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Spontaneous bioelectric activity in long term cultures of the embryonic insect central nervous system

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Ganglia of the nervous system of the cockroach embryo (*Periplaneta americana*) become interconnected by dense fibrillar networks when cultured *in vitro* for long periods in a chemically defined medium[1]. The considerable fiber outgrowth and long survival of the ganglia in culture attests to the vigor of the preparation. In the present report, we describe for the first time the presence of simple and complex forms of spontaneous bioelectric activity in these long term cultures. We also demonstrate the presence of functional interganglionic connectives which develop entirely *in vitro*.

Cultures were prepared and maintained according to the methods developed by Chen and Levi-Montalcini[1]. Ganglia were dissected from embryos removed from oothecae (egg cases) which were incubated for 16 days at 29 °C. These ganglia and frequently either leg or foregut were then transferred onto small glass coverslips which were in culture dishes containing a newly developed nutrient medium[6]. The preparations were cultured at 29 °C for periods ranging from 14 up to 90 days *in vitro* (div).

The types and numbers of cultures which were examined electrophysiologically in the present study were as follows: thoracic ganglia (237), abdominal ganglia (171), frontal ganglia and ingluvial ganglia attached to foregut (18), brain (8), cercal ganglia (23). Fifteen minutes before electrophysiological observations were begun, the nutrient culture medium was replaced by a phosphate buffered cockroach physiological salt solution which had a pH of 7.2 (see ref. 10). This was done in order to control for the possibility that the high Mg^{2+} (7.4 m*M* $MgSO_4$) and amino acid content of the culture medium may influence the bioelectric activity of the cultures. However, most of the single and multiple unit phenomena described in this paper also have been observed in ganglia remaining in the culture medium. In all cases, electrophysiological observations were carried out at room temperature, which was approximately 25 °C.

Standard electrophysiological techniques were used. In most cases, recording electrodes were 3–8 μm glass micropipettes filled with the same salt solution surrounding the cultures. A platinum wire inserted into the shaft of the pipette was connected to a PAR 113 AC coupled preamplifier which yielded a gain of 10,000. All recordings were single ended with a large platinum wire in the medium acting as the ground electrode. The band pass of the system was usually 0.3 Hz–10 kHz. However, the band

width was reduced to 100 Hz–10 kHz in order to take the slow sweep speed photos reproduced in this paper. In those cases where activity was monitored simultaneously from 2 ganglia, either 25 μm glass insulated tungsten electrodes or 5–10 μm indium core pipettes were substituted for the saline pipettes. The experiments were carried out on the stage of either a Zeiss inverted phase contrast microscope or a Wild dissecting scope. The electrodes were guided into place under visual control by means of Leitz micromanipulators.

We used several criteria to select a spontaneously active unit suitable for observation: (1) the firing pattern of a unit must remain unchanged as the electrode is advanced, and (2) a unit must be held for at least 5 min. Most units encountered easily met the above criteria. In fact, many units could be held for 20 min or more. All spike activity reported here is clearly different in discharge pattern from the rapidly firing units which were obviously the result of electrode advance. Electrical activity similar to that reported here was never observed when the recording electrode was out of contact with the cultured neurons.

Spontaneous unit electrical activity was recorded from all types of neural tissue examined. This includes embryonic brain and thoracic, abdominal, frontal, ingluvial and cercal ganglia. However, most of our observations were made upon thoracic and abdominal ganglia. All recordings were made from ganglia. No attempts have been made to record from the fibers growing out of the ganglia. Photographs of representative culture preparations which were electrically active are presented in Fig. 1. As can be seen in the figure, most of the ganglia were interconnected with their neighbors by means of nerve bundles which were formed *in vitro*. A cross section of a characteristic thoracic ganglion which was maintained for 36 div is shown in Fig. 1c. The typical insect ganglionic cellular organization which is characterized by a rind of cells surrounding a central neuropil was maintained *in vitro*. In many cases, prominent bundles of connective fibers linked adjacent ganglia. Note the case in Fig. 1b and the long interconnected chain of ganglia in Fig. 1a.

Culture bioelectric activity ranged from simple single unit discharges to complex multiunit bursts. Examples of single unit discharge patterns are shown in Fig. 2. Continuously active units with regular interspike intervals were found in all types of ganglia. Units with this pattern of firing were particularly prominent in the ingluvial (Fig. 2b) and frontal ganglia where they constituted the majority of all activity. The thoracic and abdominal ganglia had a much wider variety of firing patterns, including continuously active units with both regular (Fig. 2a) and irregular (Fig. 2f) interspike intervals. A few units present in thoracic ganglia were characterized by doublet and triplet firing patterns (Fig. 2c). Another more commonly found type of unit discharged primarily in bursts of many spikes. Units of this type were most frequently found in the thoracic (Fig. 2d, e) and abdominal ganglia. Bursting units usually discharged periodically at intervals ranging from seconds to minutes. Except during interludes of bursting, continuously active units observed in the present study had relatively low rates of firing, ranging from an upper value of about 10/sec down to 0.2/sec or less.

The record of burst activity from the thoracic ganglion shown in Fig. 3a is of special interest because it clearly depicts the discharges of both a prominent single unit

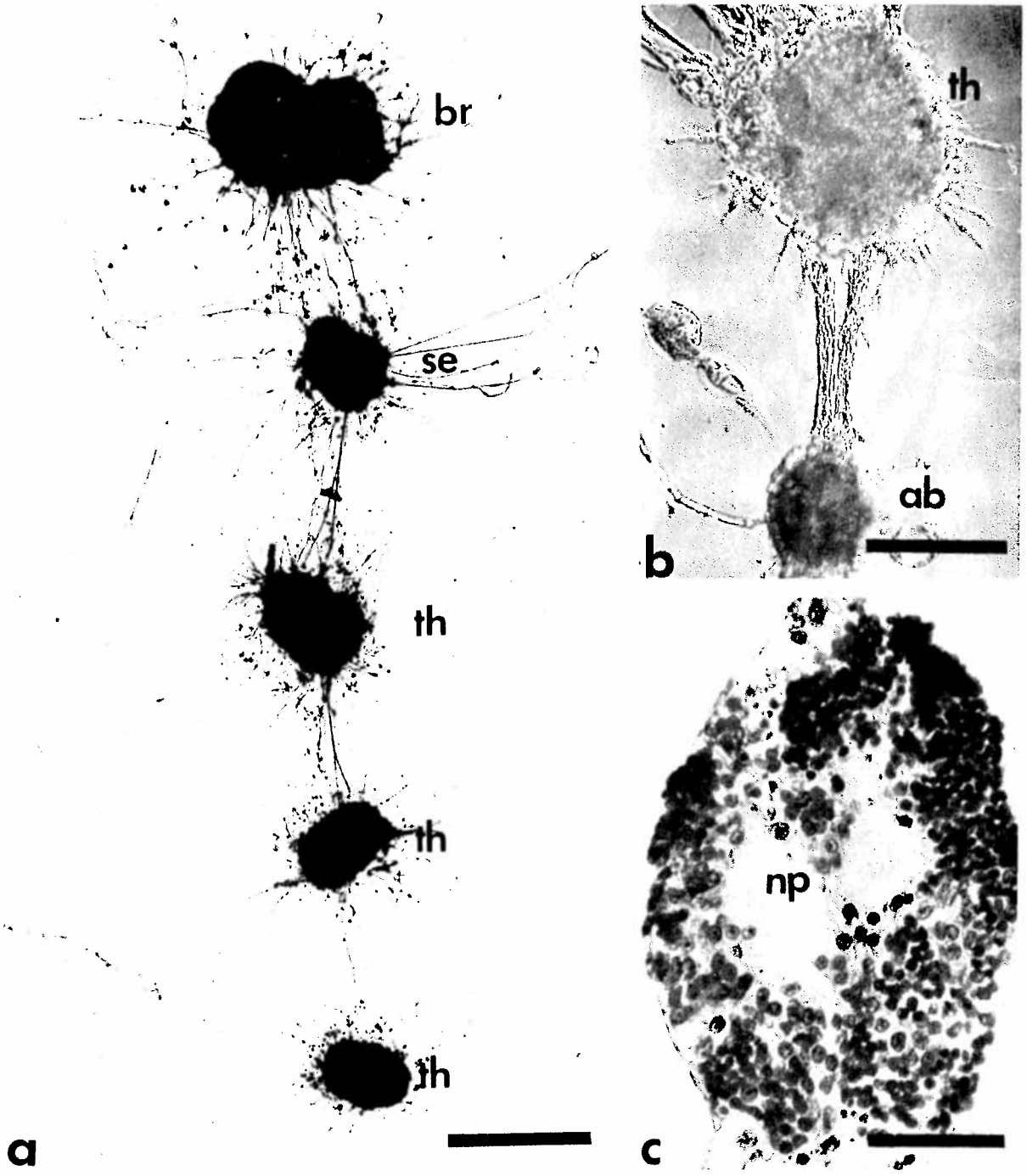

Fig. 1. Examples of bioelectrically active cultures. a: interconnected chain of brain (br), subesophageal (se), and 3 thoracic ganglia (th) maintained 23 div, silver stain. Scale is 500 μm. Fixation procedures resulted in considerable shrinkage of the interganglionic connectives. b: thoracic (th) and abdominal (ab) ganglia (23 div) coupled by large fiber bundles, Nomarski photograph. Scale is 140 μm. c: cross section of thoracic ganglion maintained 36 div, toluidine stain. Central neuropil (np) is surrounded by a cortex of cell bodies. Scale is 65 μm.

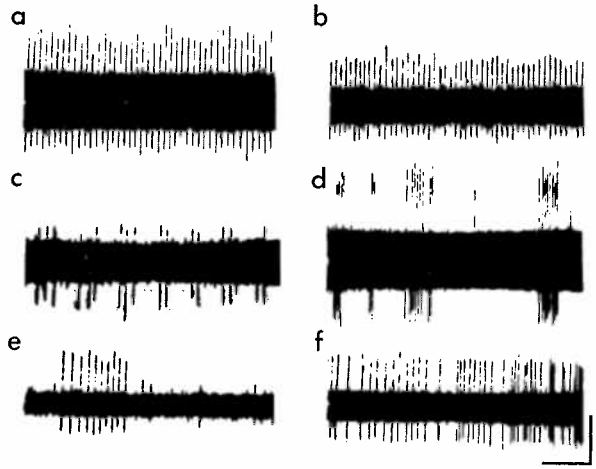

Fig. 2. Patterns of single unit discharge. a: unit with regular interspike intervals recorded from the thoracic ganglion (36 div) shown in Fig. 1c. Time scale is 2 sec. b: units with regular interspike intervals recorded from an ingluvial ganglion (45 div). Time scale is 1 sec. c: unit from 30 div thoracic ganglion with doublet and triplet firing pattern. Time scale is 4 sec. d: prominent burst firing single unit recorded from a 31 div thoracic ganglion. Time scale is 0.4 sec. e: burst firing single unit from a 38 div thoracic ganglion. Time scale is 0.4 sec. f: unit with irregular pattern of interspike intervals recorded from a thoracic ganglon (27 div). This unit initiated activity about 30 sec before the multiunit burst which is shown at the far left. After the burst, the unit became inactive for 3–4 min, after which the cycle was resumed. Time scale is 6 sec. Amplitude scale is 50 μV except in a and d, where it is 100 μV.

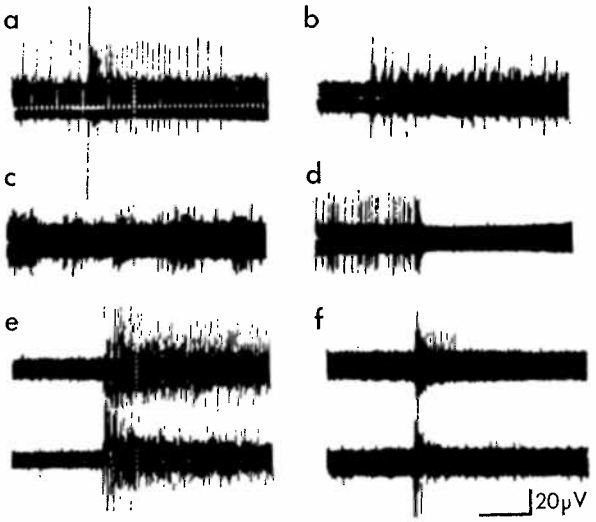

Fig. 3. Patterns of multiunit discharge. a: multiunit burst discharge with a prominent single unit which becomes active before the population discharge. Recorded from a thoracic ganglion of a chain (30 div) similar to that of Fig. 1a. Time scale is 10 sec. b: complex multiunit burst with many oscillatory after discharges recorded from a thoracic ganglion (40 div) which was a member of a ganglionic chain. Time scale is 4 sec. c: periodic burst discharges recorded from an abdominal ganglion (28 div) which was a member of a ganglionic chain. Bursts do not have the rapid onsets characteristic of the burst discharges from thoracic ganglia which are shown in a and b. Time scale is 15 sec. d: unit in thoracic ganglion (27 div) which is characterized by irregular interspike intervals. The unit began firing seconds before a multinuit burst which is shown in the center of the sweep. Activity of the unit was temporarily terminated after the burst. Time scale is 0.2 sec. e: multiunit bursts recorded at 2 points separated by 100 μm within a single thoracic ganglion (27 div). Time scale is 0.2 sec. f: multiunit burst recorded simultaneously from 2 adjacent thoracic ganglia (29 div) 650 μm apart which were interconnected by a prominent fiber bundle. Time scale is 0.4 sec.

and a multiunit burst. The unit became active a few seconds before a multiunit burst and then fired a rapid barrage of spikes in concert with the burst. The unit then remained quiescent for several minutes until it again became active before the next multiunit burst. This cycle of events repeated itself for 30 min. Two other examples of this commonly observed pattern of firing are shown in Figs. 2f and 3d. Both began firing with irregular interspike intervals about 30 sec before a multiunit burst. Also, both units stopped firing immediately after a burst. An example of a post-burst cessation of firing is shown in Fig. 3d. The finding that these units became active prior to the multiunit burst is open to several interpretations. The units may either act as pacemakers for the multiunit activity or be responding to a source of input common both to them and to the bursting population, but responding at a lower threshold. The latter explanation seems to be the most probable because several units at any given recording site often show similar post-burst refractoriness followed by a slow build-up in firing frequency until the occurrence of the next burst. This finding is most easily accounted for by assuming that a general increase in excitability within a ganglion triggers a burst when some threshold value is exceeded.

The single, relatively brief, multiunit bursts of Fig. 3a, d and f have simple burst envelopes. In contrast, other bursts had complex, multimodal envelopes and lasted from seconds to tens of seconds. A long lasting burst of the complex type recorded from a thoracic ganglion is presented in Fig. 3b. This burst is oscillatory in nature. It is initiated by a single high amplitude discharge which is followed by multiple bursts which occur at a frequency of about 1/sec and slowly decrease in amplitude until the activity dies out. Crain[2,3] has also reported oscillatory discharges in his tissue culture preparations of the vertebrate nervous system.

Experiments carried out with 2 recording electrodes indicated that there was always a high degree of correlation between multiunit burst discharges observed at different points within single abdominal and thoracic ganglia (Fig. 3e). Correlated burst discharges (Fig. 3f) were also observed in adjacent thoracic and abdominal ganglia which were connected by prominent fiber bundles which spanned distances of hundreds of microns (Fig. 1a, b). The latter finding indicates that fiber tracts which were formed entirely *in vitro* are functional in transmitting information from one ganglion to another. Similar results were obtained by investigators[2,3] in other laboratories using explants of the vertebrate central nervous system.

The correlation between discharges in interconnected ganglia ranges from high to low between different ganglionic pairs and even varies widely over time within a given pair of ganglia. For example, a high amplitude multiunit burst in one ganglion may be accompanied by only the discharge of a few spikes in its neighbor, while minutes later, both may show synchronous high amplitude discharges. In other cases, a long oscillatory discharge in one ganglion may be correlated with only a single short burst in another. Also, there is evidence of directional information flow in some ganglionic pairs. This is suggested by instances where ganglion A consistently evokes activity in ganglion B but seldom vice versa. Future, more refined studies must examine whether sophisticated interganglionic communication comparable to that

present in the adult arthropod nervous system[9] can develop and be maintained in our *in vitro* preparations.

In several experiments, the usual physiological salt solution was replaced with a similar solution containing 25 m*M* $MgSO_4$. The multiunit bursting and interganglionic transmission was eliminated in all cases. This finding is consistent with the interpretation that the synchronization of units during a multiunit burst was due to interganglionic synaptic processes. The addition of Mg^{2+} also reduced but did not eliminate single unit activity in the cultures. Several single units showed a particularly interesting response to Mg^{2+}; they switched from a bursting to a continuous discharge pattern with regular interspike intervals of about 1/sec. Although the effect is open to other interpretations, the transformation from a bursting to a continuous pattern of discharge in the presence of Mg^{2+} suggests that presynaptic processes may be influencing the patterning of activity in the recorded unit. In the absence of this supposed source of presynaptic input, the unit shows a continuous pattern of spontaneous firing. A similar switch from bursting to continuous activity in response to Mg^{2+} has also been reported in tissue culture preparations of the vertebrate cerebellum[4,8].

The present study establishes the existence of both simple and complex forms of spontaneous unit electrical activity in cultures of the embryonic insect central nervous system. It also demonstrates that spontaneous discharges may be propagated from one ganglion to its neighbors by means of neurite bundles which are formed entirely *in vitro*. These results concerning the physiological properties of our culture preparations, when considered together with previously documented findings of vigorous morphological differentiation *in vitro*[1,5], suggest that the cultures may be a promising model system in which to study problems related to the development, electrophysiology and pharmacology of the invertebrate central nervous system. Furthermore, the previously noted similarities between the present findings and those reported for vertebrate cultures suggest that certain basic properties such as the capacity of isolated parts of the central nervous system to develop and maintain spontaneous electrical discharges *in vitro* are shared by widely divergent phyla.

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