CO-CULTURE OF HYPOTHALAMIC NEURONS AND MELANOTROPE CELLS: A MODEL TO STUDY SYNAPTOGENESIS BETWEEN CENTRAL NEURONS AND ENDOCRINE CELLS

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Abstract—As a first step towards elucidating mechanisms involved in neuroendocrine synaptogenesis, we developed a model of co-culture based on hypothalamic-intermediate pituitary interactions. Dissociated hypothalamic neurons from fetal rats at embryonic day 15 were cultured in a defined medium together with melanotrope cells of the pituitary intermediate lobe from neonatal rats. In these co-cultures, establishment of synaptic contacts between GABAergic or dopaminergic neurons and an endocrine target cell, the melanotrope cell, was studied by morphofunctional approaches.

Using double immunostaining with antibodies directed against glutamate decarboxylase or tyrosine hydroxylase and α-melanocyte-stimulating hormone, we demonstrated morphological contacts between GABAergic or dopaminergic neurons and melanotrope cells as early as three days in vitro. Furthermore, using an antibody directed against synapsin I, we showed a modification of synapsin I immunoreactivity from diffuse to punctate distribution correlated with the establishment of contacts and the observation of characteristic neuroendocrine synapses by electron microscopy. These results were further confirmed by electrophysiological studies. Patch-clamp recordings demonstrated that, at six days in vitro, some melanotrope cells displayed GABAergic synaptic currents, which occurred either spontaneously and/or could be evoked chemically by 50 mM KCl or 100 µM kainate. The proportion of the melanotrope cells receiving functional synaptic inputs increased until 10 days in culture, a stage at which virtually all melanotrope cells in contact with neurons possessed functional synapses.

The results presented here describe the establishment of neuroendocrine synapses in vitro, studied by combining morphofunctional and electrophysiological approaches. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

Key words: neuroendocrine synapse, hypothalamus, intermediate lobe of the pituitary, dopamine, GABA, patch-clamp recording.

Due to the complexity of the developing nervous system and the technical difficulties involved in studying individual neurons or one neuron coupled to its target cell, mechanisms underlying synaptogenesis in vivo are poorly understood. The technical difficulties in studying individual neurons in situ are being overcome with the development of in vitro approaches. Until now, all in vitro studies concerned synaptogenesis at the neuromuscular junction and neuron-neuronal synapse formation of a limited number of neurons of the CNS.20,23 Furthermore, in the nervous systems, precise identification of the nature of the target cell has been made difficult because of the heterogeneity of the cell populations. In this work, we report a system of co-culture offering the possibility of studying the genesis of a neuroendocrine synapse. This system allows studies of the reciprocal inductive interactions between central neurons of the hypothalamus and endocrine melanotrope cells from the intermediate lobe (IL) of the pituitary.

In situ, melanotrope cells constituting the IL of the pituitary are negatively regulated by a direct innervation originating from the rostral periventricular area of the hypothalamus.5,11,39 This innervation is mainly dopaminergic2 and GABAergic.21 Dopamine and GABA are co-localized within the same axon terminals,45 synaptically released, and exert a tonic inhibition on melanotrope cells. In adult melanotrope cells, pharmacological activation of dopamine D2 receptors9,24,28 and GABA A receptors7,40,42 lead to a decrease of pro-opiomelanocortin mRNA expression and to a reduction of spontaneous electrical and secretory activities. Dopamine D2 receptors...
activate a $K^+$ conductance and inhibit a $Ca^{2+}$ current via a pertussis toxin-sensitive pathway, whereas GABA inhibits electrical and secretory activities of melanotrope cells by activating GABA$_A$ receptors and decreases the release of $\alpha$-melanocyte-stimulating hormone ($\alpha$-MSH) by activating GABA$_B$ receptors, probably by reducing the intracellular free $Ca^{2+}$ concentration.

Maturation of synapses is characterized by the increased expression of synapsin I. This protein belongs to a family that is highly specific for nerve terminals. Synapsin I is selectively associated with small synaptic vesicles and is not present on other membrane structures. Moreover, in vivo, the appearance of synapsin I in neurons during ontogenesis of the nervous system correlates with the onset of synaptogenesis and with the development of synaptic contacts. For these reasons, synapsin I immunoreactivity was used as a marker of synaptic maturation in our model. The maturation was further confirmed by using electron microscopy and patch-clamp recording.

**EXPERIMENTAL PROCEDURES**

**Tissue culture**

Hypothalamic neurons. Pregnant Wistar rats (D'près, France) at day 15 of gestation (day 0 of gestation corresponding to the day of fertilization) were anesthetized with pentobarbital and the embryos removed. The diencephalic area corresponding to the hypothalamus was isolated and incubated with trypsin–EDTA (0.05–0.02%; Gibco) for 7 min at 37°C under constant shaking. The enzymatic dissociation was stopped by adding 20% fetal calf serum. Cultures were maintained in water saturated atmosphere (95% air, 5% CO$_2$) at 37°C. Medium was first renewed after five days and every three days thereafter. Hypothalamic neurons at day 15 of gestation (day 0 of gestation corresponding to the day of fertilization) were anesthetized with pentobarbital and the embryos removed. The diencephalic area corresponding to the hypothalamus was isolated and incubated with trypsin–EDTA (0.05–0.02%; Gibco) for 7 min at 37°C under constant shaking. The enzymatic dissociation was stopped by adding 20% fetal calf serum (FCS) and followed by centrifugation for 3 min at 180 g. Mechanical dissociation was performed in Dulbecco modified Eagle's medium (Gibco, France) containing 20% FCS and 0.1% Triton X-100. After centrifugation for 7 min at 180 g, the pellet was resuspended in serum-free medium according to Bottenstein and Sato. Hypothalamic cells were seeded at a density of 400 cells/mm$^2$ in 35-mm Petri dishes for electrophysiological study, or at a density of 800 cells/mm$^2$ on 8-mm diameter coverslips in four-well multidishes (15-mm diameter) for immunocytochemistry and electron microscopy studies. In each condition, both 35-mm dishes and glass coverslips were precoated with poly-l-lysine (type VI 1B, mol. wt 60,000, Sigma; 10 µg/ml in 0.1 M borate buffer, pH 8.4) and preincubated for 1 h in phosphate-buffered saline (PBS) containing 10% FCS. Cultures were maintained in water saturated atmosphere (95% air, 5% CO$_2$) at 37°C. M edium was first renewed after five days and every three days thereafter.

**Intermediate lobe cells.** Neonatal (one- to seven-day-old) Wistar rats were killed by decapitation under deep diethyl ether anesthesia. Pituitaries were taken out, extensively washed in PBS and neurointermediate lobes were separated from anterior lobes. Melanotrope cells of neurointermediate lobes were dissociated as described above. Cells were seeded with hypothalamic neurons at a density of 80 cells/mm$^2$ for electrophysiological studies and 160 cells/mm$^2$ for immunocytochemical and electron microscopic analyses.

**Immunostaining**

After one, three, five, seven or 12 days in vitro (DIV), cultures were fixed for 10 min in PBS containing 4% paraformaldehyde (Kodak) at room temperature and subsequently rinsed with PBS. For the detection of tyrosine hydroxylase (TH) immunoreactivity, cultures were permeabilized for 10 min in PBS containing 1% Triton X-100 followed by 30 min incubation in PBS containing 5% horse serum and 0.1% Triton X-100.

For the detection of gial fibrillary acidic protein (GFAP) immunoreactivity, cultures were permeabilized for 30 s in ice-cold methanol and rinsed with PBS.

For the detection of neuron-specific enolase (NSE), vimentin, glutamate decarboxylase (GAD) and $\alpha$-MSH immunoreactivities, cultures were not pre-permeabilized. Cultures were incubated overnight at 4°C with the primary antibody diluted in PBS containing 0.1% Triton X-100. The rabbit polyclonal antibodies used were directed against NSE (1:2000, Immunotech, France), GFAP (1:1000, Dako, Denmark), synthetic $\alpha$-MSH (1:3000; the late Dr G. Schmidt, Strasbourg, France) and GAD (1:500, Chemicon: AB 108). The mouse monoclonal antibodies used were directed against vimentin (1:20; Boehringer) and TH (1:10,000; Chemicon: MAB 318). Following antibody incubation, cells were rinsed three times with PBS and incubated for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobulin G (1:500; Vector) for NSE, GFAP, $\alpha$-MSH and GAD detection, or in sheep anti-mouse immunoglobulin G (1:500; Vector) for vimentin and TH. Cultures were rinsed three times for 10 min with PBS and incubated for 1 h with an avidin–biotin–peroxidase complex (Elite kit, Vector). After three washes, peroxidase was detected with 3,3’-diaminobenzidine tetrahydrochloride (Vectastain kit, Vector). Reaction was stopped by washing with PBS. Cultures were then dehydrated in graded alcohols, cleared in xylene and mounted.

To perform the double immunostaining for TH or GAD and $\alpha$-MSH, detection of TH or GAD was performed as described above. Cultures were then incubated for 1 h at 37°C with rabbit antiserum against $\alpha$-MSH (1:3000) and rinsed with PBS. They were incubated for 1 h with goat anti-rabbit alkaline phosphatase-coupled Fab fragments (1:650; Sigma). After rinsing, $\alpha$-MSH immunoreactivity was detected with a chromogenic solution containing 340 µg/ml nitro blue tetrazolium (Sigma), 175 µg/ml bromo-chloro-indol-phosphate (Sigma), 0.1 M NaCl, 0.05 M MgCl$_2$, in 0.1 M Tris-HCl (pH 9.5). Cultures were rinsed and mounted in aqueous mounting medium consisting of 7.5% gelatin and 50% glycerol in PBS.

For the detection of synapsin I, cultures were incubated overnight in rabbit polyclonal antibody directed against bovine synapsin I (1:500; Dr J. Baudier, Strasbourg, France) diluted in PBS containing 0.1% Triton X-100. In in the western blot analysis of rat cortical neurons, the anti-synapsin I antibody used revealed only two bands (mol. wts 80,000 and 86,000), corresponding to the isoforms described by De Camilli et al. (not shown). After three washes in PBS containing 0.1% Triton X-100, they were incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G (1:200, Biosys, France), rinsed with PBS and mounted.

Cultures used for semi-thin sections (detection of synapsin I and $\alpha$-MSH on adjacent sections) were fixed for 10 min in 0.1 M phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde (Kodak). After dehydration in graded alcohols, they were conventionally embedded in Araldite–Epon mixture and semi-thin sections (1.5 µm) were made. Embedding medium was removed with sodium methoxide and sections were incubated overnight with the polyclonal antibodies directed against synapsin I or $\alpha$-MSH (see above). The following steps for immunoperoxidase detection were performed as described above.

All the antisera used in culture have also been characterized in histological sections; no non-specific labeling was observed. Moreover, cultures incubated without
primary antibody or with an irrelevant antibody showed no staining.

Electron microscopy

Cultures grown for 8 and 12 DIV on glass coverslips were fixed with 5% glutaraldehyde (Fluka) in PB for 1 h at 4°C and postfixed for 1 h with 1% osmium tetroxide in PB containing 1.6% potassium ferrocyanide. After dehydration in graded alcohols, they were flat-embedded in an Araldite–Epon mixture. The coverslips were detached by immersion in liquid nitrogen. Thin sections (50 nm), contrasted with uranyl acetate and lead citrate, were examined under a Jeol 100 CX electron microscope.

α-Melanocyte-stimulating hormone measurement

α-MSH radioimmunoassay was performed as described previously by Vuillez et al. Data are the mean ± S.D. from five cultures.

Neuron counting

Cells were counted using an inverted phase-contrast microscope. The number of NSE-, vimentin-, GFAP-, α-MSH-, GAD- or TH-positive neurons were counted in 10 randomly chosen observation fields corresponding to 0.39 mm². In the same fields, total number of neurons was counted on the basis of morphological criteria (ovoid cell body, neurites). Data are expressed either as the mean of cell type ± S.D. or as the mean percentage of GAD-positive (or TH-positive) neurons in the total neuronal population ± S.D. Data are the average from three cultures in triplicate.

Electrophysiological recordings

Electrophysiological experiments were performed at room temperature (20–22°C) three to 15 days after plating of the cells. Patch-clamp recordings were made under voltage-clamp in the whole-cell recording configuration using an Axopatch 200 A amplifier (Axon instruments, U.S.A.). Low-resistance (3–4 MΩ) electrodes were used. The external medium contained (in mM): NaCl 135; KCl 5; CaCl₂ 5; MgCl₂ 1; HEPES 5; glucose 10; pH 7.3 with NaOH. Pipettes were filled with an intracellular solution containing (in mM): KCl or CsCl 125; CaCl₂ 5; MgCl₂ 2; HEPES 10; EGTA 10; pH 7.3 (adjusted with KOH or CsOH). The estimated intracellular free calcium concentration was 10⁻⁶ M and the equilibrium potential for chloride ions (E_Cl) was −2 mV.

The competitive GABA_A receptor antagonists SR 95531 and bicuculline were prepared as 10 mM stock solutions in distilled water and stored at −20°C. Just before the recording session, these substances were diluted to their final concentrations in external medium. Local application of drugs was performed with a “U-tube”. SR 95531 was a kind gift of Sanofi-Rcherche (Montpellier, France) and bicuculline methiodide was purchased from Sigma (France).

Data storage and analysis

Synaptic currents were stored on videotape in digital form (20 kHz) after being filtered at 5 kHz by the internal filter of the Axopatch 200 A amplifier. Analysis was performed using the Axograph software (Axon Instruments, U.S.A.) on a Macintosh IIx computer. For this purpose, stored data were acquired off-line with the pclamp software (Fetcher, Axon Instruments, U.S.A.) at a 10-kHz resolution with no additional filtering. All statistical results are expressed as mean ± S.D.

RESULTS

Co-culture characterization

Immediately after dissociation, when observed under a phase-contrast microscope, hypothalamic cells as well as IL cells appeared spherical and devoid of any processes, making it impossible to indentify any type of cell. After 3 DIV, neurons have started to extend neurites, whereas melanotrope cells remain spherical. Some neurons (white arrows) have already entered into contacts with melanotrope cells (black arrowheads). Scale bar = 50 µm.
After 1, 3, 5, 7 and 12 DIV, cultures were fixed and processed for immunocytochemical detection of NSE for neurons. G F A P and/or vimentin for glial cells and fibroblasts, α-M SH for melanotrope cells, TH for dopaminergic neurons and G A D for GABAergic neurons. Immunoreactive cells were counted in 10 randomly chosen observation fields corresponding to 0.39 mm² each. Data are expressed as the mean of cell type ± S.D. per field and are the average from three cultures in triplicate.

α-M SH in culture medium at a constant rate (1.04 ± 0.38 nM per day and per well; n = 20). Usually, melanotrope cells were isolated, but occasionally they tended to reaggregate and to form small clusters.

The neuronal cells had a bright and ovoid cell body, and extended one or two thin short processes. Concomitantly, they started to establish apparent contacts with each other (Fig. 1B). The diameters of their perikarya were smaller (<10 µm) than those of the melanotrope cells (10–12 µm). A slight NSE immunoreactivity was detectable in some neurons 24 h after plating (14.7 ± 0.8%; n = 9; Table 1), but the labeling became consistent in all of them after 3 DIV (Fig. 2D). From the third DIV, all neuronal cells had processes and a dense and complex fiber network was formed. The number of NSE-positive neurons was constant until 5 DIV (Table 1), and then started to slowly decrease to about 50% of the initial population identified on the basis of morphological criteria after 1 DIV. G A D-positive neurons represented 80–85% of the total population of neurons over the time in culture (Table 1). G A D-positive immunoreactivity was found over the cell body and the proximal part of neurites (Fig. 2E). The level of G A D-positive immunoreactivity was not homogeneous between the G A D-positive cells, but the light staining observed corresponded to positive cells compared to the control reactions (not shown). In contrast to G A D-positive neurons, TH-positive neurons constituted a small subpopulation of neurons which increased until 5 DIV and represented 5–7% of the neuronal population (Table 1). They displayed a uniform TH immunoreactivity distributed over the whole cell (Fig. 2F).

Morphofunctional study of synaptogenesis

Phase-contrast observation of co-cultures shows apparent neuroneuronal contacts after only 1 DIV (not shown). Thin neurites of a few neurons made apparent contacts with melanotrope cells after 3 DIV (Fig. 1B). Both G A D-positive (Fig. 3A) and TH-positive (Fig. 3B) neurons established contacts with melanotrope cells. At 3 DIV, most of the neurons were weakly labeled with the synapsin I antibody (Fig. 4A). Immunoreactivity was diffusely distributed in the cell body and in the proximal part of neurites. Progressively, the number of neurons contacting melanotrope cells increased with time in culture. In addition, most of the TH-positive neurons sent two or more processes towards several melanotrope cells and established contact with them, as shown after 12 DIV in Fig. 3C. Numerous G A D-immunoreactive contacts were also identified (data not shown); however, due to the high density of GABAergic neurons, a clear-cut identification of the cell bodies of GABAergic neurons establishing contacts with melanotrope cells was difficult. At this time, synapsin I immunoreactivity was highly concentrated in tiny dots scattered on less intensely labeled neuronal cell bodies and extensions (Fig. 4B). Synapsin I immunoreactivity had almost disappeared from the neuronal cell bodies. By comparing synapsin (Fig. 4C) and α-M SH (Fig. 4D) immunolabeled adjacent semi-thin sections, synapsin I punctae were found in close apposition with melanotrope cells.

Electrophysiological characterization of synaptic transmission

As shown in the above sections, hypothalamic neurons formed direct contacts with melanotrope cells as well as with other hypothalamic neurons. Moreover, these contacts seemed to have the morphological characteristics of synapses. In order to test the functionality of these contacts, we have recorded from melanotrope cells and neurons which were in apparent contact with one or several neurites.
of hypothalamic neurons. The postsynaptic cells were identified on the basis of morphological and electrophysiological criteria (see below). Their electrical activities were recorded with the patch-clamp technique under whole-cell voltage-clamp, a configuration which allowed optimal resolution of synaptic currents of small amplitude. Figure 5A shows a recording obtained in a melanotrope cell. The current trace displays spontaneously and randomly occurring inward currents corresponding to
Synaptic events. These synaptic currents had variable amplitudes (10–100 pA), and were characterized by fast rise times (<1 ms) and slowly decaying phases. The total duration of these events was typically between 100 and 200 ms. These synaptic currents were completely and reversibly blocked in postsynaptic melanotrope cells after local application of 5 µM SR95531 (n = 5; Fig. 5A) or 5 µM bicuculline (n = 18; data not shown), two selective and competitive antagonists of GABA<sub>A</sub> receptors. At the same concentration (5 µM), bicuculline (n = 8) and SR95531 (n = 6) also reversibly suppressed the synaptic activity recorded in postsynaptic neurons (data not shown). These results indicated that the contacts formed between hypothalamic neurons and melanotrope cells corresponded to functional GABA<sub>A</sub>ergic synapses and that synaptically released GABA activated GABA<sub>A</sub> receptors.

Pattern of synaptic activity at neuroneuronal versus neuroendocrine GABA<sub>A</sub>ergic synapses

As shown on Fig. 5B, neuroneuronal and neuroendocrine synapses displayed distinct patterns of synaptic activity. Spontaneous synaptic events were observed only in a subset of melanotrope cells, i.e. 22.9% (19 of 83 cells), although all of these cells (n = 83) were synaptically connected to a presynaptic neuron. Indeed, synaptic events could be induced in these cells by local application of an extracellular solution containing 50 mM KCl. There was no significant change in the fraction of cells displaying spontaneous activity with time in culture. For example, this fraction was similar after eight days (23%; n = 13) and 13 days (30.7%; n = 13) in culture. The mean percentage of melanotrope cells exhibiting spontaneous synaptic events was 29 ± 11% as determined between seven and 14 days in culture in a total of 83 synaptically connected cells from six different co-cultures. Moreover, in this subset of melanotrope cells, the spontaneous synaptic currents occurred at a low frequency, i.e. 0.31 ± 0.20 Hz (n = 19). In contrast, the mean frequency of spontaneous activity recorded in neurons was about four-fold higher (1.19 ± 0.62 Hz; n = 16). In addition, the fraction of neurons displaying spontaneous synaptic events increased markedly with time in culture. For example, 12.5% (n = 8) of the neurons possessed spontaneous synaptic currents after eight days in culture, whereas all of the neurons examined (n = 13) displayed spontaneous activity after day 13.

Fig. 3. Double labeling of GAD/α-MSH (A) and TH/α-MSH (B, C). α-MSH is revealed by immunophosphatase, GAD and TH by immunoperoxidase. (A, B) After 3 DIV, few GAD-positive neurons (A) and TH-positive neurons (B) are connected to melanotrope cells by a thin, weakly labeled, neurite (arrow). (C) After 12 DIV, a single TH-positive neuron establishes contacts (arrows) with several melanotrope cells and displays a highly developed neuritic arborization. Scale bars = 20 µm (A, B); 40 µm (C).
Electrophysiological identification of cell types

As shown in the first part of this report, neurons rapidly extended neurites after plating. The size of these neurites increased with time in culture, whereas, under the same conditions, melanotrope cells kept a rounded phase-bright appearance and a constant diameter of approximately 12 µm. This suggested that the membrane surface of neurons should increase with time in culture, whereas that of melanotrope cells should stay relatively constant. Since the membrane capacitance of a cell is directly proportional to the membrane area, this parameter is a good index of the cell surface. Therefore, we determined the value of the membrane capacitance of...
neurons and melanotrope cells with time in culture. The measurements were performed in separate cultures of each cell type in order to avoid any possible error. The membrane capacitance was deduced directly from the capacitance compensation settings of the patch-clamp amplifier. Figure 5C summarizes the results obtained. The values of membrane capacitance of neurons and melanotrophs were relatively close on the fifth day in culture, when capacitance measured 13.5 ± 5.3 pF (n = 7) for neurons and 7.8 ± 0.7 pF (n = 15) for melanotrope cells. However, with time in culture, the membrane capacitance of neurons continued to increase regularly (e.g., 30 ± 8 pF, n = 15, at day 14), whereas that of melanotrope cells remained constant (7 ± 1.7 pF, n = 25, at day 14) over the same period. These results suggest that, together with morphological characteristics (presence or absence of neurites), the value of membrane capacitance and the pattern of synaptic activity are useful criteria to distinguish melanotrope cells from neurons in our co-culture system.

Development of functional synaptic connections

The pattern of development of functional neuroendocrine synapses was obtained by recording, at different days in culture, melanotrope cells displaying apparent contacts with neurites of hypothalamic neurons. The presence of both spontaneously occurring or chemically evoked synaptic activities was investigated. The latter was induced by applying locally a solution containing 50 mM KCl, which depolarized the presynaptic terminal and thereby caused a calcium-dependent release of neurotransmitter. Figure 6 shows the general time-course of functional synapse formation in our co-culture
The results illustrated represent recordings from a total of 137 melanotrope cells in 26 different co-cultures over a period ranging from the third to 14th days in culture. We never observed any spontaneous or chemically evoked synaptic activity before day 6 (n = 16). At 6 DIV, 8.3 ± 11.7% (n = 11) of the melanotrope cells displayed synaptic currents. The proportion of pituitary cells receiving synaptic inputs continued to increase until day 10, after which virtually all melanotrope cells (n = 48) in contact with neurites were found to possess functional GABAergic synapses.

**DISCUSSION**

In the present study, we show that our culture conditions in serum-free medium allow the maturation of hypothalamic neurons and the establishment of functional neuroendocrine synapses with melanotrope cells. The hypothalamic neurons, which are poorly differentiated when dissociated, displayed signs of maturation over time in culture. The number of NSE-positive cells, very low just after plating (fewer than 15% of cells with neuronal morphology), increased thereafter. From this stage, the number of neurons slowly decreased, but at 12 DIV, about 50% of the initial neuronal population still displayed the characteristics of differentiated neurons: TH or GAD immunoreactivity, development of neuritic processes and neuroneuronal or neuroglandular functional contacts, attesting to the overall health of the cells and the suitability of the co-culture for synaptogenesis studies. However, the reduction of postsynaptic activities (percentage of inhibitory postsynaptic currents; Fig. 6) from 14 DIV may indicate that the co-culture system does not allow such studies after further maintenance in culture under the prevailing conditions. As early as after 1 DIV, neurons extended neurites, formed homotypic contacts and established a dense neuritic network with a pattern comparable to those described in embryonic day (E) 16 mouse hypothalamic cultures.3,17,26 We did not investigate all possible types of neurons which could be present in our cultures. In view of the specific innervation of the IL, we first focused on dopaminergic (TH-positive) and GABAergic (GAD-positive) neurons. The increase of TH and GAD expression (until 7 DIV) is probably due to an increase in the number of TH- and GAD-immunoreactive neurons and an increase of immunoreactivities themselves, which correlates with the maturation in vivo. Indeed, during development, expression of TH increases between E13 and postnatal day 3 within the rat hypothalamus.7,33,36,37,43,44 GAD expression is first detected around E15 in the hypothalamus. However, in our system, we have a large proportion of GAD-positive neurons ( ≥ 80%) compared to that found by Wahle et al.47 (30%) in cultures of E14-E15 rat hypothalamic neurons alone. This discrepancy is not due to a non-specific labeling with the anti-GAD antibody used, because a specific labeling of GABAergic fibers and cell bodies was found on brain semi-thin and free-floating sections. However,
this discrepancy could be related to differences in tissue sampling, cell dissociation, culture conditions or to the presence of the melanotrope cells in the co-culture.

The melanotrope cells were taken from neonatal rats (one to seven days old) at the time when dopaminergic and GABAergic fibers invade the IL and contact these cells. As demonstrated by in vivo23,32,34 and in vitro experiments, at this stage, almost all of the IL cells display the main characteristics of differentiated melanotrope cells, including pro-opiomelanocortin gene expression, spontaneous release of α-MSH and β-endorphin, functional dopamine D2 receptors, and ability to form neuroendocrine synapses. Some of the phenotypic characteristics of melanotrope cells (the expression and secretion of α-MSH) were conserved during the time in culture, so it is likely that we reconstitute in vitro the neuron-target interactive system, including differentiated melanotrope cells.

The apparent formation of contacts between neurons and melanotrope cells was observed only after 3 DIV and the proportion of melanotrope cells receiving neuronal contacts increased through the time in culture, despite the decrease of the neuronal population after 7 DIV. The establishment of these neuroendocrine contacts is subsequent to the formation of the first neuroneuronal appositions, which occurred as early as 1 DIV. The delay observed in the formation of neuroendocrine versus neuroneuronal contacts might be due to the lower density of melanotrope cells compared to that of the neurons over the time in vitro (from 1:10 after 1 DIV to 1:2.5 after 12 DIV). This delay may also be related to the need of specific factors from the endocrine cells, such as neurotrophic/neurotropic factors or cell surface molecules which might require some time in culture after dissociation prior to their functioning.20

At the time of the first neuroendocrine contacts, synapsin immunoreactivity is diffusely distributed in the neurons. The diffuse synapsin I immunoreactivity in nerve cell bodies has been observed previously both in vivo14 and at early times in vitro, and corresponds, as described in this report, to an immature state where synapses are not functional. The evolution of synapsin I immunoreactivity to a punctate labeling is correlated with the appearance of morphological and functional characteristics of more mature neuroendocrine synapses. Indeed, we observed ultrastructural features of mature synapses: clustering of electron-lucent vesicles in presynaptic axon terminals and symmetrical moderate membrane thickening, as described in vivo by Baumgarten et al.2 These different morphological aspects of synapse maturation are similar to those described in cultures of striatal48 and hippocampal19 neurons.

Moreover, patch-clamp recordings revealed that the contacts formed between the neurons and the endocrine cells corresponded to functional synapses. The synaptic currents were specifically blocked by competitive antagonists of the GABA_A receptor, which demonstrated that these synapses were GABAergic. This electrophysiological approach allowed us to describe the time-course of the formation of functional synapses in culture. Synaptic currents were never observed in melanotrope cells before 6 DIV. Between 6 and 10 DIV, the number of melanotrope cells in contact with neurites, presenting spontaneous and chemically evoked synaptic currents, increased markedly, reflecting the functional maturation of GABAergic synapses. This direct evaluation of the development of functional GABAergic synapses with time in culture matched well with the morphofunctional synaptic maturation indicated by the redistribution of synapsin I immunoreactivity (from the cell body to terminals) and ultrastructural observations. Spontaneous synaptic currents were recorded only in a subset of melanotrope cells (30%) and displayed specific features (low frequency) as compared to those recorded in neurons. We have no clear explanation for this phenomenon, but it could be due to a difference in electrical activity of neurons involved in these connections, or related to an effect of the postsynaptic element (melanotrope cell or neuron), which could respond differently to, or regulate the properties of, the presynaptic neuron. In the future, this co-culture system might offer the possibility to study the effects of different targets (neurons or endocrine cells) on the last steps of presynaptic differentiation.

In situ, the innervation of melanotrope cells arises from neurons in which GABA and dopamine are co-localized.45 This situation has to be confirmed in our co-culture system. In this case, it will be interesting to study the formation of functional dopaminergic synapses as well as the possible co-release of GABA and dopamine at the synaptic level. Double immunostaining studies are currently underway to investigate the presence of GABAergic/dopaminergic neurons.

**Conclusion**

We report, for the first time, the development of a functional neuroendocrine synapse in vitro. Furthermore, we describe a model of co-culture which appears particularly well adapted to analyse the mechanisms of synaptogenesis and synaptic transmission between pairs of connected cells. In the continuity of this work, a study concerning functional GABAergic neuroendocrine synaptic transmission is currently in progress.

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