AN ELECTRON MICROSCOPIC STUDY OF THE DEVELOPMENT OF SYNAPSES IN CULTURED FETAL MOUSE CEREBRUM CONTINUOUSLY EXPOSED TO XYLOCAINE

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ABSTRACT

Explants of fetal mouse cerebral cortex, continuously exposed to the local anesthetic Xylocaine from the time of explantation to the time of fixation, were examined in the electron microscope to determine whether morphologically normal synapses and potentially functional interneuronal synaptic networks can form in the absence of electrical impulse activity. Morphological differentiation of complex synaptic networks proceeds normally, and the drug does not alter the fine structure of the formed synapses. These observations are consonant with the electrophysiological data which show that the potential for complex bioelectric activity can develop in the absence of its expression. The development and maturation of functional synaptic networks, then, is not contingent upon prior electrical impulse activity. These data support the concept that organized neuronal assemblies are formed in forward reference to their ultimate function.

INTRODUCTION

Explants of embryonic or neonatal rodent central nervous system (CNS) differentiate organotypically when maintained in tissue culture for extended periods of time (7). During the first week after explantation, cultures of rat spinal cord (10, 11) and mouse cerebral cortex (6, 8) develop complex bioelectric activities characteristic of functioning synaptic networks in vivo. At the same time many synaptic contacts are formed (2, 26).

Crain et al. (9) have explanted and grown immature CNS tissues in the presence of the local anesthetic Xylocaine or excess magnesium ions, both of which prevent spontaneous, as well as evoked, complex bioelectric activity. Subsequent removal of the blocking agent after 5–30 days in vitro allows immediate evocation of complex responses similar to those seen in control explants of comparable age.

This study was undertaken to determine whether morphologically normal synapses and potentially functional interneuronal synaptic networks can form in the absence of detectable extracellularly recorded electrical activity.

MATERIALS AND METHODS

Fragments of 17 or 18 day fetal mouse cerebral neocortex were explanted onto collagen-coated coverslips and maintained in Maximow depression slide assemblies at 35°C. Techniques for the preparation and maintenance of fetal and neonatal CNS tissue cultures have been described previously in detail (1, 8). The nutrient medium was replaced twice a week and con-

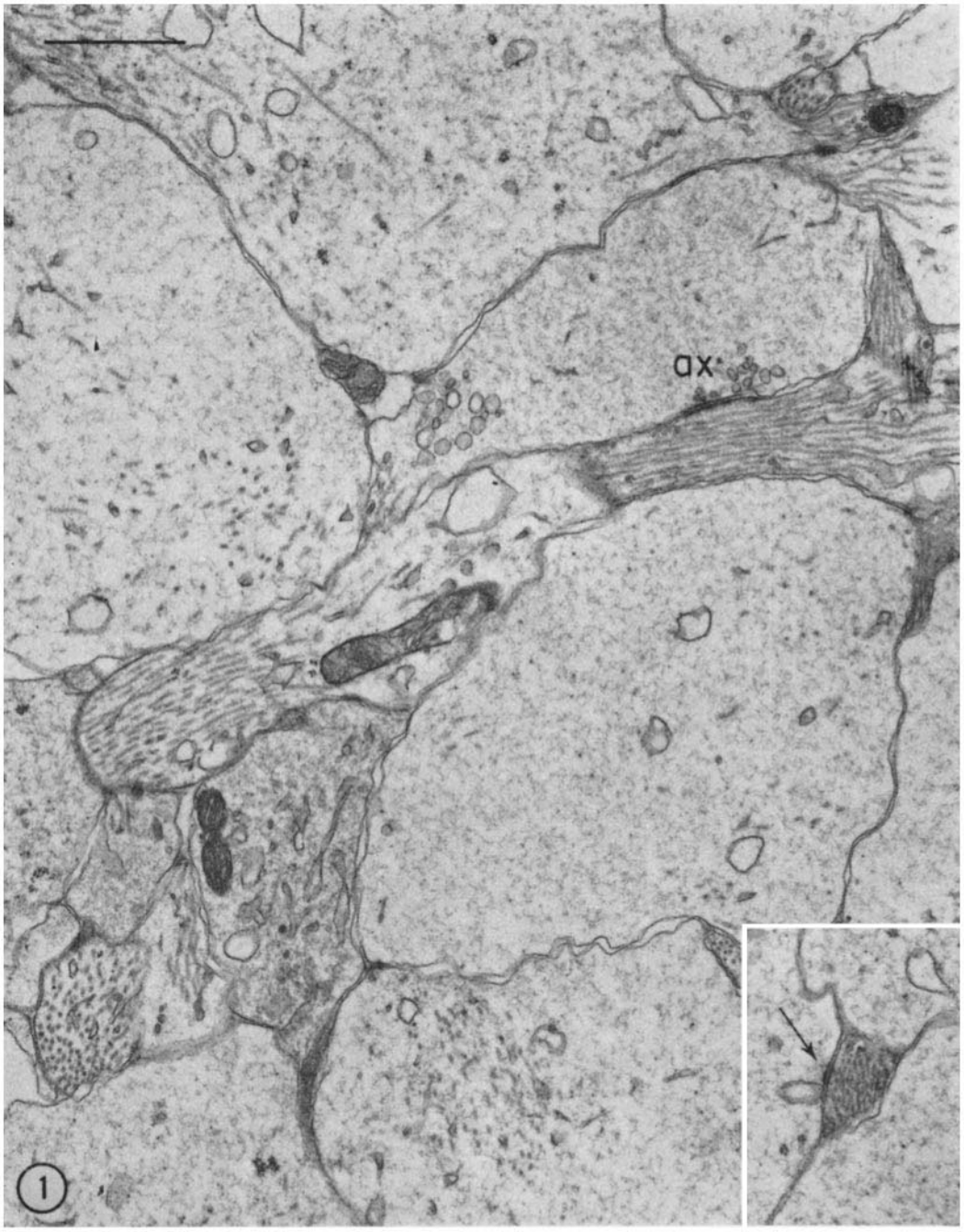


FIGURE 1 Immature fetal mouse cerebrum, 0 d.i.v. The neuropil is characterized by profiles of large cellular processes. Immature axodendritic synapses (*ax*) are few in number but desmosome-like junctions (*Inset*, arrow) are numerous. Scale, 1 μ. $\times$ 22,000.

sisted of human placental serum (25–40%) and Eagle's synthetic medium (25%) in Simms balanced salt solution (BSS), supplemented with 600 mg% glucose. In test cultures, at the time of explanation, Xylocaine (lidocaine)·HCl was included in the medium at a concentration of 100 μg/ml. This concentration of blocking agent was maintained through all subsequent feedings.

The electrophysiological methods applied to these explants have been reported (5, 8–10). In brief, the cultures were transferred to a moist chamber maintained at 35°C, and microelectrodes were positioned with micromanipulators for focal recording and stimulation within an explant, under direct visual control, at high magnification (see also legend for Fig. 6).

Tissue was prepared for electron microscopy at explantation and at 3, 10, 11, or 15 days in vitro (d.i.v.). Explant fragments and cultures were fixed by immersion in 2.5% glutaraldehyde in Millonig's buffer (pH 7.4, 430 milliosmols) at room temperature. After 10 min, the temperature was reduced to 4°C and the tissue was left in the fixative for 1–2 hr. After several rinses in cold Millonig's buffer (pH 7.4, total time 45 min), the cultures were postfixed in cold 1% OsO_4 in Millonig's buffer (pH 7.4) for 1 hr, dehydrated in a graded series of alcohols, and embedded in Epon 812. Explants were removed from the coverslips before Epon infiltration. Thin sections were stained with uranyl acetate and lead citrate and examined and photographed in an RCA EMU 3G or a Philips EM 200 electron microscope.

12 explants were prepared for electron microscope examination. The quality of preservation of this material was uniformly satisfactory. To avoid bias, micrographs were made whenever a synapse, or a portion of a synapse, was observed. Over 1000 micrographs of representative fields were studied.

RESULTS

Functionally immature and mature cultures of fetal mouse cerebral cortex were examined in the electron microscope. Immature cultures are those in which only simple spikelike responses can be evoked with electrical stimulation (8). At this stage there are none of the long-lasting bioelectric phenomena or other responses indicative of the complex synaptic interactions found in mature cultures (reference 8, and see below).

Cultures were divided into two main groups, a control group (untreated), and a second groug continuously exposed to Xylocaine from the time of explantation to fixation (Xylocaine-treated). Electron microscope observations were made on cultures after 0, 3, 10, 11, or 15 d.i.v. To assure that functional development had proceeded normally (8), 15-day cultures were examined electrophysiologically before fixation for electron microscope study. Some of the Xylocaine-treated cultures were washed free of the drug immediately before electrophysiological testing and subsequent fixation (Xylocaine-treated washed).

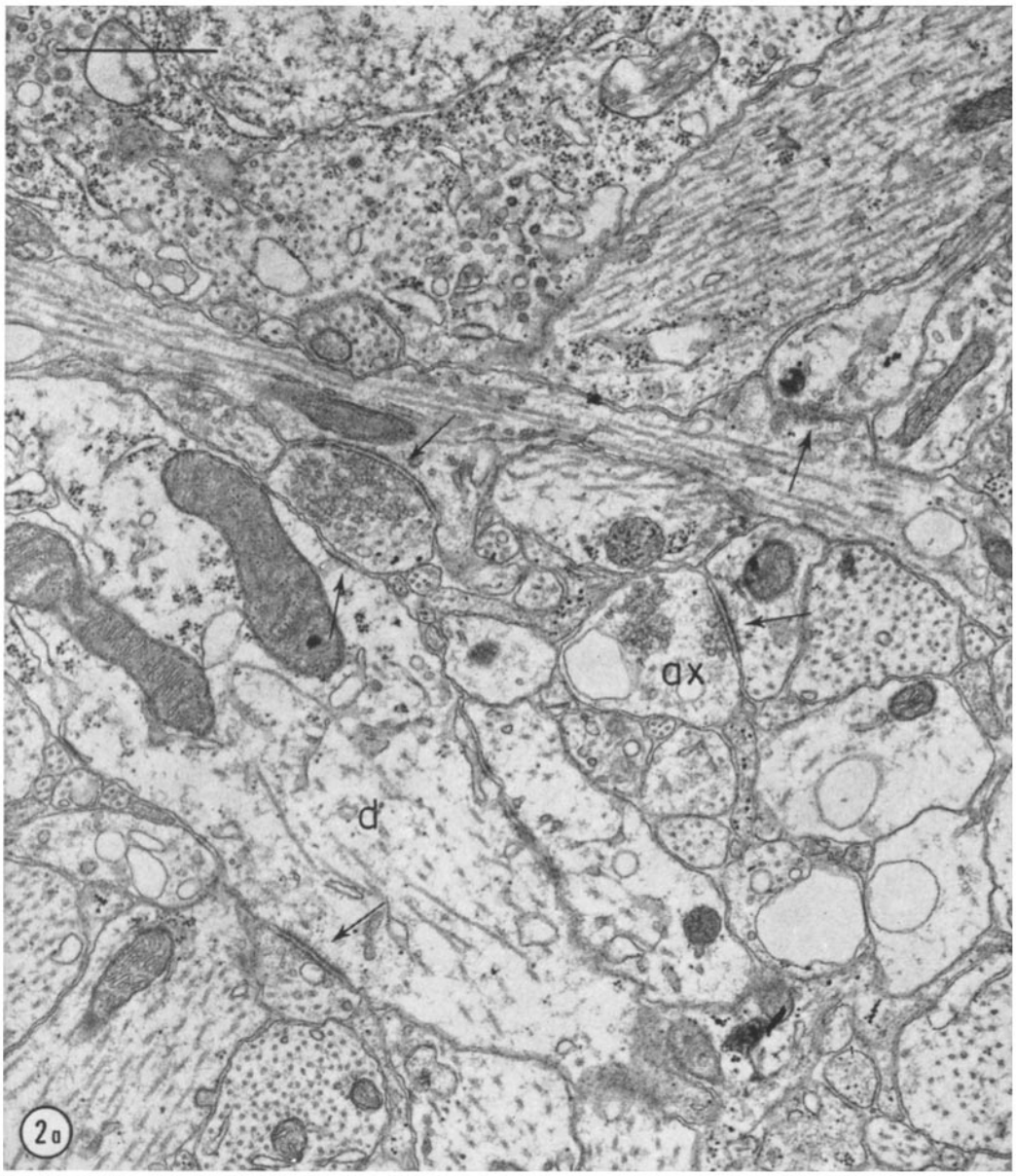
Fine Structure

IMMATURE CULTURES: The neuropil of 17 and 18 day fetal mouse cerebrum (0 d.i.v.) and of explants at 3 d.i.v. is comprised of large cellular processes (Fig. 1). Microtubules are the predominant structure in some of the processes, whereas a finely fibrillar material predominates in others. Intercellular space, for the most part, is similar to that found in micrographs of routinely prepared mature neuropil and is represented by a gap which averages 200 A across.

Desmosome-like junctions between adjacent cell somas, between soma and cellular process, or between processes (Fig. 1, inset at arrow) are numerous. The contact region consists of parallel membranes separated by an amorphous gap substance. A symmetrically distributed cytoplasmic dense material is associated with the membranes. These membrane complexes are relatively uncommon in mature cultures.

Occasionally, small groups of vesicles are found near one of the membranes of an asymmetric junctional complex (Fig. 1 at *ax*). The vesicles are structurally identical to the presynaptic vesicles of mature CNS tissue in vivo. Because the area of junction is small and mitochondria are not seen in the vesicle-containing process, these complexes are considered to be immature synaptic contacts (2). Such synapses are the only ones observed; they are infrequent and exclusively axodendritic.

FIGURE 2 Mature fetal mouse cerebrum, 15 d.i.v., untreated. This explant was examined electrophysiologically before fixation for electron microscopy (see Fig. 6 A). Fig. 2 *a*, the neuropil is comprised of many small processes as well as large dendritic trunks (*d*). Many axodendritic synapses (arrows) of conventional morphology are present. Although synaptic vesicles are usually located near the presynaptic membrane, an occasional vesicle cluster may be found distal to it (*ax*). Fig. 2 *b*, axosomatic synapses (arrow) are also present. Scale, 1 μ. × 22,000.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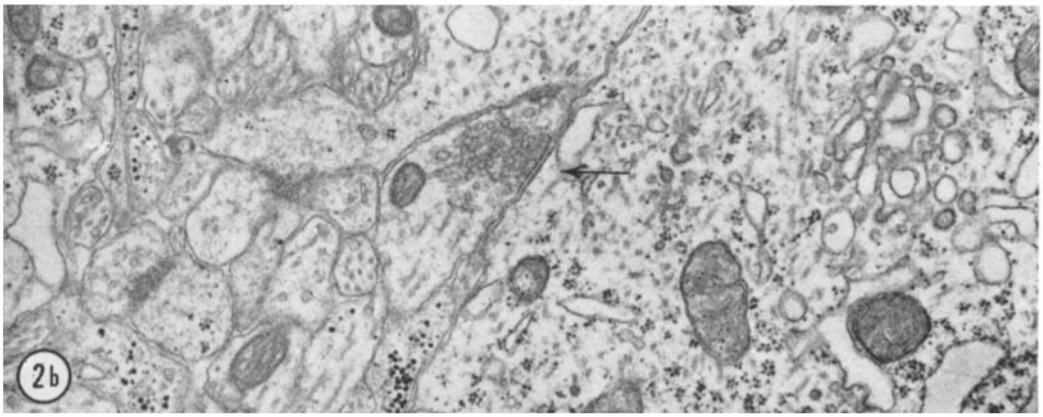
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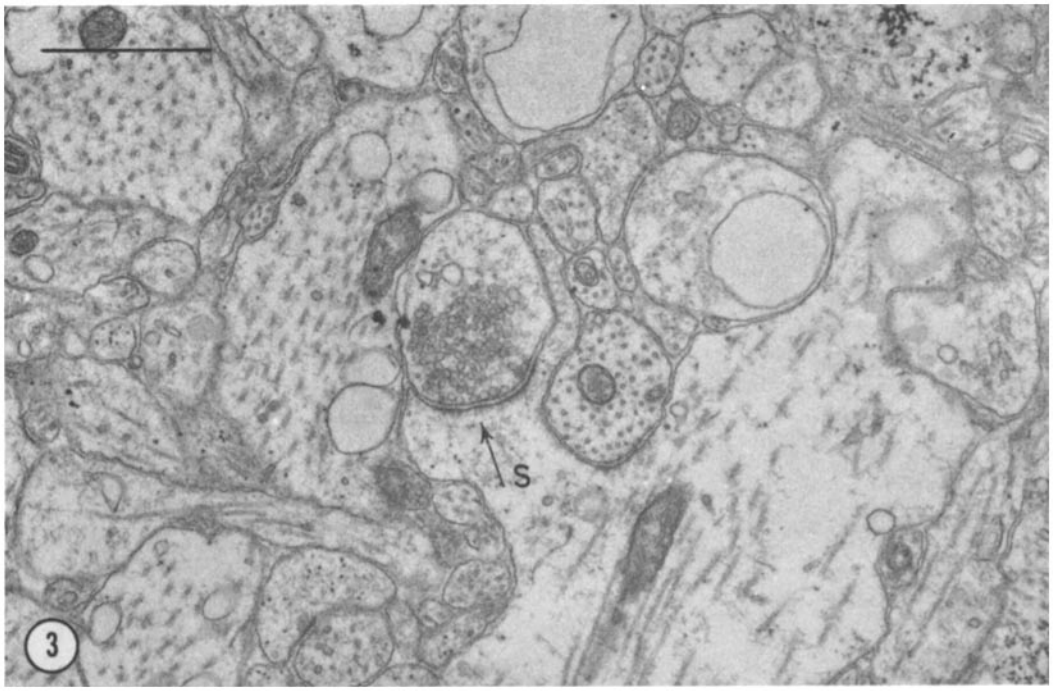


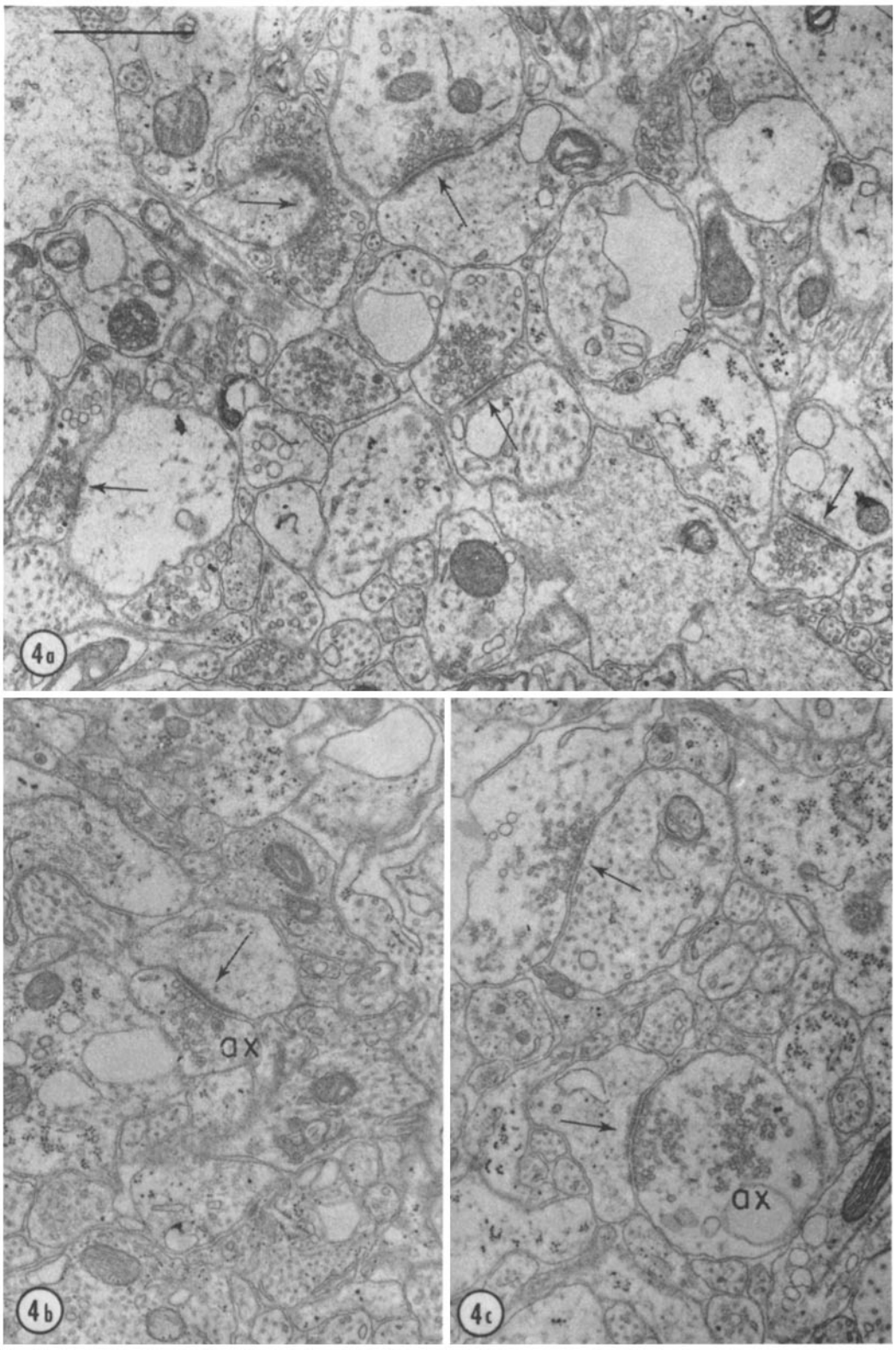
FIGURE 3 Mature fetal mouse cerebrum, 15 d.i.v., untreated. This electron micrograph is from the explant depicted in Fig. 2 and shows an axodendritic-spine synapse (arrow). Dendritic spine, *s*. Scale, 1 μ. × 22,000.

MATURE CULTURES, UNTREATED: On the 10th, 11th or 15th d.i.v., the neuropil of fetal mouse cerebrum is characterized by profiles of many small processes (Fig. 2 *a*), as well as large dendritic trunks (Fig. 2 *a* at *d*). Typically, numerous axodendritic synapses of conventional morphology (13, 15, 16, 24, 25) are present (Fig. 2 *a* at arrows). Axosomatic synapses (Fig. 2 *b* at arrow) are also present, but they are fewer in number. Some axons terminate on dendritic spines (Fig. 3 at arrow).

The synaptic vesicles are clear, round, 300–500 A in diameter, and usually clustered near the presynaptic membrane (Fig. 2 *a*). In an occasional axodendritic synapse, however, a vesicle cluster may be found in the presynaptic process in a position distal to the presynaptic membrane (Fig. 2 *a* at *ax*).

Most, but not all, of the axodendritic synapses conform to the morphological criteria of Gray's Type 1 synapses (14). The pre- and postsynaptic membranes show increased density and are separated by a widened gap (250–300 A) which contains a somewhat dense extracellular substance. A concentration of cytoplasmic dense material is closely associated with the apposing membranes of the contact region. That beneath the postsynaptic membrane is particularly well developed (Fig. 2 *a*). Other synapses, however, have a narrower gap and either lack, or show less pronounced, postsynaptic cytoplasmic specializations. Most axosomatic synapses (Fig. 2 *b*) and some

FIGURE 4 Mature fetal mouse cerebrum, 15 d.i.v., Xylocaine-treated. This explant was examined electrophysiologically before fixation for electron microscopy (see Fig. 6 B). Fig. 4 *a*, the synaptic complexes (arrows) of Xylocaine-treated cultures are morphologically undistinguishable from those of untreated controls. Fig. 4 *b*, in addition to the usual clear vesicles, profiles of presynaptic processes (*ax*) may contain one or two dense-core vesicles. Axodendritic synapse, arrow. Fig. 4 *c*, an occasional cluster of vesicles may be found at some distance from the presynaptic membrane (*ax*). Axodendritic synapses, arrows. Scale, 1 μ. × 22,000.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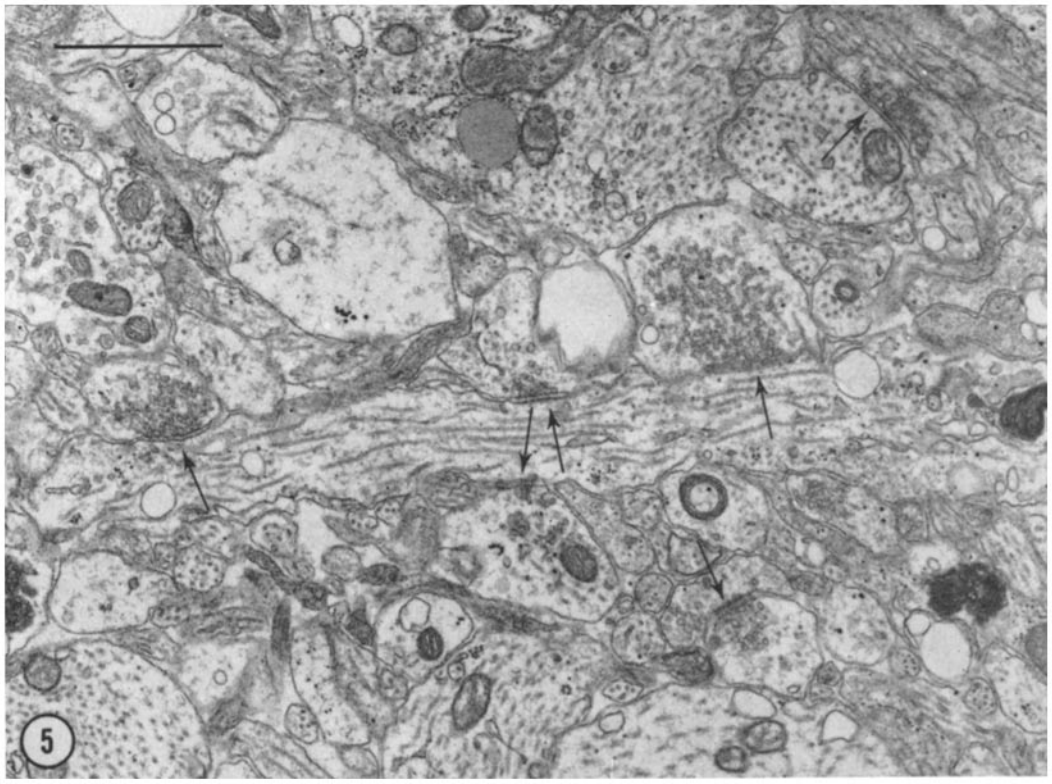

FIGURE 5 Mature fetal mouse cerebrum, 15 d.i.v., Xylocaine-treated washed. This explant was examined electrophysiologically before fixation for electron microscopy (see Fig. 6, C and D). Many axodendritic synapses (arrows) of conventional morphology are present. These cultures, like the Xylocaine-treated cultures, cannot be distinguished from the untreated controls. Scale, 1 μ. × 22,000.

axodendritic synapses (see later, Fig. 4 *c* at upper arrow) are of this type which corresponds to Gray's Type 2 (14). Intermediate forms are also observed (see later, Fig. 5).

MATURE CULTURES, XYLOCAINE-TREATED: The synaptic junctions of Xylocaine-treated cultures are morphologically indistinguishable from those of untreated controls (Fig. 4 *a*, *b*, *c* at arrows). Furthermore, the frequency and distribution of both axodendritic and axosomatic synapses are the same as the controls.

In addition to the usual clear vesicles, profiles of presynaptic processes in both treated and untreated cultures may contain one or two larger (750–1250 A) dense-core vesicles (Fig. 4 *b* at *ax*). As in controls an occasional cluster of vesicles may be located at some distance from the presynaptic membrane (Fig. 4 *c* at *ax*).

The Xylocaine-treated washed cultures, like the Xylocaine-treated cultures, cannot be distinguished from the controls (Fig. 5).

The presence of Xylocaine, then, has no effect on synaptogenesis or on the morphology of synaptic contacts in cultured fetal mouse cerebrum.

Electrophysiology

Electrophysiologic experiments were carried out on the 15-day cerebral explants which were subsequently studied by electron microscopy (cf. Figs. 2, 3, 4, and 5). In contrast to the complex, long-lasting, after-discharge responses which could be readily evoked after maturation in normal culture media (Fig. 6 A), only simple, brief, short-latency spike potentials could be elicited in a typical long-term Xylocaine-treated culture, even with high stimulus intensities (Fig. 6 B). Application of paired stimuli produced only long-lasting refractoriness following the initial spike response, instead of the facilitatory effects generally associated with repetitive activation of CNS tissues maturing in normal culture media (7). However,

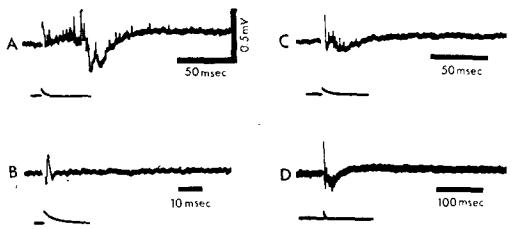

FIGURE 6 Bioelectric responses evoked in explants of fetal mouse cerebral cortex after maturation for 15 days in normal and Xylocaine-treated culture media. Fig. 6, A: Characteristic complex afterdischarge triggered by single electric stimulus applied about 0.5 mm from site of recording microelectrode in untreated explant. Note repetitive spikes and slow-wave components lasting for more than 100 msec. Fig. 6, B: Simple spike potential evoked only by larger stimulus in Xylocaine-treated explant. Note that entire response is terminated within a few msec. Fig. 6, C and D: Complex spike barrage and slow-wave responses triggered in Xylocaine-treated washed explant by much smaller stimulus, within a few minutes after transfer to physiological salt solution (BSS). Positive-negative sequence in afterdischarge lasts well over 100 msec (Fig. 6, D). Amplitude calibration applies to all records; upward deflection indicates negativity at active recording electrode; all recordings were made with 5 μ saline-filled pipettes and electric stimuli were applied via 10 μ-saline-filled pipettes; stimuli were 0.1–0.3 msec in duration and their onset and relative amplitude are indicated by signals on line below each recording.

within a few minutes after transfer of a Xylocaine-treated explant to normal physiological saline (BSS), complex, long-lasting discharges could be readily triggered with single stimuli (Fig. 6 C and D). These discharge patterns are well within the range characteristic of cerebral explants after maturation in normal media. The organotypic array of synaptically connected neurons seen after fixation and electron microscope study of this explant (Fig. 5) provides the morphological basis for these complex bioelectric after-discharges.

DISCUSSION

We have shown that in cultures of fetal mouse cerebrum the morphological differentiation of complex synaptic networks proceeds normally in the presence of Xylocaine. Furthermore, the drug does not alter the fine structure of the synapses. These observations are consonant with the earlier work of Crain et al. (9), which showed that the potential for complex bioelectric activity could develop in the absence of its expression. It therefore seems clear that the development and maturation of functional synaptic networks is not contingent upon prior electrical impulse activity.

Sperry (32–34), on the basis of extensive studies of the regeneration of severed optic nerves, concluded that the specification of interneuronal connections could be ascribed to a selective chemoaffinity between the growing presynaptic process and the neuron with which it will synapse. Jacobson (20, 21) showed that, during the development of the visual system in amphibians, complex neuronal circuits are organized in advance of their use by a predetermined stepwise specification of progressively finer details in the pattern of neuronal connections. The classic experiments of Harrison (18), Carmichael (4), and Matthew and Detwiler (22) showed that neither neuromuscular activity nor proprioceptive input are essential to the development of coordinated swimming behavior in amphibians. Thus there is by now considerable evidence suggesting that specific interneuronal contacts are determined by genetic mechanisms during development, and that organized neuronal assemblies are formed in forward reference to their ultimate function (see reviews in references 17, 20 and 33).

The development of fetal mouse cerebral cortex in tissue culture closely approximates the analogous process in vivo (23, 35). During perinatal cortical ontogenesis both in vitro and in vivo, there is a decrease in the average size of the processes which make up the neuropil. This is the result of the proliferation of fine dendritic processes from large dendrites and the growth of small axonal and glial elements. At the same time, there is an increase in the number and type of synaptic contacts. Axodendritic synapses appear before axosomatic synapses. The earlier development of axodendritic synapses has been observed in other regions of the CNS as well (2, 30).

There is some disagreement concerning the differentiation of extracellular space in immature CNS. This may be due to the well-known difficulty in obtaining good preservation of fetal and immature, as opposed to mature, CNS material. Some authors (3, 12, 28) have reported that in the immediate perinatal period many irregular gaps or lacunae, often more than 1000 A wide, exist between cellular processes. They further report that as the age of the animal increases there is a progressive reduction in the size of the extracellular space until the 200 A gap characteristic of mature neuropil is attained. Voeller et al. (35), on the

other hand, observed that no prominent extracellular spaces could be found in the immature cortex of the cat, and that the distance between cellular elements remains constant (about 200 A) during maturation (27). We also find the gap distance to be constant in maturing fetal mouse cerebrum (see Results). Since the maturation of cortical tissues is characterized by the elaboration of numerous small cellular processes and the gap distance between cellular processes does not change significantly during maturation, there is necessarily an increase in the total volume of extracellular space commensurate with the increase in number of elements (19).

It has been reported that under conditions in which there is a failure of synaptic transmission produced by ischemia, electroanesthesia, or glucose deprivation, vesicle depletion (36, 37), vesicle proliferation (31), or vesicle clumping and retraction away from the presynaptic membrane occurs (37). In fetal mouse cerebrum, however, there is no apparent difference in the number or distribution of synaptic vesicles present in the presynaptic processes in Xylocaine-treated and untreated cultures. Furthermore, the clustering of vesicles away from the presynaptic membrane is observed in both treated and untreated cultures. We would suggest, then, that although the same agents may cause a failure of synaptic transmission and the clumping of vesicles, there need not be a relationship between these phenomena. Ross and Bornstein (29), for example, have observed that bioelectric activity in mature cultures of fetal mouse CNS tissues exposed to serum from animals with allergic encephalomyelitis is blocked immediately, while degeneration-like morphological changes do not appear until long after. Since Xylocaine-treated cultures of fetal mouse cerebrum develop normally from the standpoint of potential function, we must conclude that the presence of morphologically normal synaptic junctions need not imply normal impulse activity, and that functional alterations are not necessarily reflected in morphology at the electron microscope level.

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REFERENCES

1. BORNSTEIN, M. B. 1964. Morphological development of neonatal mouse cerebral cortex in tissue culture. *In* Neurological and Electroencephalographic Correlative Studies in Infancy. P. Kellaway and I. Petersen, editors. Grune and Stratton, Inc., New York. 1.
2. BUNGE, M. B., R. P. BUNGE, and E. R. PETERSON. 1967. The onset of synapse formation in spinal cord cultures as studied by electron microscopy. *Brain Res.* **6**:728.
3. Caley, D. W., and D. S. Maxwell. 1970. Development of blood vessels and extracellular spaces during postnatal maturation of rat cerebral cortex. *J. Comp. Neurol.* **138**:31.
4. CARMICHAEL, L. 1926. The development of behavior in vertebrates experimentally removed from the influence of external stimulation. *Psychol. Rev.* **33**:51.
5. CRAIN, S. M. 1956. Resting and action potentials of cultured chick embryo spinal ganglion cells. *J. Comp. Neurol.* **104**:285.
6. CRAIN, S. M. 1964. Development of bioelectric activity during growth of neonatal mouse cerebral cortex in tissue culture. *In* Neurological and Electroencephalographic Correlative Studies in Infancy. P. Kellaway and I. Petersen, editors. Grune and Stratton Inc., New York. 12.
7. CRAIN, S. M. 1966. Development of "organotypic" bioelectric activities in central nervous tissues during maturation in culture. *Int. Rev. Neurobiol.* **9**:1.
8. CRAIN, S. M., and M. B. BORNSTEIN. 1964. Bioelectric activity of neonatal mouse cerebral cortex during growth and differentiation in tissue culture. *Exp. Neurol.* **10**:425.
9. CRAIN, S. M., M. B. BORNSTEIN, and E. R. PETERSEN. 1968. Maturation of cultured embryonic CNS tissues during chronic exposure to agents which prevent bioelectric activity. *Brain Res.* **8**:363.
10. CRAIN, S. M., and E. R. PETERSON. 1964. Complex bioelectric activity in organized tissue cultures of spinal cord (human, rat, and chick). *J. Cell. Comp. Physiol.* **64**:1.
11. CRAIN, S. M., and E. R. PETERSON. 1967. Onset and development of functional interneuronal connections in explants of rat spinal cord-

ganglia during maturation in culture. *Brain Res.* **6**:750.

12. DEL CERRO, M. P., R. S. SNIDER, and M. L. OSTER. 1968. Evolution of the extracellular space in immature nervous tissue. *Experientia.* **24**:929.
13. DE ROBERTIS, E. D. P. 1968. Submicroscopic morphology and function of the synapse. *Exp. Cell Res.* **5**:347.
14. GRAY, E. G. 1959. Axo-somatic and axo-dendritic synapses of the cerebral cortex: An electron microscope study. *J. Anat.* **93**:420.
15. GRAY, E. G. 1966. Problems of interpreting the fine structure of vertebrate and invertebrate synapses. *Int. Rev. Gen. Exp. Zool.* **2**:139.
16. GRAY, E. G., and R. W. GUILLERY. 1966. Synaptic morphology in the normal and degenerating nervous system. *Int. Rev. Cytol.* **19**:111.
17. HAMBURGER, V. 1968. Emergence of nervous coordination. Origins of integrated behavior. *In* The Emergence of Order in Developing Systems. M. Locke, editor. *Develop. Biol.* Suppl. **2**:251.
18. HARRISON, R. G. 1904. An experimental study of the relation of the nervous system to the developing musculature in the embryo of the frog *Amer. J. Anat.* **3**:197.
19. HORSTMANN, E., and H. MEVES. 1959. Die Feinstruktur des molecularen Rindengraues und ihre physilogische Bedeutung. *Z. Zellforsch. Mikrosk. Anat.* **49**:469.
20. JACOBSON, M. 1966. Starting points for research in the ontogeny of behavior. *In* Major problems in Developmental Biology. M. Locke, editor. Academic Press Inc., New York. 339.
21. JACOBSON, M. 1968. Development of neuronal specificity in retinal ganglion cells of Xenopus. *Develop. Biol.* **17**:202.
22. Matthew, S. A., and S. R. Detwiler. 1926. The reaction of Amblystoma embryos following prolonged treatment with chloretone. *J. Exp. Zool.* **45**:279.
23. MELLER, K., W. BREIPOHL, and P. GLEES. 1968. Synaptic organization of the molecular layer in the motor cortex in the white mouse during during postnatal development. A Golgi- and electronmicroscopical study. *Z. Zellforsch. Mikrosk. Anat.* **92**:217.
24. PALAY, S. L. 1956. Synapses in the central nervous system. *J. Biophys. Biochem. Cytol.* Suppl. **2**:193.
25. PALAY, S. L. 1958. The morphology of synapses of the central nervous system. *Exp. Cell Res. Suppl.* **5**:275.
26. PAPPAS, G. D. 1966. Electron microscopy of neuronal junctions involved in transmission in the central nervous system. *In* Nerve as a Tissue. K. Rodahl and B. Issekutz, Jr., editors. Harper and Row, Publishers, New York. 49.
27. PAPPAS, G. D., and D. P. PURPURA. 1964. Electron microscopy of immature human and feline neocortex. *Progr. Brain Res.* **4**:176.
28. PYSH, J. J. 1969. The development of the extracellular space in neonatal rat inferior colliculus: An electron microscopic study. *Amer. J. Anat.* **124**:411.
29. ROSS, L. L., and M. B. BORNSTEIN. 1969. An electron microscopic study of synaptic alterations in cultured mammalian central nervous tissues exposed to serum from animals with experimental allergic encephalomyelitis. *Lab. Invest.* **20**:26.
30. SCHWARZ, I. R., G. D. PAPPAS, and D. P. PURPURA. 1968. Fine structure of neurons and synapses in the feline hippocampus during postnatal ontogenesis. *Exp. Neurol.* **22**:394.
31. SIEGESMOND, K. A., A. SANCES, and S. J. LARSON. 1969. Effects of electroanesthesia on syanptic ultrastructure. *J. Neurol. Sci.* **9**:89.
32. SPERRY, R. W. 1963. Chemoaffinity in the orderly growth of nerve fiber patterns and connections. *Proc. Nat. Acad. Sci. U.S.A.* **50**:703.
33. SPERRY, R. W. 1965. Embryogenesis of behavioral nerve nets. *In* Organogenesis. R. L. Haan and H. Ursprung, editors. Holt. Rinehart, and Winston Inc., New York. 161.
34. SPERRY, R. W., and E. HIBBARD. 1968. Regulative factors in the orderly growth of retinotectal connexions. *Growth Nervous System Ciba Found. Symp.* 41.
35. VOELLER, K., G. D. PAPPAS, and D. P. PURPURA. 1963. Electron microscope study of development of cat superficial neocortex. *Exp. Neurol.* **7**:107.
36. WEBSTER, H. DEF., and A. AMES. 1965. Reversible and irreversible changes in the fine structure of nervous tissue during oxygen and glucose deprivation. *J. Cell Biol.* **26**:885.
37. WILLIAMS, V., and R. G. GROSSMAN. 1970. Ultrastructure of cortical synapses after failure of presynaptic activity in ischemia. *Anat. Rec.* **166**:131.