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Short Communications

Emergence of geometric patterns in insect nerve nets: an *in vitro* analysis

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Ganglia from the central nervous system (CNS) of cockroach embryos produce an initial radial pattern of nerve fiber outgrowth after being dissected free from the ganglionic chain and explanted on clean glass coverslips submerged in a chemically defined liquid nutrient medium[1–3,6]. A previous study from our laboratory indicated that interactions between fibers growing out from adjacent ganglia in conjunction with an increase in fiber tension play an important role in fasciculation and the formation of substantial, straight, interganglionic connectives[6]. It was also shown that connectives are formed between rows of ganglia without regard to the axial orientation of adjacent ganglia or whether the ganglia normally form connections *in vivo*. In the present paper, we demonstrate further that striking geometrical patterns emerge from two-dimensional arrays of abdominal and thoracic ganglia explanted and incubated on clean microscope coverslips in a liquid medium.

Cultures were prepared using either thoracic or abdominal ganglia which were dissected from the ganglionic chains of 16-day-old cockroach embryos of the species *Periplaneta americana*. (Hatching occurs at 30 days when embryos are incubated at 29 °C.) Individual ganglia were gently pressed upon a clean cover glass at the bottom of a small culture vessel containing a chemically defined liquid medium[3] and incubated for from 1 to 6 weeks at 29 °C in an atmosphere of 5% CO_2 and 95% air, according to the method of Chen and Levi-Montalcini[1]. The various patterns in which ganglia were arranged are described in the text. Explanted ganglia were usually separated by 300–700 μm, a distance sufficient to prevent fusion of adjacent ganglia and reduce, but not eliminate, the number of interganglionic connectives which are formed. At the end of the incubation period, cultures were fixed and stained using a modified silver technique[2].

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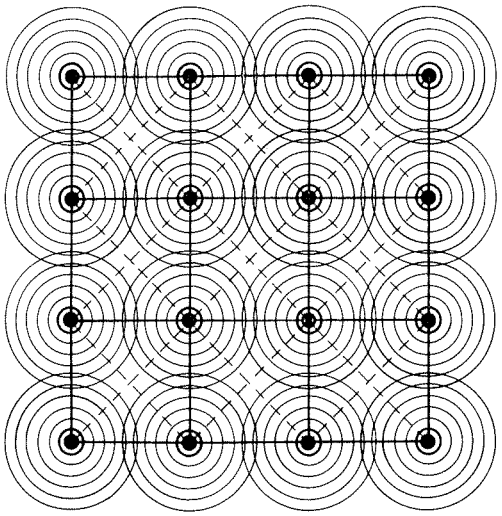

Fig. 1. Estimate of strong (solid lines) and weak (broken lines) interactions between nerve fibers randomly growing away from ganglia arranged in a 4 × 4 array.

Previous investigations indicated that isolated embryonic ganglia produce a radial, random pattern of nerve outgrowth[6]. The radial pattern is also the first phase of outgrowth from ganglia which will later form connections with neighboring ganglia. If embryonic ganglia are explanted in a 4 × 4 matrix and initial fiber outgrowth takes place in a random, radial pattern as represented by the series of concentric circles in Fig. 1, the probability of contact being made between fibers growing out from adjacent ganglia or between a given fiber and ganglion would be proportional to the degree of overlap between intersecting circles. In Fig. 1, strong (high probability) interactions are represented by solid lines, while weaker (low probability) interactions are indicated by broken lines. The diagram shows that connections between horizontally and vertically aligned ganglia are more probable than between more distant, diagonally opposed ganglia. We will now examine how well this probability model fits the experimental results.

Fiber outgrowth was monitored daily with a Zeiss inverted phase contrast microscope. The preliminary radial outgrowth phase of a 10-day *in vitro* array of thoracic ganglia arranged in the 4 × 4 pattern diagrammed in Fig. 1 is shown in Fig. 2a. Note the absence of linear fiber processes between adjacent ganglia. A more mature 21-day *in vitro* culture of abdominal ganglia which is also arranged in a 4 × 4 array is shown in Fig. 2b. Observe how a matrix configuration emerges out of the intermingling fibers, as illustrated in Fig. 2a. Slowly, as a function of development, cultured ganglionic arrays come to approximate the result suggested by the model of Fig. 1. More striking examples of emergent geometric patterns are shown in the 28- and 34-day *in vitro* preparations of abdominal ganglia presented in Fig. 2c and d. The 'square' shown in Fig. 2d is a small part of an equally impressive complete 4 × 4 matrix which unfortunately detached from the cover glass during fixation. Initial fiber outgrowth from the ganglia of the latter culture as well as those shown in Fig. 2b and c showed the same apparent disorder as the early result depicted in Fig. 2a. A secondary

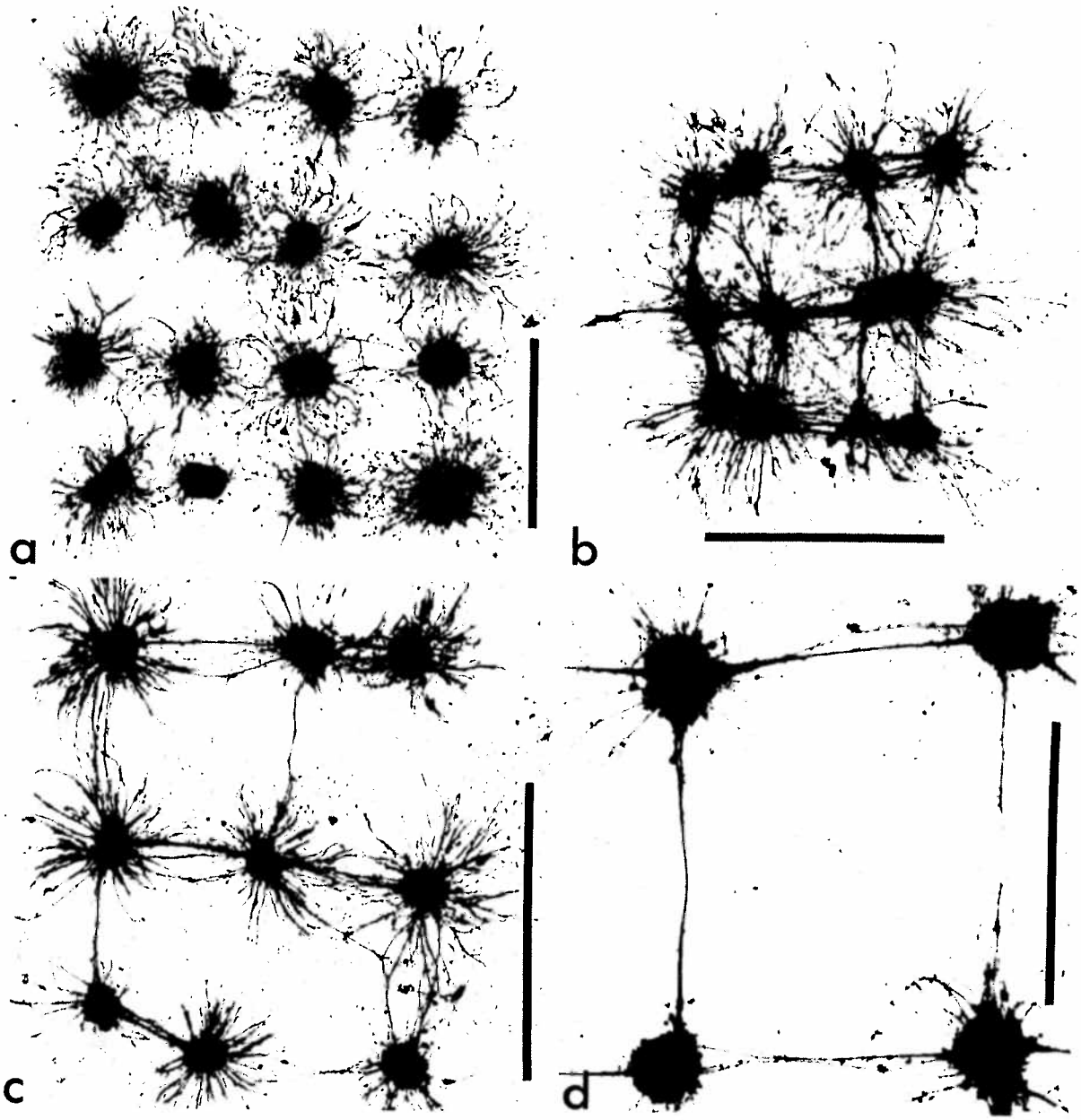

Fig. 2. Stages in nerve outgrowth in ganglionic arrays. a: early, random period of outgrowth in thoracic ganglia, 10-day *in vitro*. b: beginning of matrix formation in an array of abdominal ganglia 21-day *in vitro*. c: matrix formed from 3 × 3 array of abdominal ganglia, 28-day *in vitro*. d: 'square' formed between abdominal ganglia, 34-day *in vitro*. Fibers not forming connections were resorbed. Scale equals 1 mm except in d where it equals 0.6 mm.

process helps to make the geometric configurations such as that shown in Fig. 2d stand out more clearly. Many fibers which do not form connections with adjacent ganglia are retracted, or, in the case of some older cultures, degenerate.

The patterns of interganglionic connectives shown in Fig. 2 represent a very small sample of the immense variety of possible open and closed configurations. Other examples are shown in Fig. 3a and b. The 'triangle' of Fig. 3a demonstrates that connectives can be formed in a configuration of ganglia different from the columns

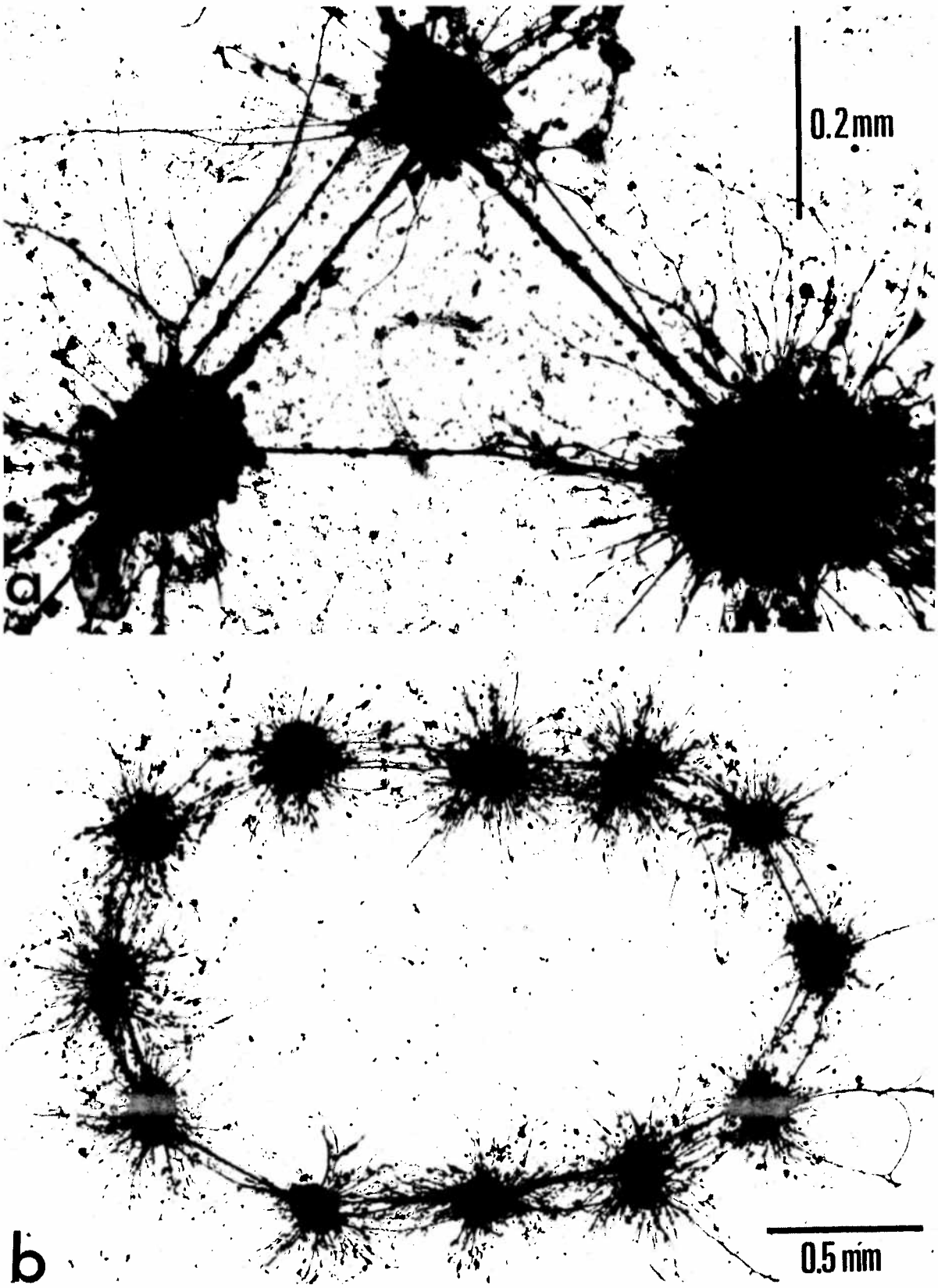

Fig. 3. a: a 'triangular' configuration of neuronal connectives which was formed between abdominal ganglia after 30 days *in vitro*. b: an 'ellipse' formed from 12 interconnected abdominal ganglia after 30 days *in vitro*.

and rows of the 4 × 4 matrix (Fig. 2) which bears at least a distant resemblance to the *in vivo* chain arrangement of ganglia in the embryonic nerve cord. The 'ellipse' of Fig. 3b is another possible ganglionic configuration.

The steps involved in the formation of linear interganglionic connectives are similar to those described for connective formation between single rows of ganglia[6]. An initial phase of random, radial nerve fiber outgrowth leads to chance fiber–fiber interactions between ganglia or to contacts between single fibers and ganglia. The radial fiber pattern serves as an ideal 'fiber catcher' for fibers growing out from neighboring ganglia. Contacting fibers adhere and grow toward each others' perikarya in the adjacent ganglion. An increase in fiber tension is correlated with the pulling in of initially bowed connectives to form substantial, straight fascicles. An increase in fiber tension is indicated by the findings that straightened connectives lift up off the growth surface and directly span the liquid medium between ganglia and that such straightened fibers vibrate when their culture dish is tapped. In fact, it is difficult to imagine how flexible, slender fascicles which often consist of only a few fibers could span distances between ganglia as great as 1 mm and remain perfectly straight if they were not taut. The required fiber tension may be provided by a shortening of connective fibers, a process which has been observed in *Lepidoptera*[4].

A previous study has indicated that most insect ganglia are capable of forming structural connections with other ganglia regardless of their type or axial orientation[6]. Different types of ganglia have also been shown to produce an initial radial pattern of fiber outgrowth and go through similar steps in the formation of connectives. While we do not wish to equate all outgrowth properties of different types of ganglia, their many similarities in regard to fiber outgrowth and connective formation make it useful to consider them as *fiber sources*. Likewise, target ganglia may be thought of as *anchorage points*. Fibers growing out from a given source must make contact with an anchorage point (terminal adhesion), which in the present paper is another ganglion, or they will not undergo straightening. Suitable anchorage points are also often made by a terminal fiber plexus on the glass growth surface or between a fiber and any other tissue. Fibers which do not form such contacts are reabsorbed. The combined processes of straightening of those fibers, which form connections and reabsorption or degeneration of those which do not, yield substantial linear connectives which stand out in bold relief on the coverslip, leaving few clues as to their relatively disordered beginnings.

In the system described above, linear processes gradually emerge which link fiber sources and anchorage points with the initial placement pattern of ganglia determining the range of permissible connections. Thus, straight lines, squares, triangles, and circles are a few of the possible results. However, fibers from the ganglia of many cultures do not resolve into the relatively simple and well defined patterns shown in this paper. If ganglia are spaced very closely, so many adhesions are formed between fibers impinging from different directions that long, straight processes seldom occur. Instead, much more complex geometrical structures emerge with single fibers having many crooks and bends which were formed in response to the different fibers pulling on them. If, in contrast, ganglia are placed too far apart to permit connective forma-

tion, the pattern of fiber outgrowth never advances beyond the radial stage, and the fibers are eventually reabsorbed or degenerate.

The present experiments clearly show that geometrical patterns need not be forbidden, *a priori*, as products of developing nerve nets. The patterns of outgrowth which we present are particularly impressive because the embryonic cockroach nervous system *in vitro* has the capacity to form well defined long and tightly packed fascicles. Weiss[8] has previously reported that linear connectives develop between embryonic chick spinal ganglia which are explanted in plasma clots. However, the patterns of connectives which are produced by the vertebrate material are less well defined than those on which we report. In other experiments, Weiss[7] produced more clearly defined geometrical figures, but these were achieved by growing nerves along grooves on a scratched mica surface instead of on smooth glass which was used in the present experiments.

The culture preparation which we describe has several properties which may be of interest to the neuronal modeler. Since the explanted ganglia are electrically active and discharges are propagated from ganglion to ganglion[5,6], the effect which different patterns of connectivity have upon the spatial and temporal patterning of electrical activity in nerve nets can be examined. The cultures also provide an interesting model system for developmental processes. In the present study, we examined the effect of spatial patterning of ganglia upon the formation of connectives. Several related problems pertaining to spatial patterning deserve further attention. One such problem is the effect which temporal phasing of fiber outgrowth has upon connective formation. For example, if ganglia were arranged in a matrix pattern such as that in Fig. 1 and different ganglia in the pattern produced maximal outgrowth at different times, the pattern of connectives would probably be very different from that obtained in Fig. 2b–d, where outgrowth from ganglia was relatively synchronous. Differential phasing of neuron outgrowth could be achieved by explanting ganglia at different times. The results from such studies may provide insight into developmental processes *in situ* which usually have different phase relationships. Another problem worthy of further study is the effect which substrate properties have upon the pattern of nerve outgrowth. In the present study, the use of an obstruction-free, glass growth surface allowed stressed nerve fibers to move toward mechanical equilibrium and assume the geometric tension structures which we describe in Figs. 2 and 3. If we would impose constraints upon the system in the form of a stickier substrate or a substrate with more texture or with mechanical obstructions, we would probably produce nerve nets very different from those which we presently describe, even if we would begin with identical patterns of explanted ganglia.

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