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Excitatory amino acids, monoamine, and nitric oxide synthase systems in organotypic cultures: biochemical and immunohistochemical analysis

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Summary. The nigrostriatal and mesolimbic systems of the rat have been re-constructed using the organotypic culture model, whereby neonatal brain tissue is grown *in vitro* for approximately one month. The nigrostriatal cultures consisted of tissue from the substantia nigra, dorsal striatum and frontoparietal cortex; while the mesolimbic cultures included the ventral tegmental area, ventral striatum and cingulate cortex.

The cultures were grown at 35°C in normal atmosphere, using a tube-roller device placed in a cell incubator and changing the medium every 3–4 days. The *in vitro* development was evaluated with an inverted microscope equipped with a variable relief contrast function. Samples were taken directly from the medium in the culture tube and analysed for several amino acids with HPLC. After a month the cultures were fixed and processed for immunohistochemistry.

High levels of glutamate and aspartate were observed every time the medium was changed, but the levels rapidly decreased reaching a steady state after approximately 24 h. A decrease in the levels was also observed along development, reaching stable values (~2 μM and ~0.12 μM for glutamate and aspartate, respectively) at approximately two weeks, but only when the cultures showed an apparently healthy development. The levels were approximately 10 times higher in deteriorating or apparently damaged cultures. Glutamine levels were in the mM range and remained stable along the entire experiment. No differences were observed among nigrostriatal and mesolimbic cultures.

Immunohistochemistry confirmed the impressions obtained from microscopic and biochemical analysis along the *in vitro* development, revealing apparently healthy neuronal systems with characteristics similar to those observed *in vivo*, when tyrosine hydroxylase and nitric oxide synthase, markers for dopamine and nitric oxide containing neurons, respectively, were

analysed. In the substantia nigra, nitric oxide synthase-positive networks surrounded tyrosine hydroxylase-positive neurons, while in the striatum nitric oxide synthase dendrites were surrounded by tyrosine hydroxylase-positive nerve terminals, suggesting a reciprocal interaction among dopamine and nitric oxide containing neurons.

Thus, the organotypic model appears to capture many of the neurochemical and morphological features seen *in vivo*, providing a valuable model for studying in detail the neurocircuitries of the brain.

Keywords: Amino acids – Basal ganglia – Dopamine – Nitric oxide – Excitatory amino acids – Organotypic culture – Immunohistochemistry

Introduction

Neurocircuitries of the basal ganglia have been associated with several neurodegenerative disorders, such as Parkinson's and Huntington's diseases, as well as dementia of Alzheimer's type. Neurodegeneration probably leads to re-arrangements of the remaining neurocircuitries, preserving and/or impairing further the affected functions. Similar re-arrangements may perhaps provide a background for functional alterations, such as those observed in psychosis and drug addiction. Thus, further characterisation of the neurocircuitries of the basal ganglia may lead to a better understanding of the pathophysiology of disorders affecting this region, and hopefully to identify new therapeutic targets.

Gähwiler and collaborators have proposed an organotypic culture model for studying the neurocircuitries of the brain (Gähwiler et al., 1997), consisting in growing neonatal brain tissue on a coverslip immersed in a medium-containing culture tube, which is stored in a roller-tube device, providing a continuous change between liquid and gas interfaces. In such cultures, the major neuronal components of the basal ganglia can develop for long periods, re-establishing many of the morphological and electrophysiological properties observed *in vivo* (Plenz and Aertsen, 1996a,b; Plenz and Kitai, 1998).

Thus, in the present study the organotypic culture model was used to investigate how the interactions among several brain regions develop and to what extent the *in vivo* neurotransmitter phenotype of the basal ganglia is preserved, focusing on mesostriatal and mesolimbic dopaminergic systems. Triple cultures consisting of substantia nigra, dorsal striatum and frontoparietal cortex, or of ventral tegmental area (VTA), ventral striatum and cingulate cortex from newborn rats were grown for approximately one month. The *in vitro* development was evaluated with an inverted microscope equipped with a variable relief contrast function. The medium was analysed for several amino acids with HPLC. After a month the cultures were fixed and processed for immunohistochemistry, using antisera against tyrosine hydroxylase (TH) and/or nitric oxide synthase (NOS), markers for neurons containing dopamine (see Hökfelt et al., 1984) and nitric oxide (see Vincent and Kimura, 1992).

Materials and methods

Preparation of organotypic cultures

The preparation of chemicals and tissue was performed in the sterile environment of a laminar flow cabinet. Instruments and glasses were autoclaved and further sterilised in a hot bead steriliser. Newborn rats (postnatal day 2–3, body weight ~8 g; Sprague-Dawley, B&K, Stockholm, Sweden) were killed by decapitation, the brain rapidly removed within the laminar flow hood and stored in a small Petri dish containing Dulbecco's modified Eagle medium (DMEM; Life Tecnologies AB, Täby, Sweden) with 0.1% of an antibiotic/antimycotic solution. The brains were then apposed to an agar block and fixed with the frontal pole onto the specimen stage of a microslicer with a cyanoacrylate glue. Coronal sections were cut at mesencephalic (300 μm thick) and telencephalic (350 μm thick) levels and stored further in cold DMEM. Tissue samples were dissected according to the planes 203 and 204 of the atlas by Foster (1998), for the substantia nigra and VTA, and to the planes 193–195 and 193–194, for dorsal and ventral striatum, respectively, using a stereomicroscope within the laminar flow cabinet. Samples from frontoparietal and cingulate cortices were also taken. The dissected areas containing either substantia nigra, dorsal striatum and frontoparietal cortex, or VTA, ventral striatum (including fundus striati, accumbens nucleus, olfactory tubercle, lateral septum, ventral pallidum, piriform cortex) and cingulate cortex were placed on a coverslip with a spread layer of chicken plasma (25 μl) and bovine thrombine (20 μl, 1,000 NIH units in 0.75 ml DMEM; Sigma-Aldrich Sweden AB, Stockholm, Sweden). After ~20 min the coverslips containing the cultures were set into Nunc flat CT-tubes (Nunc, Naperville, IL, USA), containing an un-buffered culture medium (50% Basal Medium Eagle, 25% Hanks Balanced Salt Solution and 25% horse serum, 0.5% glucose and 0.5 mM of L-glutamine; Life Technologies AB). The cultures were grown at 35°C in normal atmosphere using a tube-roller device placed in a Heraeus incubator (Heraeus Instruments GmbH, Hanau, Germany), exposing the cultures to gaseous or water phase every 0.75 minute. At the third day *in vitro* (DIV 3), 10 μl of a mitosis inhibitor cocktail was added for 24 h. The medium was changed every 3–4 days.

In vitro monitoring

Growth was periodically monitored with an Inverted Axiovert-25 Zeiss microscope equipped with a variable relief contrast (VAREL) function (Carl Zeiss AB, Stockholm, Sweden).

Biochemical samples were taken from the waste when the medium was changed, or directly from the culture tube (20 μl) by carefully inserting a suitable micropipette in the laminar flow cabinet, preserving the sterile conditions. The samples were analysed in a HPLC system with *o*-phthaldialdehyde/mercaptoethanol precolumn derivatization and fluorescence detection (see Herrera-Marschitz et al., 1996).

Immunocytochemistry

After approximately one month, the cultures were washed in 0.1 M cold phosphate buffer (PB, pH 7.4) and fixed in 4% paraformaldehyde containing picric acid. After washing in phosphate buffered saline (PBS) and preincubation in 2% bovine serum albumin with 0.3% Triton X-100 in PBS, the cultures were processed with antiserum for TH, and afterwards with a second antiserum for NOS (see Gomez-Urquijo et al., 1999 for details). The tissue-bound antibodies were detected by incubating the sections at 37°C for 30 min with fluorescein isothiocyanate (FITC)- and lissamine-rhodamine-labelled antibodies. The culture containing coverslips were examined in a Nikon Microphot FX-fluorescence microscope Nikon, Tokyo, Japan) equipped with proper filter combinations, and then scanned in a confocal laser scanning microscope.

Confocal laser scanning microscopy

Organotypic cultures double-labelled for TH and NOS were analysed using a Biorad Radiance Plus (Biorad, Hemel Hempstead, UK) confocal scanning module, installed on a Nikon eclipse E600 fluorescent microscope. The FITC and rhodamine signals were detected separately. The FITC labelling was excited using the 488 nm line of the Argon ion laser and detected after passing a HQ530/60 (Biorad) emission filter. For the detection of the rhodamine signal the 543 nm line of the green HeNe laser was used in combination with the E570LP (Biorad) emission filter. Images of double-labelled cultures were merged in Adobe PhotoShop 5.0 (Adobe Systems Inc. San Jose, CA, USA). After optimising image resolution, brightness and contrast, images were printed using a Fuji Pictrography 3000 colour laserprinter (Fuji, Tokyo, Japan).

Statistics

Levels are expressed as the concentrations found in the samples, or as the percentage of levels detected in the stored medium (means ± S.E.M.).

The experimental protocol was approved by a Local Committee for Ethic in Experiments with Laboratory Animals.

Results

Evaluation of survival and development during culturing

The development and survival of the cultures was monitored with an inverted microscope at regular periods after culturing. During the first 24 hours *in vitro*, the tissue had a compact appearance when observed under dark field conditions. When inspected using the VAREL function at DIV 4, some bright cells could be observed on the surface of the tissue and fibre rich areas were slightly more white and amorphous. Some expansions and contacts could already be observed (Fig. 1a). During the first week (DIV 8), the cultured tissue became more transparent and cells became apparent on the surface of the tissue (Fig. 1b). The general appearance of the individually dissected tissue was still maintained, although fibre rich regions started to degenerate. At DIV 12 (Fig. 1c), the tissue was thinner and many cells could be observed on the surface. Cells could also be seen lying outside the originally cultured tissue. The tissue appeared to be more integrated, but the features of the individual tissues could still be distinguished. Deteriorating tissue left holes in what could represent fibre rich regions. This general impression was further confirmed when the cultures were examined at DIV 24 (Fig. 1d).

Survival was also assessed by the colour change of the phenol red included in the culture medium as pH indicator; a mild acidic change in pH indicated high metabolic activity, while a very alkaline pH indicated a poor culture condition, which could be confirmed by direct microscopic inspection or, after fixation, by immunocytochemistry.

Biochemical evaluation

Samples were first taken from the waste when the medium was changed, and assayed for glutamate, aspartate and glutamine.

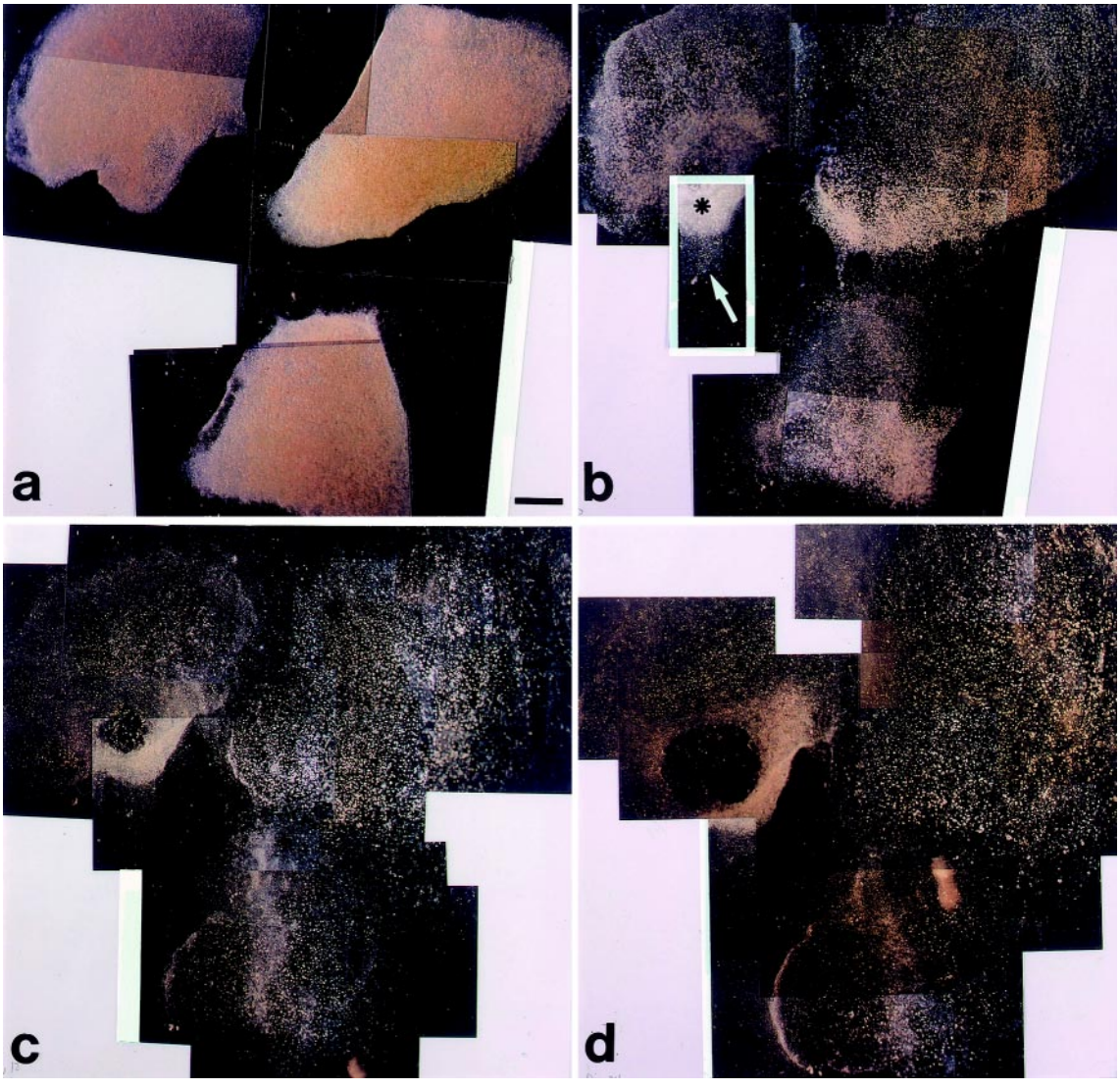

Fig. 1a–d. Montage of microphotographs taken from different stages of the same triple culture including tissue from the frontoparietal cortex (left), substantia nigra (right) and dorsal striatum (bottom), at DIV 3 (**a**), DIV 8 (**b**), DIV 12 (**c**) and DIV 24 (**d**) with an Inverted Zeiss Microscope equipped with a VAREL function. Note the merging, and the thinning of the tissue along development. The different compartments can still be distinguished at DIV 24. Scale bar: 200 μm. The picture within the white frame in **b** was over exposed (asterisk) in order to magnify the processes indicated by the arrow

As shown in Fig. 2, glutamate (a) and aspartate (b) levels were high during the first week after culturing ($>4\mu$M and $>1.5\mu$M, respectively), but decreased to stable "steady state" levels after approximately 2 weeks of culturing. Glutamate and aspartate levels were systematically high in cultures showing obvious deterioration (approximately 10 fold higher than that in apparently healthy cultures; data not shown). Glutamine levels were at a relatively stable mM concentration along the experiment (0.8–1.2 mM).

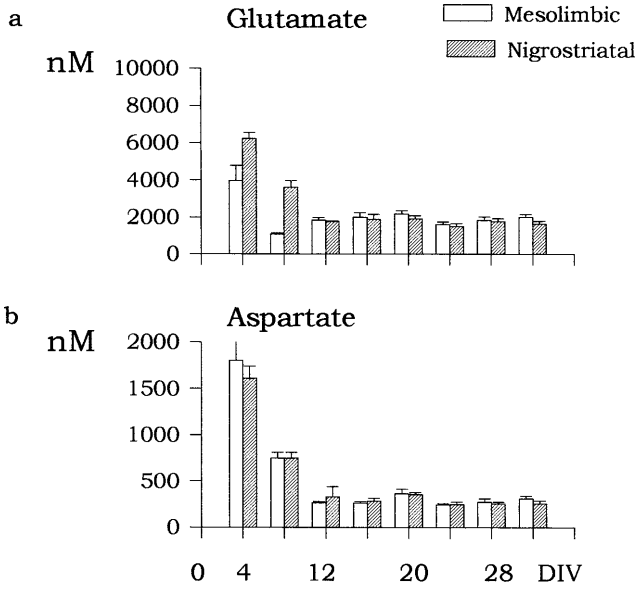

Fig. 2a,b. Glutamate (**a**) and aspartate (**b**) levels measured in the waste medium of mesolimbic (N = 6) and nigrostriatal (N = 6) organotypic cultures with a HPLC system. DIV, days *in vitro*. Levels are expressed as the concentrations (nM) determined in the samples (Means ± S.E.M)

Chromatographic analysis revealed ~1 mM glutamine, ~80 μM glutamate and ~12 μM aspartate in the stored medium, and ~40 μM glutamine, ~50 μM glutamate and 12 μM aspartate in the stored horse serum. No significant traces of glutamine, glutamate or aspartate were found in the Eagle or in the Hanks balanced salt solutions.

Glutamine levels measured in 20 μl culture samples taken at a one-hour interval after the medium change, both in nigrostriatal and mesolimbic cultures at DIV 12–14, were roughly the same as that measured in the stored medium (Fig. 3). In contrast, glutamate and aspartate levels rapidly decreased after the medium change, both in nigrostriatal and mesolimbic cultures, to levels representing less than 1% of that measured in the stored medium (Fig. 3).

Figure 4 shows the daily variation in glutamate and aspartate levels following medium change in a six week old nigrostriatal culture series, showing that glutamate and aspartate levels were largely stable on successive days following medium change (~2 μM and ~0.12 μM for glutamate and aspartate, respectively).

Immunohistochemical evaluation in fixed tissue

A series of cultures was fixed at DIV 24, and treated for immunohistochemistry, using antibodies against TH and NOS. In the region corresponding to the VTA of a mesolimbic culture there were many TH positive

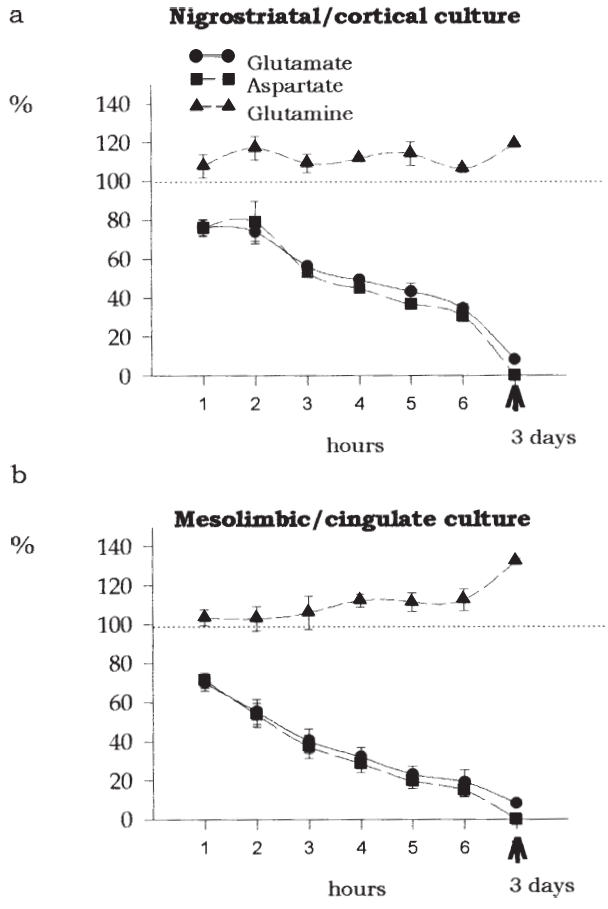

Fig. 3a,b. Glutamate, aspartate and glutamine levels in 20μl samples (in percentage, means ± S.E.M, of the concentrations detected in the stored medium; glutamine, 1,005μM; glutamate, 82.1μM; aspartate 15.4μM), taken at successive hours (1–6 hours) after the medium change from nigrostriatal (**a**) (N = 4) and mesolimbic (**b**) (N = 4) 12–14 days old organotypic cultures. Samples were also taken 3 days after, before the medium was again changed, arrow. Dotted lines indicate 100%

cells, showing a healthy appearance and many local arborizations and fibres innervating the co-cultured tissue, mainly the region corresponding to the ventral striatum. TH positive neurons and fibres intermingled with NOS positive cells (Fig. 5a). Fibres from NOS positive cells apposed to TH positive processes, possibly indicating synaptic interactions. In the ventral striatum, many NOS positive cell groups were observed intermingling with TH positive axons, also suggesting synaptic interactions. No TH positive neurons could be seen in ventral striatum (Fig. 5b).

Discussion

In the present study attempts were made to reconstruct the major neurocircuitries of the basal ganglia *in vitro* by using the organotypic culture

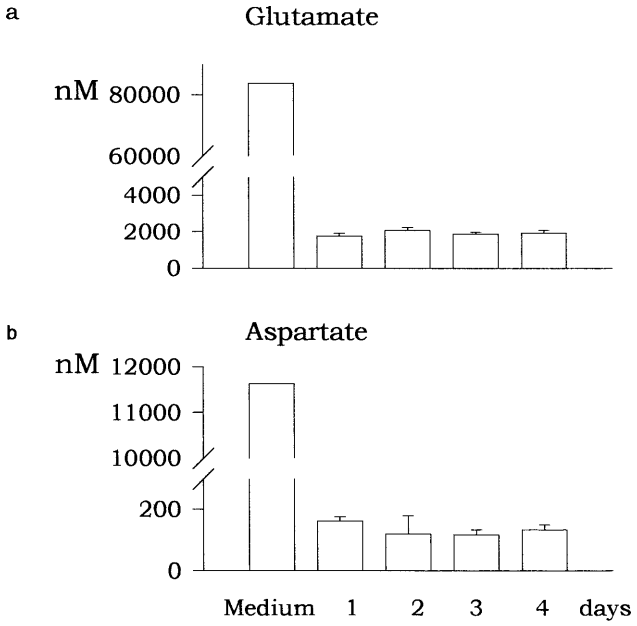

Fig. 4a,b. Daily variation of glutamate (**a**) and aspartate (**b**), 1 to 4 days after medium change in a six week old nigrostriatal culture series (N = 4). The first column represents the concentrations (*nM*) found in the stored medium

model of Gähwiler and co-workers (1997). The focus was on a morphological and biochemical characterisation *in vitro*, and on an immunocytochemical analysis of TH-and NOS-systems in fixed material. Several putative neurotransmitter and metabolism substances could be characterised, e.g. amino acids, glucose, pyruvate, lactate, glycerol, hypoxanthine and xanthine (Kohlhauser et al., in preparation). In this study, however, glutamate, aspartate and glutamine were used to exemplify and discuss some general problems emerging when this kind of biochemical characterisation is attempted.

The nonessential amino acid glutamine, a major nitrogen donor, contributing to the biosynthesis of a wide range of important compounds, including glutamate and γ-aminobutyric acid (GABA), but also aspartate, was directly added at a relative high concentration (0.5 mM) to the medium, and it was also found in the purchased horse serum. *In vivo*, glutamine is kept at a relative constant equilibrium by a tight regulation of the activity of glutamine synthetase and glutaminase, and here, *in vitro*, we found that glutamine levels were very stable, representing ~100% of that in the stored medium.

Glutamate, a precursor of glutamine, proline and arginine, is also produced when the citric acid cycle intermediate α-ketoglutarate accepts an amino group. Glutamate can also be produced from glutamine via glutaminase. In the present preparation, there were already high levels of glutamate in the stored medium, but the levels rapidly decreased in samples

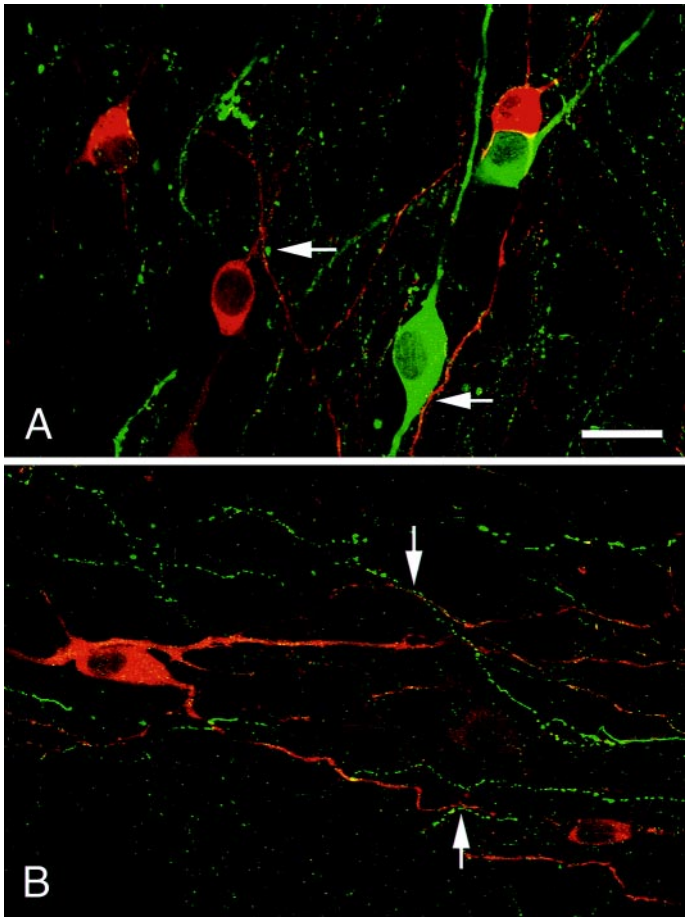

Fig. 5a,b. Confocal laser scanning microphotograph of a mesolimbic 24 days old culture, double labelled for tyrosine hydroxylase (TH, green) and nitric oxide synthase (NOS, red) (see Materials and methods). The arrows indicate apposition among TH and NOS positive fibres in the ventral tegmental area (**A**) and NOS fibres and TH positive terminals in the ventral striatum (**B**), possibly indicating synaptic contacts. Scale bar: 20 μm

taken from healthy cultures, indicating transport and/or metabolism, to levels similar to those found in the extracellular compartment of the brain (see Herrera-Marschitz et al., 1996). Glutamate levels were always high in cultures showing obvious deterioration. The issue whether steady-state glutamate levels also reflect neuronal activity in organotypic cultures is still under investigation.

Aspartate, derived from the citric acid cycle intermediate oxaloacetate, and closely associated to glutamate, was found at high concentrations in the stored medium, and it appeared to be transported and/or metabolised to steady state levels similar to those observed *in vivo* (Herrera-Marschitz et al., 1996). Aspartate levels were high in deteriorating or dead cultures. Thus, as for glutamate, a decrease in aspartate levels appears to indicate an active

transport by membrane bound proteins and metabolism, probably by healthy neuronal and/or glial cells.

As previously shown, we found that the nigrostriatal (Östergaard et al., 1990; Plenz and Kitai, 1996; Schatz et al., 1999; Gomez-Urquijo et al., 1999), and mesolimbic (Gomez-Urquijo et al., 1999) dopamine systems develop well in the organotypic preparation, preserving many of the features observed *in vivo*. Dopamine cell bodies were concentrated in the pars compacta of the substantia nigra, or in the VTA, and only a few TH-positive neurons were displaced out from the original tissue. As recently reported (Gomez-Urquijo et al., 1999), the expression of NOS-like immunoreactivity was particularly impressive. Both large and small NOS-positive neurons were observed in the cultured mesencephalon. The small neurons were more abundant, very similar in morphology to TH positive neurons, and often grouped in their vicinity. NOS positive fibres could appose to TH dendrites, suggesting synaptic interactions. In the striatum, many NOS-positive, medium sized, multipolar neurons were seen, with morphological characteristics similar to those described *in vivo* with NOS immunohistochemistry and diaphorase staining (Vincent et al., 1983; Dawson et al., 1991; Vincent and Kimura, 1992; de Vente et al., 1998). NOS-positive neurons and processes were surrounded by TH-positive fibres, suggesting a reciprocal interaction among dopamine and nitric oxide releasing neurons, both in mesencephalon and telencephalon.

In conclusion, the nigrostriatal and mesolimbic dopaminergic systems can be re-constructed *in vitro* using the organotypic culture model. The *in vitro* development provides an opportunity for functional studies. Some neuronal systems can be particularly enhanced in the organotypic model, revealing unexpected interactions, which perhaps may lead to novel pharmacological targets.

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