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Synaptogenesis in Reaggregating Brain Cell Culture

(embryonic mouse/electron microscopy/axons/synapses/myelin)

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ABSTRACT **Dissociated cells from embryonic mouse brain reassociate in rotation culture to form highly organized aggregates. Electron microscopic observations of aggregates show the presence of synapses that mature and increase in number during culture. Apparent myelination of axons is observed in the aggregates after 5 weeks of culture. Thus, brain cell aggregates perform two highly specialized morphological events that are characteristic of mouse brain differentiation.**

In vitro studies of synapse formation and myelination of brain tissues have been limited to explants, where established cell–cell contacts are maintained (1–3). The genetic and environmental factors that govern synaptogenesis and myelination may be more easily resolved by the study of events occurring in cultures of dissociated cells. This approach has been used in the study of neuromuscular junctions (4, 5) and retinal synapses (6).

Dissociated embryonic brain cells reassociate in rotation culture to form aggregates. Reaggregating mouse brain cell cultures show a high degree of cellular organization (7) and patterns of biochemical differentiation (8) that are very similar to the *in vivo* counterpart. The specific activities of choline acetyltransferase (EC 2.3.1.6), acetylcholinesterase (EC 3.1.1.7), and neuronal glutamate decarboxylase (EC 4.1.1.15) increase up to 20-fold during culture of the aggregates (8). These phenomena suggest that brain cell cultures may also undergo morphological differentiation characteristic of brain maturation. In this report, the development of morphologically mature synapses and myelinated structures in brain cell aggregates is described.

METHODS

Cell Dissociation. C57B1/6 mice, 16-days pregnant, were the source of fetuses for cell culture. The whole brains were removed and finely diced. The tissue was dissociated in 0.25% trypsin (Difco 1:250) in Saline 1 (138 mM NaCl, 54 mM KCl, 1.1 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 0.01% $CaCl_2$, and 0.4% glucose) and incubated at 37°C with constant rotation for 20 min. The tissue was triturated several times and incubation was continued for an additional 15 min. The suspension was triturated several times and passed through a nylon screen to complete its dissociation into single cells. After the addition of 10% fetal-calf serum, the cells were collected by centrifugation.

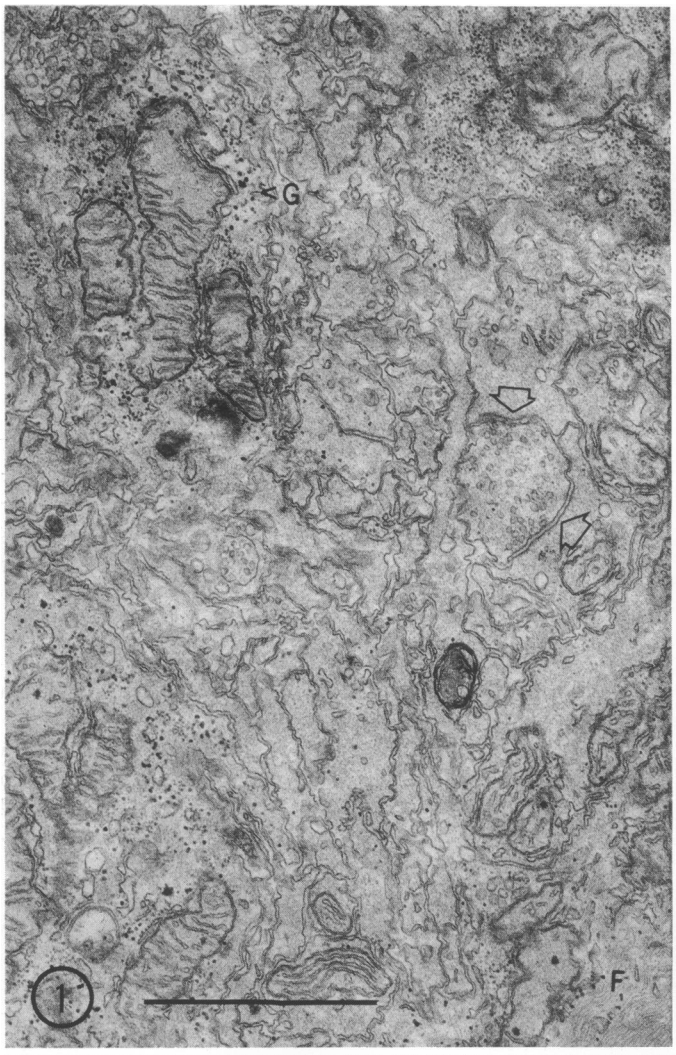
Cell Culture. 25-ml Erlenmeyer flasks containing 3.5 ml of basal Eagle's medium with 0.4% glucose and 10% fetal-calf serum were inoculated with 1–2 $\times$ 10^7 cells. The flasks were gassed with CO_2–air 5:95 and incubated at 37°C with constant rotation (70 rpm). After 3 days, the cultures were transferred to 50-ml flasks and 5 ml of fresh medium, containing 15% fetal-calf serum, was added. The culture medium was replaced with fresh basal Eagle's medium containing 0.4% glucose and 15% fetal-calf serum at 2-day intervals.

Electron Microscopy. Tissue cultured aggregates and suspensions of cultured cells were treated initially by the addition of 0.5 ml of cold fixative to 2 ml of culture medium containing the specimen. The cell preparations were preserved with a freshly prepared mixture of 2.5% glutaraldehyde–1% osmium tetroxide 1:2 in cacodylate buffer (pH 7.4) (9). Suspended cells were immediately sedimented. The pellet remained intact during subsequent procedures and was treated in the same manner as the aggregates. After the initial 4-min fixation in the presence of culture medium, the mixture was removed and fresh fixative was added. The specimens were fixed for 40 min, rinsed in cold physiological saline, and post-fixed for 1 hr in cold 1% aqueous uranyl acetate. They were then dehydrated in a series of hydroxypropyl methacrylate–water mixtures and embedded in Epon. Sections were stained with lead acetate for 2 min and examined in a Phillips EM-300 electron microscope.

RESULTS

Cell suspensions of whole fetal brain from 16-day-old mice examined after trypsinization and screening contained fewer than 1% of the cells in clusters of two or more. Suspensions of these dissociated cells prepared for electron microscopy showed that regions of contact between cells were without any specialization of cellular membranes suggestive of synapse formation. Most of the clusters are probably cells that have aggregated subsequent to their separation.

Cell reaggregation is completed within 24 hr, and is followed by a period of cell migration (7) and biochemical differentiation (8). Fig. 1 is a section through a portion of an aggregate of cells that has been cultured for 12 days. Asymmetrical junctional complexes that appear morphologically normal are found scattered in the cell aggregate. These junctional complexes contain relatively few synaptic vesicles (250–350 Å). A synapse typical of those at this stage is shown in Fig. 1. A 200-Å gap separates the pre- and post-synaptic membranes. The concentration of opaque material usually associated with the faces of opposing synaptic membranes is absent, and the cell membranes are wavy. These features suggest that the junctional complexes are not completely formed. Glycogen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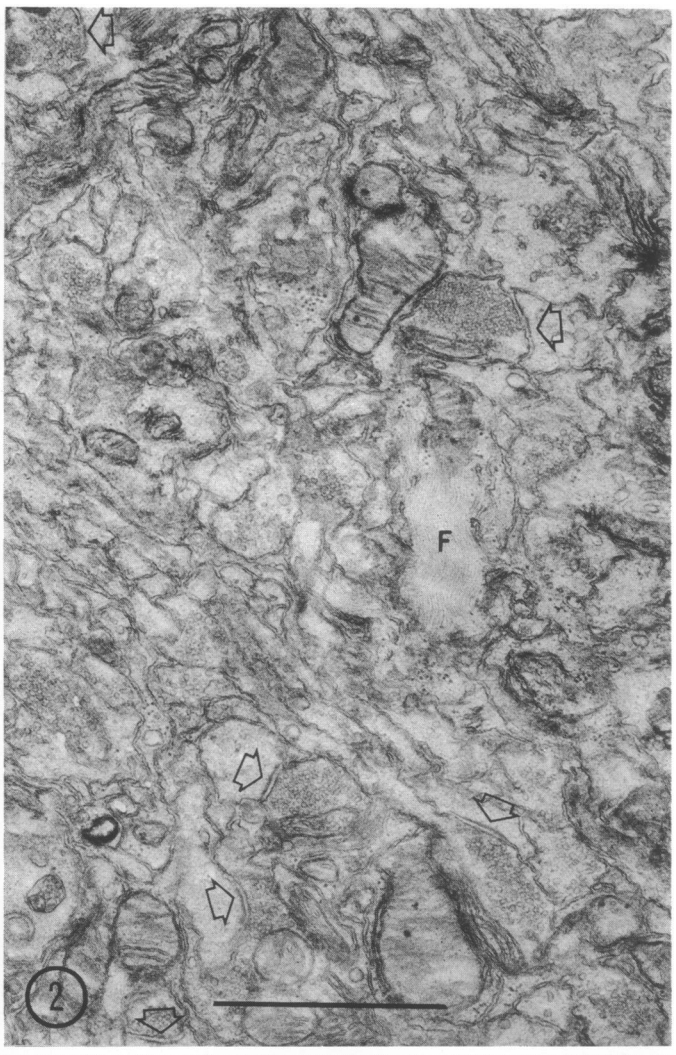
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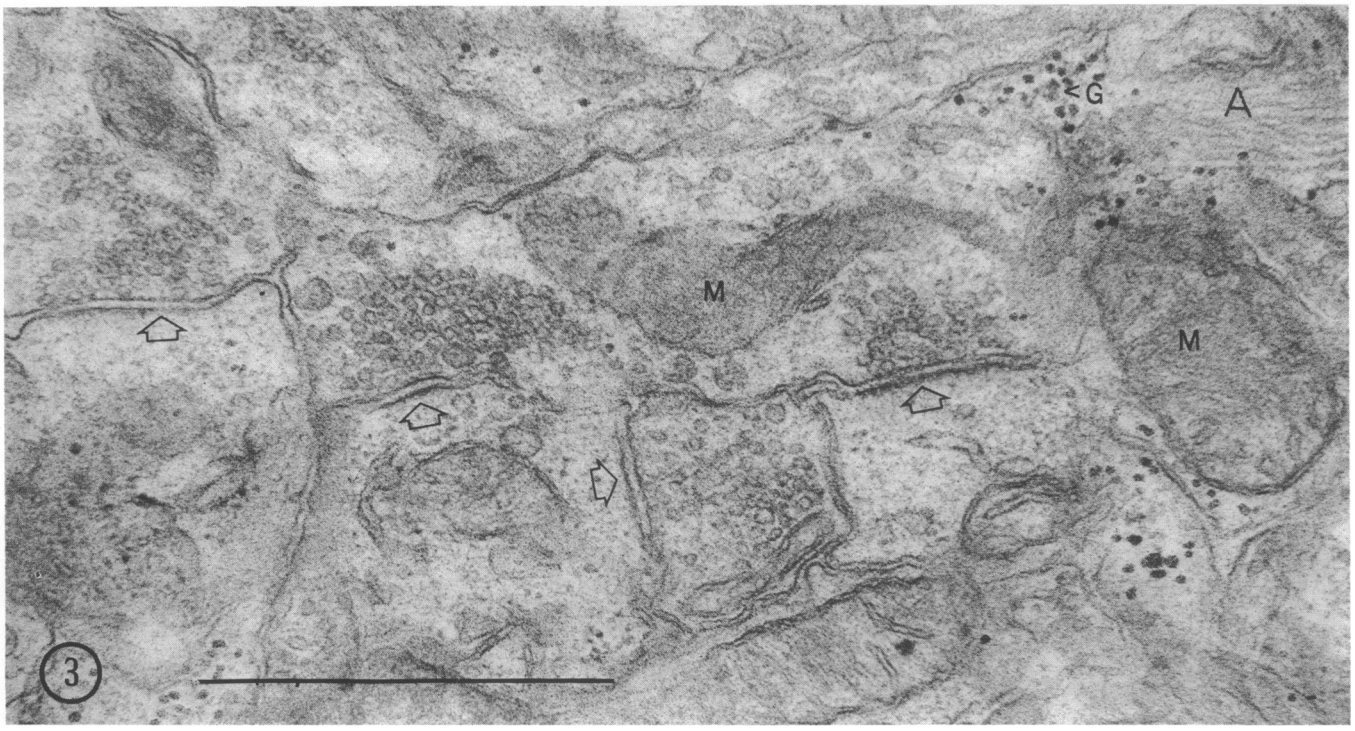
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granules and filaments are present in the cytoplasm of many cells; however, there is no evidence of myelination.

After 33 days of culture the aggregates contain increased numbers of synapses filled with synaptic vesicles (Fig. 2). The synaptic gap of many terminals is wider (250 Å) and the cytoplasmic face of the post-synaptic membrane has associated with it a more opaque material. The cells appear to be more organized and the membranes forming the junctional complex are less wavy. These features and the presence of mitochondria in some of the presynaptic terminals are more apparent in Fig. 3, where several synaptic boutons branch from a single axon. The synapses seen in Figs. 2 and 3 are similar to the mature axo-dendritic synapse of adult mouse brain (10). An additional feature noted in the 33-day-old cultures was that of myelination. Myelin-like structures were found in the older cultures (Fig. 4). These myelinated neurites are identical to those found in adult mouse brain.

DISCUSSION

We have shown that completely dissociated embryonic mouse brain cells can reaggregate and undergo morphological development similar to brain cells *in vivo*. Since the formation of many synapses in the central nervous system is thought to be under direct genetic control (11, 12), the ability of the dissociated cells to form synapses suggests that cell–cell interaction during reaggregation is very specific. Alternatively, if reaggregation is not highly specific, then synapse formation between neurons is more plastic than has been previously thought. The extent to which synaptogenesis is governed by genetic information and the essential cell types required for this phenomenon may be elucidated by the fractionation of various cell types before reaggregation.

The absence of electron microscopic evidence of synapses in the dissociated cell suspensions rules out the possibility that synapses from embryonic tissue were carried through the dissociation procedure. Also, there are very few synapses in 16-day-old fetal mouse brain. This is in accord with the observation that the greatest increase of synapses in mouse brain takes place between the second and third weeks after birth (13). The time sequence for synaptic development correlates well with eye opening, maturation of the electroencephalograph, and development of adult behavioral patterns (14).

The absence of myelin in the young cultures is not surprising, since myelination is one of the last steps of brain differentiation. Myelinated axons are first observed in the mouse brain about 10 days after birth, and the process is not complete until six weeks after birth (15).

Since synapses are not evenly distributed within the aggregate, but are found in groups, a direct comparison of the number of synapses in the 12- and 33-day aggregates is difficult. However, we estimate that there is about a 5-fold increase from day 12 to day 33. In addition, the synaptic complex of 33-day cultures shows increased numbers of synaptic vesicles, a slight widening of the synaptic gap, and increased opacity of postsynaptic, membranes as well as mitochondria in the presynaptic terminals, all of which are characteristic of *in vivo* synaptic maturation.

The biochemical development of enzymatic activities related to nerve transmission is complete by 15–17 days of culture (8, 16). Thus, biochemical differentiation appears to precede both the morphological development of synapses and myelination. Further studies are necessary to verify this conclusion, so we are presently determining the bioelectric activity of the aggregates.

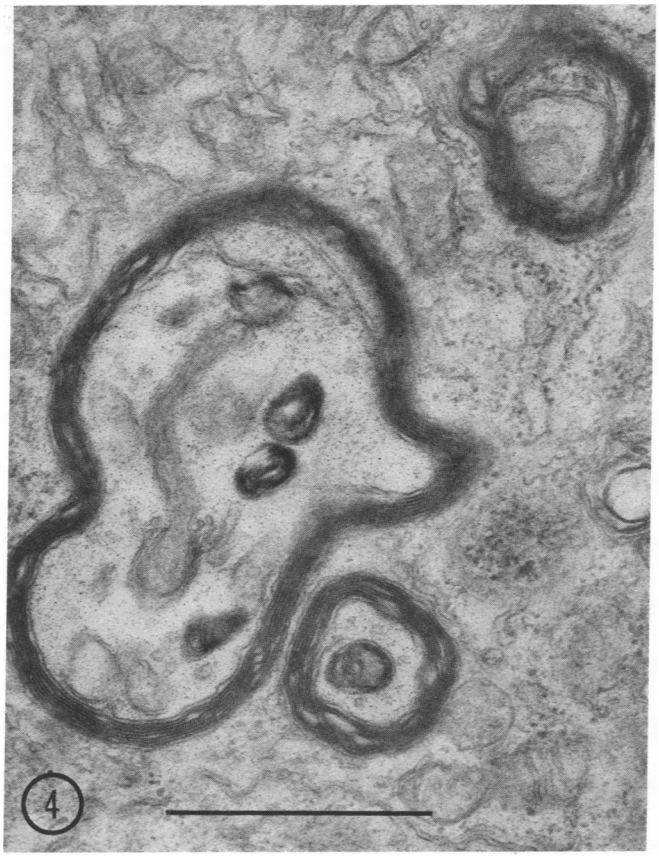

FIG. 4. A transverse section through three myelinated complexes. Note the inner loop, the presence of mitochondria, and the compactness and periodicity of the membranes, all of which are similar to mature myelinated axons. Bar, 1 μm.

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FIG. 1. A section through a 12-day aggregate showing an immature synapse (*arrow*). There is only a small amount of cytoplasmic opaque material associated with the cytoplasmic face of the postsynaptic membrane. The lumen of the presynaptic complex contains a small number of vesicles. Synaptic junctions occur infrequently at this age of culture. Glycogen granules (*g*), neurofilaments (*f*). Bar, 1μm.

FIG. 2. 33-day cell aggregate. Functional complexes are more frequent, and usually clustered. Some of the junction complexes appear morphologically mature (*arrow*), while others are either less developed or the plane of the section passes outside of the junctional region. Note the greater number of vesicles within the neurites. Bar, 1 μm.

FIG. 3. An enlarged view of a synapse-rich area that shows a number of boutons (*arrows*) at the terminus of an axon (*A*). Mitochondrion(*M*). Bar, 1 μm.

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