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# MATURATION OF DOPAMINERGIC NEURONS IN DISSOCIATED CULTURES OF MOUSE SUBSTANTIA NIGRA

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Organotypic and dissociated types of tissue cultures are widely used for study of the development and neurochemical differentiation of dopaminergic neurons of substantia nigra. Several recent publications have shown that embryonic neurons of substantia nigra taken from mouse embryos could successfully develop *in vitro* up to the stage of morphological and neurochemical maturity (Sclumpf et al. 1977; Prochiantz et al. 1979; Berger et al. 1982). However, at an early stage after explantation *in vitro* the substantia nigra neurons did not show fluorescence reactivity of endogenous dopamine while, at the same time catecholamine uptake was taking place. The absence of fluorescence of endogenous catecholamine did not allow to trace the dopamine content in substantia nigra in the early stage of neuron culture under the experimental conditions. In the preliminary report Victorov and Shashkova (1984) reported that dissociated cultures of substantia nigra taken from 18—19-day-old fetuses showed fluorescence of dopaminergic character from the beginning of explantation.

The purpose of the present study was to investigate the following steps of development and differentiation of dopaminergic neurons *in vitro* during 2 weeks under culture conditions.

## MATERIAL AND METHODS

The experiments were performed on 18—19-day-old mouse embryos (C 57 Bl/6J). The explants of substantia nigra were dissected under microscopic control from the rostral mesencephalon and cut into small pieces (1 × 1 mm). The tissue fragments were transferred to 0.25% trypsin in calcium— and magnesium-free balanced salt solution (BSS)

for 10 minutes at 37°C and then carefully rinsed in the following solutions: first in BSS and later in a mixture of equal parts of Simms solution, minimal essential medium (MEM) and fetal calf serum (FCS). Afterwards, the tissue fragments were mechanically dispersed in complete culture medium by brisk flushing through a fine bore pipette. The complete culture medium consisted of: 15% heat-inactivated FCS, 15% human placental serum, 50% MEM and 20% Simms solution, supplemented with 4 mM of glutamine, 600 mg% of glucosse and 0.2 Uml of insulin. The cell suspensions were centrifuged (3000 g) for 1 minute and resuspended in complete culture medium. The number of cells in the suspensions counted in a hemocytometer was $1.5 \times 10^6$/1 ml. One drop of the suspension was plated on a glass coverslip coated with collagen, placed in Maximow assembly and incubated at 36°C. The nutrient medium was changed every 3rd day. The cultures were grown for periods up to 14 days *in vitro* (DIV).

From 2 DIV part of the cultures was processed for fluorescence histochemistry. A modification of the glyoxylic acid technique was used (Victorov, Szaszkowa 1984). The culture medium was removed and replaced by medium containing pargyline chlorhydrate $10^{-4}$ M for 15—20 minutes at 37°C. The cultures were then fixed by dipping the coverslips for 5—7 minutes at 2°C in a mixture of 2% glyoxylic acid and 1% paraformaldehyde in a solution containing (in g/1): NaCl 8.0, $NaH_2PO_4$ 0.005, $NaHCO_3$ 1.0, KCl 0.2, $MgCl_2$ 5.0, sucrose 66.9, pH 6.7—6.9. After drying in a warm air stream the cultures were exposed to 80°C for 5 minutes. The coverslips were then mounted on glass slides with Entellan and observed with a UV microscope.

## RESULTS

Under the light microscope, the process of cell aggregation was initiated very early and could be observed during the first hours *in vitro*, although the aggregates were still rather small, consisting of few cells only. Gradually, the number of cells forming the aggregates increased and the process of aggregates adhesion to the collagen-coated glass coverslips proceeded. From the 2 DIV, two types of cell aggregates could be distinguished: the flat ones and the spherical ones. Both of them exhibited different patterns of neuronal process outgrowth. In the flat aggregates, the neuronal processes outgrowing ahead in a linear manner, were accompanied by a rapid and extensive glial migration and formed together with glial processes thick, intermingled bundles of fibers dividing into numerous branches at a certain distance from the aggregate margins. In the spherical type of aggregates, the neuronal processes surrounded radially the aggregates, emerging as isolated, thin fibers with

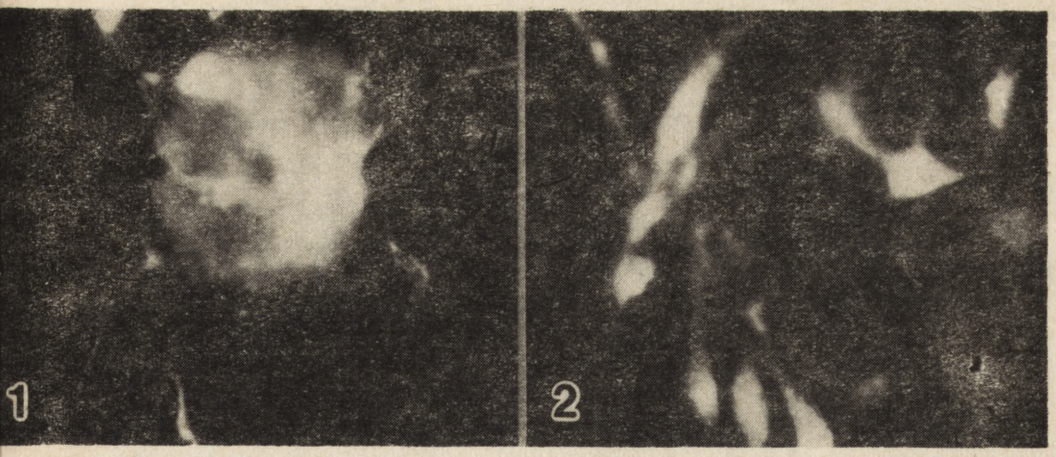


*Fig. 1.* Aggregate with single, highly fluorescent dopaminergic cell and fine, thin fibers around the edges of the aggregate. 3 DIV. × 225

*Ryc. 1.* Skupienie komórek z pojedynczym neuronem dopaminergicznym, wykazującym silną fluorescencję oraz delikatnymi, cienkimi włóknami na brzegu agregatu. 3 dni *in vitro*. Pow. 225 ×

*Fig. 2.* A group of dopaminergic neurons in an aggregate. 5 DIV. × 225

*Ryc. 2.* Grupa komórek dopaminergicznych zgromadzonych w pojedynczym skupieniu. 5 dni *in vitro*. Pow. 225 ×

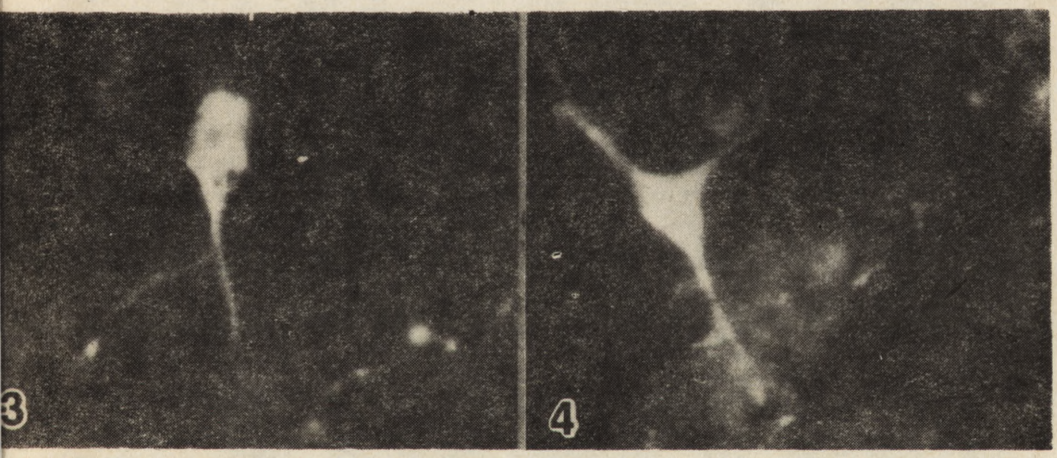


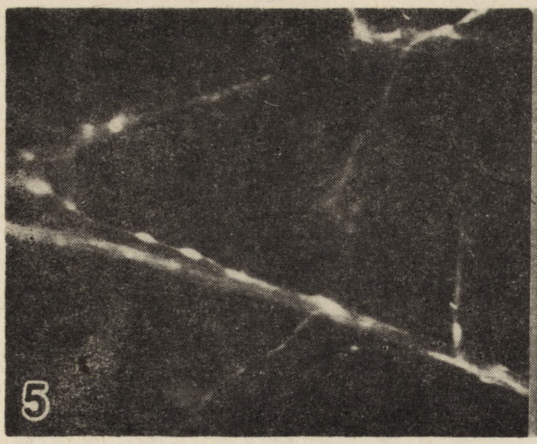
*Fig. 3.* Bipolar neuron showing strong fluorescent reaction. 3 DIV. × 225

*Ryc. 3.* Dwubiegunowy neuron dopaminergiczny, wykazujący silną fluorescencję. 3 dni *in vitro*. Pow. 225 ×

*Fig. 4.* Multipolar neuron revealing fluorescent reaction. 5 DIV. × 225

*Ryc. 4.* Wielobiegunowy neuron dopaminergiczny z silną fluorescencją. 5 dni *in vitro*. Pow. 225 ×

characteristic swelling along their long axis. At the same time, delicate neuro-glial bridges appeared between the neighbouring aggregates. From the second week *in vitro* part of the neuronal processes started to de-



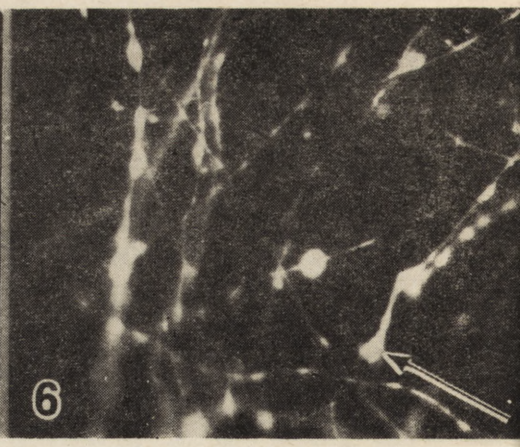




*Fig. 5.* Thin neuronal process with typical varicosities. 5 DIV. × 225

*Ryc. 5.* Cienka wypustka nerwowa z paciorkowatym zarysem. 5 dni *in vitro*. Pow. 225 ×

*Fig. 6.* Numerous neuronal processes with varicosities along their long axis and characteristic growth cones (arrow). 5 DIV. × 225

*Ryc. 6.* Liczne wypustki nerwowe z paciorkowatym zarysem i charakterystycznymi stożkami wzrostowymi (strzałka). 5 dni *in vitro*. Pow. 225 ×

generate and disrupt into small, spherical fragments. The persisting fibers were grouped into bundles and branched into delicate processes forming axon terminals. Around the flat aggregates very extensive glial migration could be seen leading to formation of a glial monolayer. Glial cells migration from the spherical aggregates was significantly less pronounced.

Fluorescence histochemistry performed on the 3, 5, 6 and 7 DIV confirmed the presence of two different patterns of aggregates outgrowth. Neurons exhibiting brigth green fluorescence of the cell cytoplasm and processes seen from the 3 DIV were only sporadically present in the flat aggregates, always as single cells (Fig. 1). In the spherical aggregates they were more numerous and formed clusters of cells (Fig. 2). They were observed rarely also outside the aggregates. The neurons visible in the youngest cultures were poorly differentiated, rather small and bipolar (Fig. 3). During further observation *in vitro*, larger, multipolar, highly fluorescent cells with brigthly green processes and characteristic varicosities along their axis could be recognized (Fig. 4). Two types of the cell processes could be distinguished: thinner ones with typical varicosities, probably representing axons, and shorter processes, also exhibiting distinct green fluorescence with all probability corresponding to dendrites (Fig. 5).

Fluorescence histochemistry revealed that only part of the fibers observed in the light microscope were of dopaminergic nature. Most of

them outgrew from the spherical aggregates and only few, forming neuro-glial bundles, from the flat aggregates. In older cultures some of these processes contained more complex terminals with typical growth cones revealing delicate, filopodial processes (Fig. 6). There was no difference in fluorescence properties between neuronal fibers from both types of cell aggregates.

## DISCUSSION

It results from our observations that mouse embryos aged between 18—19 days *in utero* from which substantia nigra was taken for a dissociated type of cultures exhibited the presence of neurons positive for dopamine. This feature observed *in vitro* allowed to use the cultures both for study of dopaminergic neurons maturation as well as of their properties in experimental conditions.

Our results are different from the maturation of dopaminergic neurons previously observed by Prochiantz et al. (1979) and Berger et al. (1982). These discrepancies might be connected with the different mouse strains used for tissue culture. After our observations of substantia nigra *in vitro* we also suggest that most important for dopaminergic fluorescence of substantia nigra neurons is the age of the embryos taken for tissue culture. Our results did not confirm that dopaminergic fluorescence appears after 3 weeks of substantia nigra maintainance *in vitro*. In our cultures the neurons which were dopamine-positive showed, moreover, some typical features of catecholaminergic cells described both *in situ* (Björklund, Lindvall 1975) and *in vitro* (Berger et al. 1982). At the beginning of culture most of the positive neurons were of bipolar type. On the following days the neurons had most numerous processes, representing branching dendrites, growth cones and axons. During further morphological development and differentiation *in vitro* numerous varicosities along the axons and dendrites appeared. All these structures revealed positive fluorescence of dopaminergic nature. Similar fluorescence in these structures was observed in cultures after 3 weeks *in vitro* by other authors (Berger et al. 1982). This fluorescence is commonly observed in all kinds of nerve cells processes and does not seem to be confined to axons of dopaminergic cells as observed by Sumners et al. (1983). Fluorescence was observed both in spherical and flat types of the aggregates. There were no differences in the fluorescence reaction between accumulated and dispersed cells and their processes. Most intensive fluorescence observed by Levitt et al. (1976) at the edges of the aggregates was not noted by us. To summarize our results it is expected that cultures of both one or more weeks *in vitro* can serve as a good model for the studies on neurotoxin action on the substantia nigra.

## DOJRZEWANIE DOPAMINERGICZNYCH NEURONÓW ISTOTY CZARNEJ W HODOWLI TKANKOWEJ TYPU ZDYSOCJOWANEGO

Streszczenie

W zdysocjowanych hodowlach tkankowych istoty czarnej, pobranej z 18—19 dniowych zarodków mysich stwierdzono obecność fluorescencji swoistej dla dopaminy w licznych wypustkach komórek nerwowych. W przeciwieństwie do opisów innych autorów, występowała ona już we wczesnych stadiach hodowli i dotyczyła zarówno wypustek osiowych, jak i dendrytów. Stwierdzano ją również w perykarionach neuronów zgromadzonych zarówno w płaskich, jak i kulistych skupień komórkowych. Autorzy sugerują, że obecność odczynu fluorescencyjnego, swoistego dla dopaminy, już we wczesnej fazie hodowli zależeć może od odrębności szczepowej użytych do doświadczeń zwierząt. Uzyskane wyniki wskazują, iż uzyskany typ hodowli stanowić może dobry model do oceny wpływu swoistych neurotoksyn na dopaminergiczne neurony istoty czarnej.

## РОСТ ДОФАМИНЕРГИЧЕСКИХ НЕВРОНОВ ЧЕРНОГО ВЕЩЕСТВА В ДИССОЦИРОВАННОЙ ТКАНЕВОЙ КУЛЬТУРЕ

Резюме

В диссоцированных тканевых культурах чёрного вещества взятого от 18—19-дневных эмбрионов мышей была установлена во многих отросках невронов флюоресценция, специфическая для дофамина. В противоположность данным, полученным другими авторами она появлялась уже в ранних стадиях культуры охватывая так аксоны как и дендриты. Она была заметна тоже в телах невронов скопленных так в плоских как и в шарообразных группах тканей. Авторы прежполагают что специфическая для дофамина реакция флюоресценции уже в ранних стадиях культуры может зависеть от штаммовой особенности экспериментальных животных. Результаты исследований показывают, что полученный тип культуры можно считать хорошей моделью оценки воздействия специфических невротоксин на дофаминергические невроны чёрного вещества.

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