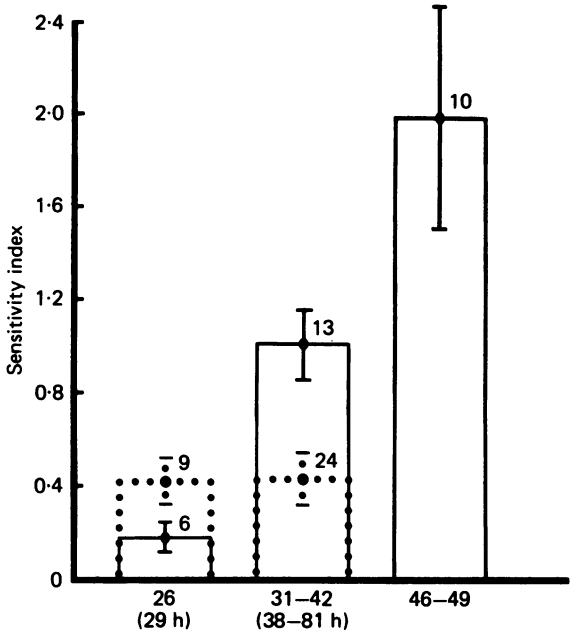

Fig. 6. Development of GABA sensitivity on the cell bodies of cultured spinal neurones. The histogram of the sensitivity index (sensitivity (mV/nC)/input resistance (MΩ); dotted lines) is compared with the values obtained for the cell bodies of Rohon–Beard neurones *in vivo* (continuous lines). Ages are given as the Nieuwkoop & Faber stages and equivalent hours since fertilization. Each point is the mean ± S.E. of mean for the numbers of cells indicated.

seems to be no change in the s.i. as the neurones develop *in vitro*, in contrast to a monotonic increase as the cells develop in the spinal cord. The neurones did not live beyond 4 days in culture, so no measurement corresponding to stages 46–49 (the latest times tested *in vivo*) was possible.

The lower limit of sensitivity is the same as that of mature Rohon–Beard neurones *in vivo*. At 1 day *in vitro*, the spinal neurones responded with a detectable change in both conductance and membrane potential to concentrations of GABA as low as 1 μM (one out of six cells) or, more often, 5 μM (five out of six cells). Again, the two populations (Rohon–Beard-like and motoneurone-like) seemed to behave similarly.

DISCUSSION

Identities of neuronal populations in spinal cord cultures

We believe that the majority of neurones developing in these cultures are either Rohon–Beard cells or motoneurones. These two populations comprise the vast majority of spinal cord neurones that have undergone their final round of DNA synthesis by the end of gastrulation (Lamborghini, 1980), and there is evidence that approximately 80% of the nerve cells in the cultures are 'early birthdate' neurones (Spitzer & Lamborghini, 1976). In addition, the Rohon–Beard-like neurones have a

profile of chemosensitivity which matches that of Rohon–Beard cells *in vivo*, even to the fraction of neurones at early times which respond to glycine, and have a reversal potential for GABA which is not significantly different from that found for Rohon–Beard neurones. The motoneurone-like cells, by the same token, exhibit sensitivity to the same spectrum of neurotransmitters as is reported for motoneurones *in vivo*, with appropriate desensitization characteristics, and a reversal potential for GABA consistent with this identity. It is possible that another population (or populations) of neurones develops in these cultures (e.g. one with ACh sensitivity), but they are in the minority. This evidence suggests that we can identify the majority populations, and distinguish between them on the basis of chemosensitivity.

Comparison of development in vivo and in vitro

The development of chemosensitivity in Rohon–Beard-like cells in dissociated cell culture closely resembles the same process in Rohon–Beard neurones differentiating in the spinal cord. GABA sensitivity is acquired at the same time, and no significant difference was detected in GABA reversal potential, pharmacology, or desensitization between the neurones *in vivo* and *in vitro*. In addition, Rohon–Beard-like neurones acquired sensitivity to GABA (and sometimes to glycine), but not to any transmitters to which they are insensitive *in vivo*. These findings complement earlier results, which showed that the change in ionic dependence of the action potential *in vitro* is the same as that seen *in vivo* (Spitzer & Lamborghini, 1976; Willard, 1980).

The correlation observed between a change in spike morphology (indicating the acquisition of a significant Na^+ component) and the acquisition of GABA sensitivity is essentially the same as that seen in the Rohon–Beard neurones *in vivo* (Bixby & Spitzer, 1982). We suggested previously that a causal relationship might exist which tied together the acquisitions of these two phenotypes. However, as the number of observations increased, so did the number of exceptions. It now seems likely that there is no obligatory relationship between the appearance of these two membrane properties.

The lack of an increase in the level of ionophoretic sensitivity in GABA during development of cells in culture stands in striking contrast to the tenfold increase in the sensitivity index observed in Rohon–Beard neurones *in vivo*. This result is an exception to the general pattern in which the cultured neurones mimic their *in vivo* counterparts. In retrospect, however, this exception might be expected. In the best-studied example of developmental changes in receptor density, the vertebrate neuromuscular junction, it is clear that such changes are under the control of extrinsic influences, such as the externally imposed pattern of activity and the presence of intact innervation (Purves, 1976). This is true even though the initial acquisition of sensitivity (ACh receptors in the muscle membrane) is apparently independent of these influences (Fambrough & Rash, 1971; Ritchie & Fambrough, 1975; Harris, 1981). Thus, the increase in sensitivity may be like later events such as cell death and synapse elimination, which are apparently dependent on cell–cell interactions.

The recapitulation in dissociated cell culture of many normal differentiative events in these neurones implies cellular autonomy in the expression of these phenotypes. Since the neurones are grown at such low densities that neurone–neurone contacts are infrequent, cell–cell surface interactions seem unnecessary for this early development.

Although the culture medium is a fully defined salt solution, a possible effect of diffusible trophic factors has not been excluded. However, the results suggest that the 'program' for early differentiation of these membrane properties is present soon after the birthdate of these neurones.

Properties of motoneurone-like cells during development

If, as seems likely, most of the cells responding to GABA, glycine and glutamate in these cultures are motoneurones, our results afford an opportunity to compare the development of motoneurones *in vitro* to that of Rohon–Beard cells. Development seems to occur in parallel with respect to time of onset of sensitivity, and the level of GABA sensitivity and susceptibility to pharmacological blockade are also quite similar for the two populations. Parallel development of the action potential is suggested by previous studies (Spitzer & Lamborghini, 1976; Willard, 1980). These results imply that the schedule of early differentiation described for Rohon–Beard cells is not unique to these neurones, but may be more generally applicable to early-appearing embryonic spinal neurones.

It is perhaps surprising that the measured ionophoretic sensitivity of the motoneurone-like cells was not detectably different from that of Rohon–Beard-like cells. All the neurones examined were held at −80 mV, which should provide roughly a twofold greater driving force for the Rohon–Beard GABA response than for the motoneurone GABA response. This might not be reflected in the final sensitivity index because motoneurone-like cells have a higher density of receptors than Rohon–Beard-like cells, or because of differences in the properties of single channels. A small difference would not be detected in the scatter of these measurements.

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