PRINCIPLES OF DEVELOPMENT OF DOPAMINERGIC NEURONS IN DISSOCIATED CULTURES OF EMBRYONIC MOUSE SUBSTANTIA NIGRA

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KEY WORDS: dissociated and reaggregated cultures of substantia nigra; dopaminergic neurons; growth of nerve fibers.

Dissociated and reaggregated brain cell cultures have been successfully used to study the principles of cytogenesis of the nervous system [1, 5, 11]. They can be used to study the process of differentiation of neurons under conditions of total deafferentation, the formation of synaptic connections, and the role of the glia for survival and growth of neurons in culture. The use of dissociated and reaggregated cultures of the substantia nigra (SN) enables dopaminergic (DA) neurons to be selectively demonstrated by histofluorescence methods.

The aim of the present investigation was to study the principles governing formation of cell aggregates in a suspension of SN cells, the distribution of DA neurons in them, and the differentiation of these neurons and growth of their processes.

EXPERIMENTAL METHOD

Experiments were carried out on 18-19-day mouse embryos of line C57B1. The central region of the midbrain containing SN was isolated, and a cell suspension of SN was obtained by methods of enzymic [11] or mechanical dissociation. In the latter case the cell suspension was obtained after repeated pipeting. The degree of dissociation was verified and the number of cells in the suspension counted in a hemocytometer. The cell density in the suspension was about 1.5×10^6 cells/ml.

A drop of suspension was placed on a slide with a collagen well and cultured in Maximov's chambers [1, 13]. Starting from the 2nd day of culture some cultures were taken for histochemical analysis. DA neurons were detected by a modified histofluorescence method [10]. The fixative (2% glyoxylic acid and 1% paraformaldehyde) was made up immediately before use in a salt solution of the following composition (in g/liter): NaCl 8.0, NaH_2PO_4 0.005, $NaHCO_3$ 1.0, KCl 0.2, $MgCl_2$ 5.0, glucose 2.0, sucrose 66.0; pH of the fixative 6.7-6.9. To inhibit monoamine oxidase activity the cultures were incubated in medium containing 10^{-4} M pargyline hydrochloride (15-20 min, 37°C). Some cultures were incubated in a culture medium containing 10^{-6} M dopamine. The cultures were fixed for 5-7 min at 2°C, dried in a jet of warm air, kept for 5 min at 80°C, and mounted in nonfluorescent immersion oil. The specimens were examined in the MBI-15 luminescence microscope.

EXPERIMENTAL RESULTS

Intravital microscopic observations showed that development of dissociated and reaggregated cultures of SN was similar to the development of corresponding cultures of other brain structures [13]. During the first few hours of culture aggregation of the dissociated brain cells began and, as a result, aggregates consisting of several cells were formed in the suspension. Later the number of cells in the aggregates increased progressively, and they adhered to the collagen substrate. During the first day in culture compact spherical and flattened aggregates with a well-marked cellular organization, and also local concentrations of cells forming a monolayer could be distinguished.

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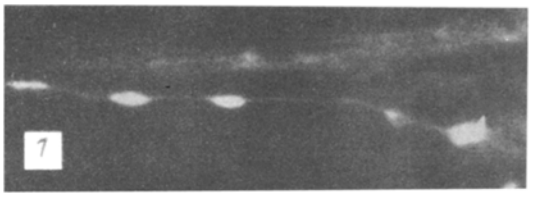

Fig. 1

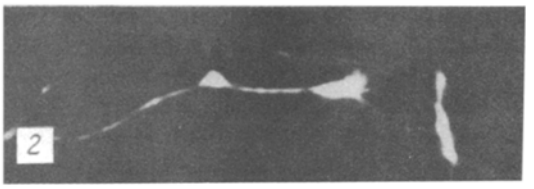

Fig. 2

Fig. 1. DA fiber with varicosities in composition of glio-axonal bundle (3 div).

Fig. 2. Cone of growth of DA fiber (3 div).

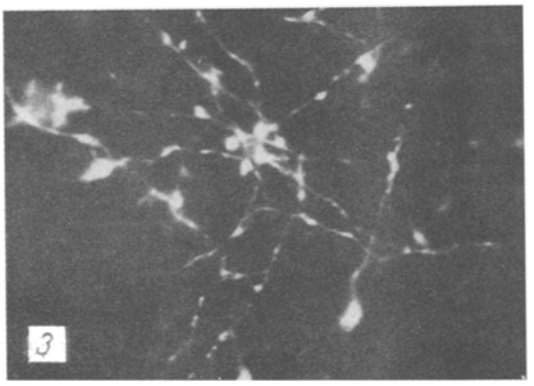

Fig. 3

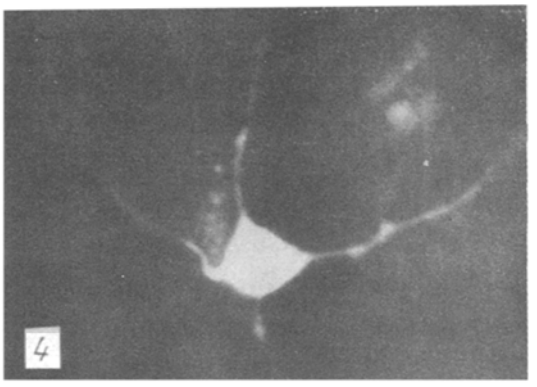

Fig. 4

Fig. 3. Plexus of thin DA fibers (5 div).

Fig. 4. DA neuron in aggregate (3 div).

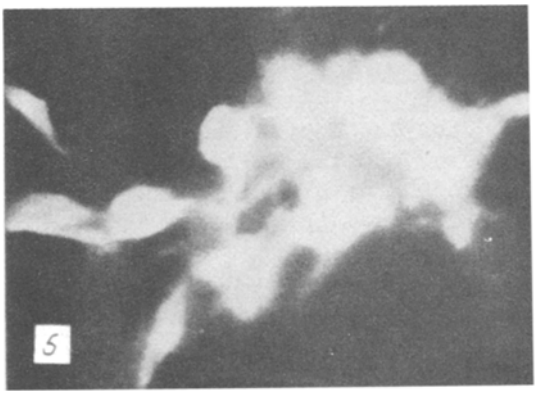

Fig. 5. Aggregate of DA neurons (5 div).

After completion of formation of compact aggregates growth of isolated radially oriented fibers with characteristic varicosities began from their peripheral zones. Growth of this type was more characteristic of spherical aggregates. At the beginning of the second week of culture some isolated axons degenerated and broke up into separate punctate fragments. A more characteristic feature of the flattened aggregates was the formation of glio-axonal fibrous bundles, consisting of processes of neurons and glial cells. These bundles, connecting adjacent aggregates, formed what have been called glio-axonal bridges [1]. Some glio-axonal bridges divided a short distance from the aggregates into single fibers, which formed plexuses of terminal ramifications. Glioblasts migrated actively from the flattened aggregates and formed a glial monolayer. Migration of glia from spherical aggregates was much less marked. Histofluorescence studies of reaggregated cultures in the early stages of development also showed differences in the character of growth of the single fibers and those gathered into bundles. In preparations of cultures 2-3 days old isolated DA fibers were seen with characteristic varicosities (Fig. 1) and cones of growth (Fig. 2). In more

mature cultures some axons leaving the glio-axonal bundles formed complex terminal plexuses (Fig. 3). As a rule single DA neurons were found in the flattened aggregates (Fig. 4). Concentrations of DA neurons, forming what resembled a nuclear structure (Fig. 5), were more characteristic of spherical aggregates. Single neurons, located outside aggregates of any kind, were found rarely in these cultures. Most DA neurons consisted of infrequently branching bipolar cells with a small, fusiform body. However, differentiated multipolar neurons in which, besides thin axons with typical varicosities, it was also possible to detect short smooth dendrites, forming branches of the second and third order and also giving bright green fluorescence.

The results of this investigation thus show that aggregation of neurons and glial cells takes place actively in dissociated cultures of SN cells, cultured in collagen wells. The aggregates thus formed are fixed to the collagen substrate, and differentiation of neurons takes place in them under conditions of total deafferentation, as shown by the formation of dendritic systems and by growth of axons which form plexuses mainly outside the aggregates. The distribution of SN neurons among aggregates in the culture, incidentally, was irregular: Whereas nuclear concentrations of DA-neurons were found in some aggregates, others had none whatever, and only a few aggregates contained single DA-neurons. This finding agrees with observations by other workers who showed that neurons in reaggregated suspension cultures of SN are capable of selective adhesion [9]. The histochemical investigations showed that active synthesis of dopamine is a characteristic feature of SN neurons of 18-19-day mouse embryos after the first day in culture. According to other workers [8, 12], the presence of endogenous dopamine can be detected in monolayer dissociated SN cultures from 13-15-day mouse embryos only after the 2nd-3rd week of culture. The results of the present investigation thus suggest that even in the earliest stages of aggregate formation, optimal glio-neuronal relations more favorable for development and differentiation of DA neurons than reaggregated cultures of SN from early mouse embryos are formed in reaggregated cultures of brain from late mouse embryos, which contain more mature DA neurons from the beginning.

Axons detectable in dissociated SN cultures by methods of intravital and fluorescence microscopy were identified by us as typical DA fibers with their characteristic varicosities, described during studies of the whole brain [2] and observed in tissue cultures of SN and other monoaminergic brain structures [14]. However, whereas synchronous growth of axons and glial processes is more characteristic of tissue cultures, in dissociated SN cultures growth of isolated fibers from aggregates, unaccompanied during the first days of culture by migration of glia, was observed more frequently. This was probably because a longer time is required for adequate glio-neuronal interactions to be established in the aggregate after complete dissociation. In some cases, incidentally, isolated axons, like fibers in the composition of glio-axonal bundles, formed terminal branches similar to the terminals of DA fibers in the cerebral cortex *in vivo* [3]. According to our observations, dopamine was present not only in the bodies and axons of SN neurons in culture, but also in their dendrites. These observations agree with the results of investigations that showed the presence of dopamine in dendrites of SN neurons *in situ*, suggesting the presynaptic nature of these dendrites [4]. Independence of formation of dendrite systems and axon branches, and also the discovery of differentiated isolated DA neurons in the cultures suggest that development and differentiation of DA neurons are genetically determined processes and are relatively independent of the influence of specific target cells. Other workers have made a similar suggestion [7]. The disagreement between our data and those of investigations showing that maturation of DA neurons depends on the presence of target cells in the cultures, can evidently be explained on the grounds that the program of differentiation of neurons is not yet realized at the beginning of culture, in neurons in the earlier stages of development such as were used in these investigations, and as a result the development of DA neurons is determined to a greater degree by the influence of their specific target cells.

Our data show that compact aggregates, in which adequate alio-neuronal interrelations are formed during culture, are most viable in dissociated SN cultures. Aggregates containing a few neurons or no nerve cells whatever are transformed during culture into a glial monolayer, confirming data published previously [13], according to which aggregates consisting mainly of glial cells are unstable in culture.

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QUANTITATIVE EVALUATION OF CELL PROLIFERATION AND DEATH IN AMMON'S HORN AND THE DENTATE GYRUS OF THE DEVELOPING MOUSE HIPPOCAMPUS

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Ammon's horn and the dentate gyrus of the hippocampus are known to differ significantly in the duration of their cytogenesis [4, 5, 9]. The source of cell production in both parts of the hippocampus is the ventricular zone, and after its exhaustion, the role is assumed by local proliferation of cambial cells and blioblasts [5, 8. 9]. Local cambial zones have been described and studied in the dentate gyrus [2, 5, 6, 9] but no quantitative analysis of age changes in the proliferative processes has been undertaken. Local cell proliferation has not been studied at all in Ammon's horn. Moreover, the age changes in mitotic cell death, which can have a significant effect on the over-all balance of cell production [2, 7], have not been studies in either part of the hippocampus. Yet we know that pycnosis, discovered in the cambial zones of the hippocampus, arises, as a result of mitotic cell death [1].

The aim of the present investigation was accordingly a quantitative analysis of mitosis and pycnosis in zones of cell proliferation (local cambial zones) of Ammon's horn and the dentate gyrus of the developing hippocampus.

EXPERIMENTAL METHOD

Experiments were carried out on 30 CBA mice aged 14, 16, 18, and 20 days of intrauterine and 1, 3, 7, 14, 21 and 60 days of postnatal life. Three animals were sacrificed at each time of development. The brain was fixed in Carnoy's fluid and embedded in paraffin wax. Frontal serial sections 5 μ thick were stained with cresyl violet. Histological sections for analysis of the location of the cambial zones in the rostral hippocampus were studied under the microscope (magnification 25). Indices of mitosis and pycnosis were counted in the local cambial zones thus revealed, by the use of an immersion objective. The quantitative analysis was undertaken on mice aged 18 and 20 days of intrauterine and 1, 3, 7, 14, 21, and 60 days of postnatal life. The indices were counted in Ammon's horn in the identified suprafimbrial cambial zone. In the dentate gyrus, mitoses and pycnoses were counted in

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