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Cross-talk between luteinizing hormone-releasing hormone (LHRH) neurons and astroglial cells: developing glia release factors that accelerate neuronal differentiation and stimulate LHRH release from GT_{1-1} neuronal cell line and LHRH neurons induce astroglia proliferation

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Recent evidences indicate that the bidirectional flow of informations governing neuron-astrocyte interactions plays a crucial role during the development and in the adult brain. In the present study, we have used the immortalized hypothalamic luteinizing hormone-releasing hormone (LHRH) neuronal cell line (GT_{1-1} subclone) to investigate LHRH-astroglial cell interactions, and addressed the following questions: (a) does the astroglial cell compartment influence GT_{1-1} neuron morphology, LHRH secretion and/or proliferation?; (b) does the bidirectional flow of informational molecules released during neuron-astroglia interactions influence one or both cell compartments?; (c) are receptor-mediated cell-cell interactions between neurons and astroglia involved in such crosstalk? In this experimental design, GT_{1-1} neuronal cells were grown either: (1) in Dulbecco's modified eagle's medium (DMEM); (2) in the presence of conditioned medium from astroglial cell (ACM) cultures at different stages of glia differentiation and maturation *in vitro*; (3) in the presence of astroglial cells, in co-cultures or mixed-cultures; and (4) in the absence or the presence of antibodies (Abs) for neural cell adhesion molecule (N-CAM) receptor. This work shows that during its maturation and differentiation *in vitro* (8–40 days, DIV), astroglial cells in primary culture release factors able to markedly influence GT_{1-1} cell morphology and accelerate LHRH cell secretory potential, with a potency depending on both the 'age' of astroglia and the degree of GT_{1-1} neuron differentiation *in vitro*. Regional differences in glial-derived factors that promote LHRH neuronal differentiation and secretion were observed, with hypothalamic astroglia being the most potent neurotrophic stimulus. Such effects were specific for astroglia conditioned medium (CM), since oligodendrocyte CM was without effect. Boiling of the ACM for 10 min completely abolished stimulatory activity on neuronal cells. When immature astroglial cells (12 DIV) were co-cultured with GT_{1-1} neurons, LHRH release increased by about 2- to 3-fold over basal levels and GT_{1-1} neuron proliferation was doubled. Astroglial cells responded to GT_{1-1} neuronal signals with an almost doubling of the [^{3}H]-thymidine incorporation and DNA synthesis. Extensive neurite outgrowth and establishment of cell-cell contacts between the two cell compartments were observed in the mixed culture preparation, accompanied by a marked stimulatory effect on both cell proliferation and LHRH secretion. Addition of N-CAM-Ab in the GT_{1-1}-astroglial cell mixed cultures resulted in a dramatic disruption of GT_{1-1}-astroglia morphology and a 95% suppression of the stimulatory effect on both cell proliferation and LHRH release, suggesting the local adhesive mechanisms are importantly involved in the crosstalk between GT_{1-1} neurons and astroglial cells *in vitro*. This work shows for the first time the presence of a bidirectional interaction between the LHRH neurons and astroglial cells and suggest a potential interplay between the two compartments in the regulation of LHRH neuronal physiology.

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Introduction

The development and functioning of the nervous system depends upon the extensive and intimate coupling between neuronal cells and the astroglial cells (Yu *et al.*, 1992; Arenander & de Vellis, 1992; Geisert & Stewart, 1992). Axon guidance and target recognition are achieved by highly specific chemical mechanisms using diffusible trophic factors, cell surface and extracellular matrix molecules which allow tropism and cell-cell interactions (Hatten, 1993; Voutsinos *et al.*, 1994; Wang *et al.*, 1994). Luteinizing hormone-releasing hormone (LHRH) is the key regulator of the hypophyseal-gonadal axis and is essential for reproductive competence. The LHRH neuronal cell system appears to be unique among all neuropeptide expressing genes in the CNS, to make a migration pathway from the epithelium of the medical olfactory pit into the developing basal forebain (Swanzel-Fukuda *et al.*, 1990, 1992a,b). Failure of LHRH neuronal migration as in Kallmann's syndrome results in a suppression of the pituitary-gonadal axis (Schawanzel-Fukuda *et al.*, 1989). Glial-derived neuronal migration in different regions of the developing mammalian brain is a well recognized phenomenon (see Hatten, 1990) and studies on the molecular basis of LHRH-guided migration suggest a crucial participation on the neural cell adhesion molecule, N-CAM (Swanzel-Fukuda *et al.*, 1992a,b). That glial elements contributed to LHRH axonal targeting was suggested by the early experiments of Kozlowski & Coates (1985) demonstrating the existence of ependymal tunnels and their association with LHRH axons. More recently, relationships of glia with LHRH axonal outgrowth have been described by Silverman & coworkers (1991). Of special interest are the studies of Ojeda & collaborators (Ojeda *et al.*, 1990, 1993; Ma *et al.*, 1992; Junier *et al.*, 1993) indicating a key role of astroglia-derived factors in the stimulation of LHRH release and induction of precocious puberty after lesions of the female rat hypothalamus. Therefore, it seems likely, that besides offering a generic pathway for neuronal migration, the glial network might provide other functional informations modulating neuronal maturation and differentiation. Indeed, astroglial cells represent a possible target and source of signals for the LHRH neuronal machinery (Olmos *et al.*, 1989; Garcia-Segura *et al.*, 1989; Torran-Aleman *et al.*, 1991; Langub *et al.*, 1992; Ma *et al.*, 1992; Junier *et al.*, 1993; Ojeda *et al.*, 1993; Duena *et al.*, 1994). The modulation of astroglial cells and LHRH neurons by a common set of molecules, including a number of neurotransmitters (see Murphy & Pearce, 1987; Weiner *et al.*, 1988; Murphy *et al.*, 1992), growth factors (GFs) (Avola *et al.*, 1988, 1993, 1994; Ojeda

et al., 1990, 1993; Ma *et al.*, 1992; Gallo *et al.*, 1992; Junier *et al.*, 1993; Duenas *et al.*, 1994; Gallo *et al.*, 1994, 1995a,b), and various cytokines (Yamaguchi *et al.*, 1991; Rettori *et al.*, 1991; Rivest *et al.*, 1993) has been variously documented. On the other hand, no direct evidence for a 'crosstalk' between astroglia and LHRH neurons *in vitro*, has yet been provided. In the present study we have used astroglial cells in primary culture and the immortalized hypothalamic LHRH neuronal cell line GT1 (GT_{1-1} subclone, Mellon *et al.*, 1990), to investigate LHRH-astroglia interactions. The present results provide the first evidence for the bidirectional interaction of glia and LHRH neurons.

Results

Astroglial conditioned medium alters GT_{1-1} neuronal cell morphologic appearance

GT_{1-1} cells have a neuronal morphology, with neurite extending from the perikarya of cells (Mellon *et al.*, 1990). As observed (Figure 1), control GT_{1-1} neuronal cells at 2(a), 4(b) and 6(c) days of culture show a classical morphological pattern characterized by a progressive shift from the ovoidal shape after 1–2 days of culture to progressively reach the neuronal phenotype. At 2 days of culture and in the absence of ACM (a), the majority of the cells are rounded, and neurite are almost absent. Panels d, e and f show the morphological appearance of GT_{1-1} neurons when cultured in the presence of astroglial cell conditioned medium (ACM) from immature (10–12 DIV) astroglia. As observed, GT_{1-1} neurons grown in ACM show an initial extension of neurite (d). ACM causes the cells to assume a flattened appearance, and extensive neurite outgrowth at 4(e) and 6(f) DIV, when this arborization become more pronounced, and GT_{1-1} treated neurons clearly manifest neurite formation, and establish together with cell-cell contacts (see arrows, Figure 2).

Astroglial conditioned medium alters GT_{1-1} neuronal cell secretion

Figures 3 and 4 show the effects of ACM from 'immature' (8–12 DIV) and 'aged' (16–40 DIV) astroglia on GT_{1-1} neurons secretion and proliferation as a function of time of culture (2–8 DIV). At 2 days of culture, from basal LHRH levels of 2.445 ± 0.1 pg/ml, an almost 50% increase of LHRH released in the medium (3.39 ± 0.19 pg/ml, $P < 0.05$ *vs* basal) was observed when GT_{1-1} cells were cultured in the

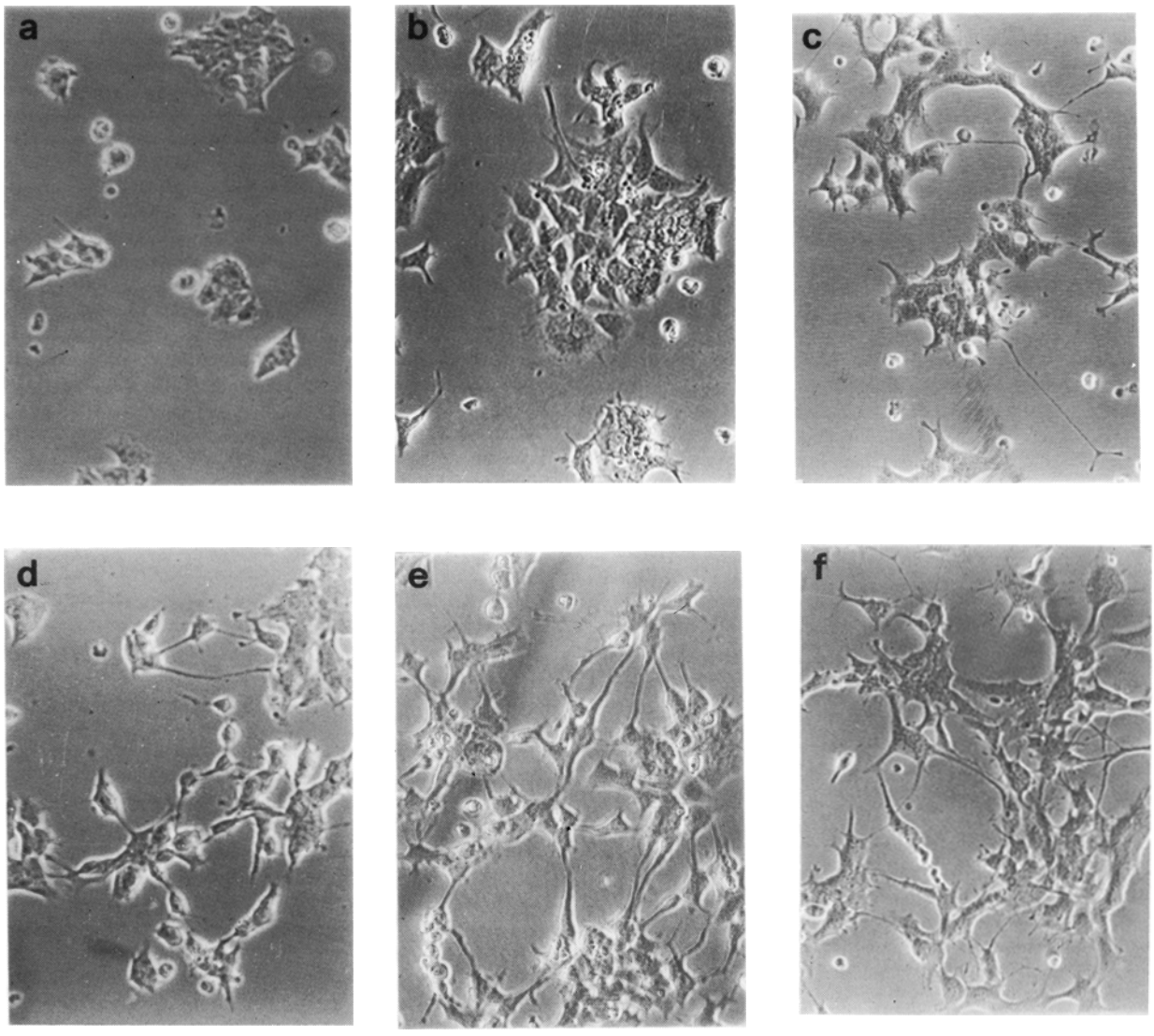
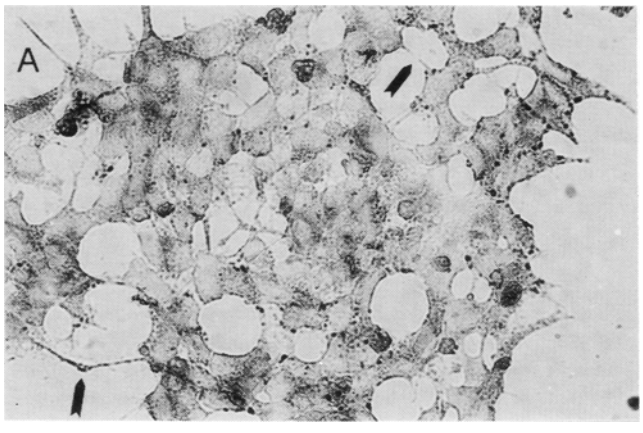


Figure 1 Phase-contrast micrograph demonstrating the time-course effect of astroglial cell conditioned medium (ACM) on GT_{1-1} cell morphologic appearance. GT_{1-1} cells were grown in DMEM (a–c) or astroglial conditioned medium (ACM, d–f), and analysed at different time intervals (2–6 days). GT_{1-1} neurons grown in DMEM were rounded and extend few neurite after 2 days of culture (a). Note neurite extension in GT_{1-1} cells grown for 2 days in ACM (d), the flattened appearance of the cells, extensive neurite outgrowth, growth cones and cell-cell contacts after 4(e) and 6(f) days of culture in ACM, compared with GT_{1-1} neurons grown in DMEM at 4(b) and 6(c) days of culture. (Magnification × 160)

presence of 8 DIV ACM, while a significantly higher (+230%) increase (7.58 ± 0.049 pg/ml, $P<0.01$ *vs* control and *vs* ACM 8 DIV), was observed with ACM of 12 DIV (Figure 3A). On the other hand, factors released from astroglia after 16 and 40 days of maturation *in vitro*, were less active ($P<0.01$ *vs* 12 DIV ACM), but still inducing a 2.5–3-fold (+130–170%) increase in basal LHRH release ($P<0.01$ *vs* control). After 4 days of culture, spontaneous LHRH release reaches 12.755 ± 0.99 pg/ml and a 60–100% increase of basal LHRH production was observed in the presence of ACM (Figure 3B). The order of potency of ACM in stimulating LHRH release in the medium was 12 DIV = 16 DIV > 40 DIV > 8 DIV. No major changes were observed on GT_{1-1} neuron proliferation (Figure 3C–D). After 6 days of culture (Figure 4A), GT_{1-1} cells release 24.38 ± 0.2 pg/ml in basal conditions and a 30 to 100% increase in peptide production followed incubation of GT_{1-1} neurons in the presence of ACM, with the maximal effect reached with ACM of 12 DIV ($P<0.01$ *vs* control and *vs* 8, 16 and 40 DIV ACM), while 40 DIV ACM failed to stimulate LHRH release (Figure 4A). No significant effects on neuronal proliferation were measured (Figure 4C). Control basal release of LHRH reaches 64.7 ± 0.8 pg/ml after 8 days of culture (Figure 4B). At this stage of LHRH neuronal organization *in vitro*, the stimulatory effect of ACM is either absent (ACM 8 DIV), weak (12 DIV, $P<0.05$), or significantly inhibitory at 16 and 40 ($P<0.01$) DIV. Neuron proliferation was significantly inhibited at 16 and 40 DIV (Figure 4D).



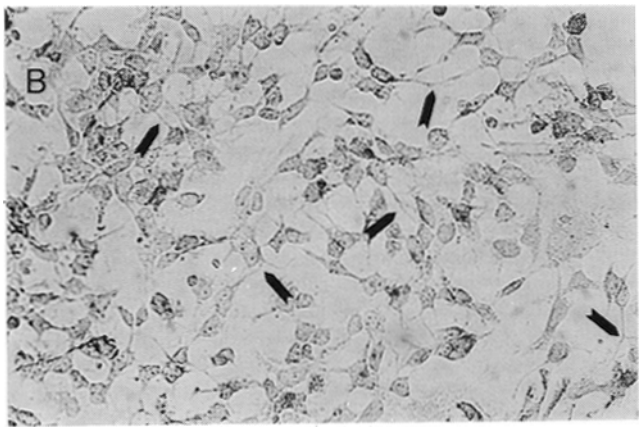


Figure 2 Immunocytochemical preparation showing the effects of astroglial conditioned medium (ACM) on GT_{1-1} cell morphologic appearance. LHRH immunoreactive cells growing in ACM for 4 DIV (B) extend neurite and establish numerous cell-cell contacts, compared with GT_{1-1} neurons grown in DMEM (A). Magnification × 300

Astroglial conditioned medium specifically accelerates GT_{1-1} neuronal differentiation

To assess the specificity of ACMs in altering LHRH neuronal function, 12 DIV astroglial CM was prepared from different regions including hypothalamus, striatum, olfactory bulb and spinal cord. Moreover to test whether it is specifically astroglia, or other cell types are endowed with such differentiating properties, the effect of oligodendrocyte CM was tested. As observed in Figure 5, hypothalamic glia appeared to sharply stimulate LHRH output in the medium, with the highest potency, followed by cortical and olfactory bulb glia. Striatum and spinal cord glia were less effective in stimulating LHRH release, while oligodendrocyte CM failed to alter the peptide release in the medium. A similar order of potency of the different ACMs in accelerating neuronal differentiation was observed (not shown). The major effect was observed in undifferentiated (2 DIV) neurons, while decreasing in magnitude with the establishment of the LHRH neuronal phenotype. After 8 DIV, the stimulatory effect was either absent (spinal cord CM), or significant stimulatory, with an amplitude varying according the region of astroglia CM (Figure 5).

Astroglial conditioned medium contains themolabile peptides derived growth factors

In order to elucidate whether the factors released by glia during its differentiation *in vitro* are of peptide nature, 12 DIV ACMs prepared from the different regions were boiled for 5–10 min at 100°C and then tested on 4 DIV GT_{1-1} neurons to assess LHRH differentiating activity. The region-specificity in stimulating LHRH release was confirmed in this experiment, where hypothalamic ACM potently stimulated LHRH output in the medium (Figure 6). Boiling the different ACMs completely abolished the effects on both morphology and secretion of the GT_{1-1} neurons. On the other hand, the presence of peptidase inhibitor (bacitracin, 2×10^{-2} M) in the different ACMs to control for nonspecific effects due to different degress of peptidase activity, did not effect ACM-induced LHRH stimulation (not shown).

Effects of GT_{1-1}-astroglial cell co-culture in LHRH secretion and cell proliferation

As a further step to verify the possible bidirectional communication between astroglial cells and GT_{1-1} neurons, a co-culture system was established, as described. As observed in Figure 7, when immature (10–12 DIV) cortical astroglial cells were co-cultured with GT_{1-1} cells, a highly significant stimulation of spontaneous LHRH secretion was measured at each time-interval (2–8 DIV) studied. In such co-culture condition the highest increase (+88%) in LHRH release was observed in relatively differentiated (6 DIV) neurons, compared to 2, 4 and 8 DIV (+44–52%). The bidirectional communication between GT_{1-1} neurons and astroglial cells is emphasized when the [^{3}H]-thymidine incorporation was measured in both astroglial and neuronal cells (Figure 8). In fact, an almost doubling of astroglial cell and GT_{1-1} cell proliferation potential (Figure 8), and RNA synthesis were observed.

Effects of GT_{1-1}-astroglial cell mixed-culture in the absence or the presence of anti-neural cell adhesion molecule (N-CAM) antibody (Ab) on LHRH neuron morphology, secretion and proliferation

Figure 9 shows the LHRH immunoreactive neurons at 1 and 4 DIV grown without (Figure 9A and C) or in the presence (B and D) of immature cortical astroglia (10–12 DIV). As observed, after 1 DIV LHRH immunoreactive neurons are rounded and neurite are almost absent. On the contrary in mixed cultures, the initial extension of neurite is apparent

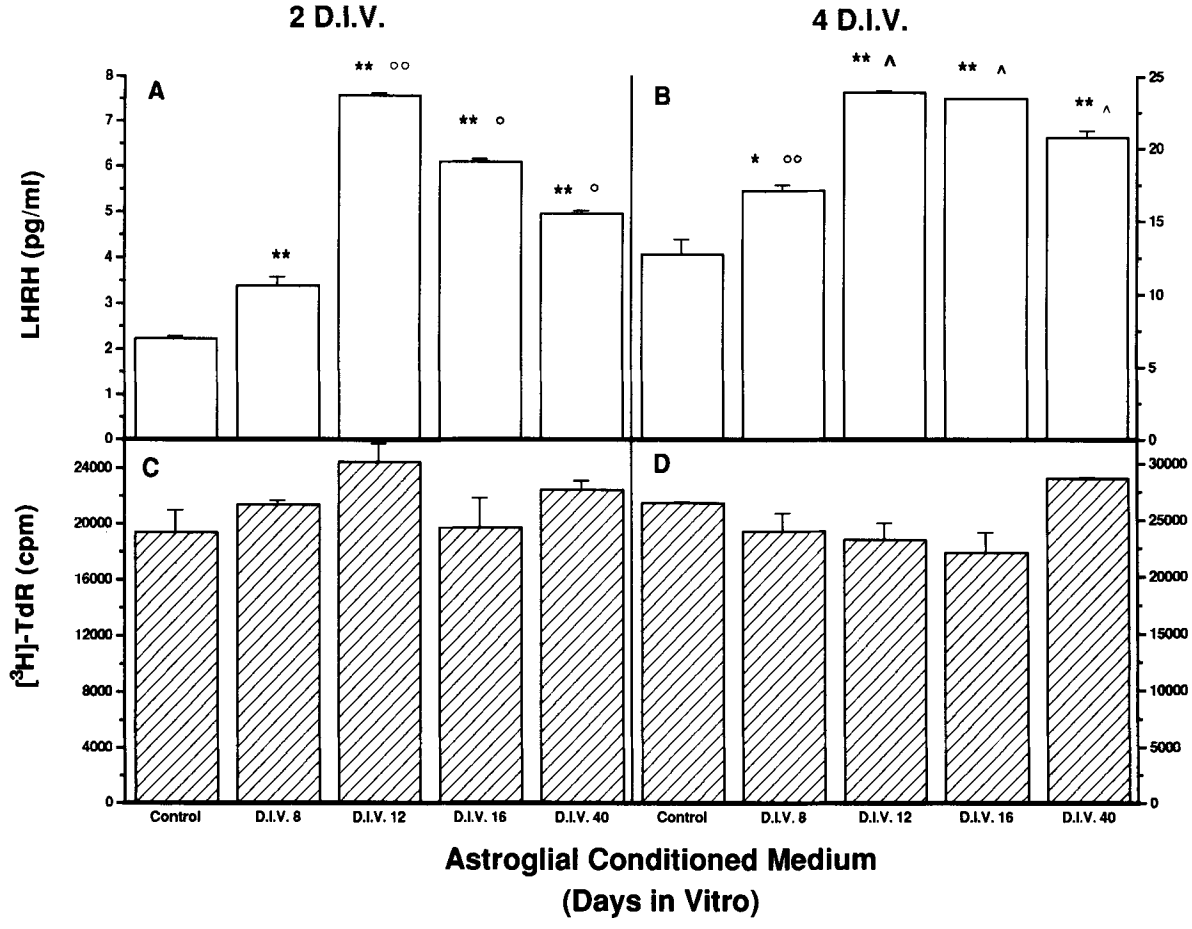


Figure 3 Effect of astroglial conditioned medium (ACM) at different times (8, 12, 16 and 40 days *in vitro*, DIV) of glia maturation and differentiation on the *in vitro* maturation of GT_{1-1} LHRH secretory (A,B) and proliferative potential (C,D). LHRH secretion and proliferation at 2 and 4 days. For measurement of LHRH release by RIA, the medium was replaced every two days, collected, centrifuged to remove cellular debris, and frozen at −80°C. Proliferation of GT_{1-1} neurons was monitored by the incorporation of Methyl-$_3$H]Thymidine (1 μCi/ml of culture medium) for 2 h at 37°C, as described in the Materials and methods section. Results are the mean ± SEM of 2–3 different experimental manipulations. $*P<0.05$. $**P<0.01$ *vs* control; $\wedge P<0.01$ *vs* 8 DIV; $^{\circ}P<0.01$ *vs* 8 and 12 DIV; $^{\circ\circ}P<0.01$ *vs* all ages

together with the establishment of contacts with glial compartment (B). After 4 DIV (C) LHRH immunoreactive cells grow in clusters and extend neurite, while in mixed cultures (D) LHRH immunoreactive cells are dispersed and clearly exhibit a distinct neuronal phenotype, including the extension of multiple lengthy neurite that contact distant cells, or end in apparent growth cones, together with the establishment of cell-cell contacts between the two cell compartments (see arrows). Figure 10 shows that also the astroglial cell morphology changes from process-bearing (A) to polygonal and flat shapes (B), in the mixed culture preparation. In B, glial cells (arrows) appear to be stained, while GT_{1-1} cells (arrowheads) are not. In GT_{1-1} neuron-astroglial cell mixed culture conditions (Figure 11), a sharp stimulation ($P<0.01$) of spontaneous LHRH release over basal levels was observed at each time point studied, with the highest effect observed in relatively differentiated (6 DIV) neurons. When compared with LHRH release in the co-culture condition, a significantly higher stimulation of LHRH ouput in the medium accompanied the mixed culture condition with a 68, 180 and 88% increase in peptide release at 4, 6 and 8 DIV, respectively. Although it is difficult to distinguish between the two cell compartments, the incorporation of [^{3}H]-thymidine after 8 days of mixed culture was even greater than the proliferative activity measured in the co-culture condition (Table 1).

To test whether receptor-mediated cell adhesion has a specific role in the cross-talk between GT_{1-1} neuron and astroglia in our *in vitro* model, two N-CAM antibodies were tested during the establishment of GT_{1-1} neurons in culture, and in the mixed culture preparations, while a nonsense antibody was included in the control medium to test for nonspecific effects (see Material and method). As observed in Figure 12 (panel A), an intense reaction of the LHRH cell-bodies was present after 4 DIV of LHRH-astroglial mixed cultures. Moreover, contacts between LHRH neurons, and LHRH neurons with the astroglial compartment were observed, with neurite contacting either neighboring LHRH cell bodies/axons, or astroglial cells (see arrows). On the other hand, in panel B, a general atrophy and degeneration of GT_{1-1} neurons followed N-CAM-Ab treatment. In particular, a sharp reduction of the immunocytochemical reaction together with cytoplasmatic degeneration, nuclear vacuolization and chromatolysis (tigrolysis) were observed (see arrows). The axons that were longer and thinner, were seeking to contact other neurons (arrows). No visible contacts between the GT-1 neurons and glial cells were observed. At a functional level (Figure 11), a dramatic inhibition of LHRH release was observed at all time-point studied. After 8 days in culture, this inhibitory effect on secretion was paralleled by almost 95% inhibition of GT_{1-1} and astroglia proliferation (Table 1). On the other hand, treatment of the control GT_{1-1} neuronal preparation with the N-CAM-Ab produced a progressive decrease of LHRH output, dependent upon time of culture, that reached a statistical significance ($P<0.05$) after 8 days of culture (Figure 11).

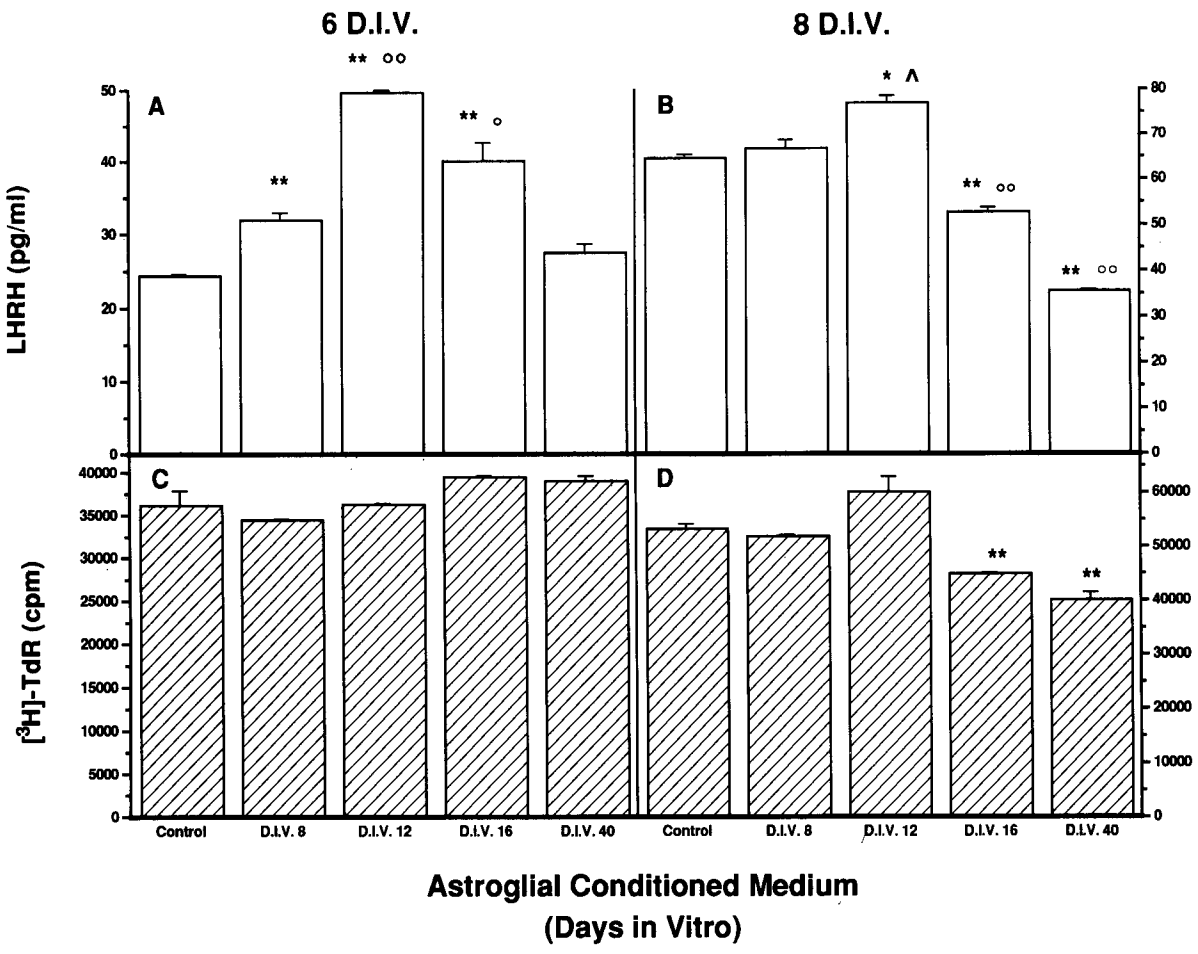


Figure 4 Effect of astroglial conditioned medium (ACM) at different times (8, 12, 16 and 40 days *in vitro*, DIV) of glia maturation and differentiation on the *in vitro* maturation of GT_{1-1} LHRH secretory (A,B) and proliferative potential (C,D). LHRH secretion and proliferation at 6 and 8 days. Cultures were processed as reported in legend for Figure 3. Results are the mean ± SEM of 2–3 different experimental manipulations. $^{*}P<0.05$, $^{**}P<0.01$ *vs* control; $\wedge P<0.01$ *vs* 8 DIV; $^{\circ}P<0.01$ *vs* 8 and 12 DIV; $^{\circ\circ}P<0.01$ *vs* all ages

Discussion

All together the information presented provide compelling evidence for the concept that a significant degree of communication between neurons and their associated glial cells contributes to the developmental regulation of LHRH neuronal function. This work, indeed, shows that according to: (1) the specific stage of maturation and differentiation; (2) the specific brain region examined, and (3) the degree of neuronal differentiation, astroglia may play a crucial regulatory function through the release of products able to alter LHRH neuronal morphology, the LHRH intracellular secretory machinery and/or proliferation. As a corollary, astroglial cells can respond to GT_{1-1} neuronal signals, and this mutual trophic and functional interaction is likely to occur via paracrine, 'intercrine' and/or autocrine mechanism(s). While further studies are required to characterize the nature of astroglial cell-derived factors, the mechanism(s) responsible for the observed effects, as well as their relevance *in vivo* for LHRH neuronal physiology, our preliminary observations would support the contention that glial-derived, peptide growth factors are involved in LHRH-astroglia crosstalk. On the other hand, this study further shows for the first time that the hypothalamic decapeptide, LHRH, may act as a growth factor for astroglia.

The immortalized GT1 neuronal cell line derived by targeting the expression of the oncogene, simian virus-40 T-antigen, to the LHRH-expressing hypothalamic neurons of transgenic mice (Mellon *et al.*, 1990) has provided a model system to study the mechanisms involved in LHRH regulation at multiple levels (see Lipositz *et al.*, 1991; Martinez de La Escalera *et al.*, 1992a,b,c; Wetsel *et al.*, 1992, 1994; Weiner & Martinez de la Escalera, 1993; Milenkovic *et al.*, 1994). In this study, we have used the GT1 cell line and primary cultures of astroglial cells and assessed different dynamic models, as a first step to investigate LHRH-astroglia interactions. The present study shows that in controlled *in vitro* conditions, and with the limitations of a tumor-derived immortalized LHRH neuronal cell line, astroglial cells during their *in vitro* differentiation and maturation, produce factors that significantly accelerate the acquisition of the neuronal phenotype and sharply stimulate the spontaneous release of the decapeptide in the medium. It seems noteworthy to notice that such stimulatory effects are strictly dependent upon the stage of both glia and LHRH neuron differentiation. In fact, while 8 DIV ACM (representing the less differentiated stage of astroglia in this *in vitro* maturational profile, see Wang *et al.* 1994), is the less active condition stimualting LHRH release in 2, 4 and 6 DIV GT_{1-1} established cultures, 12 DIV ACM (representing a relatively immature glia which initiates to differentiate), is a highly potent neurotrophic stimulus for the LHRH neuron. Such stimulatory effect is however dependent upon the stage of LHRH differentiation (see Figures 3–5). Similarly, at later stages of glia maturation and differentiation (16–40 DIV), glial-derived factors differentially affect LHRH release depending on the stage of LHRH neuron differentiation, being highly stimulatory in GT_{1-1} undifferentiated neurons (2 DIV), and gradually loosing this activity with LHRH neuron differentiation. These informations suggest a possible

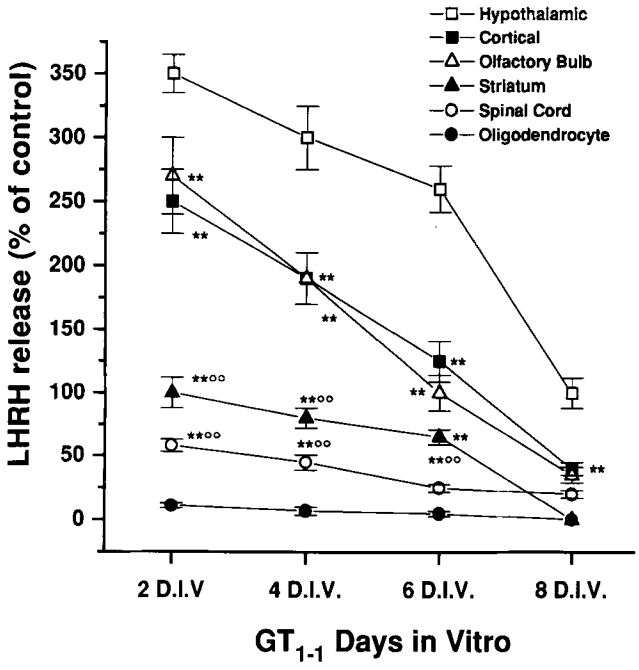


Figure 5 Regional differences of glial-derived factors that promote LHRH release from the GT_{1-1} neuronal cell line. Astroglial conditioned medium from the different regions was prepared as indicated in the Materials and methods section and 12 DIV ACMs or oligodendrocyte CM were tested during *in vitro* LHRH neuron differentiation (2–8 DIV). LHRH release in the medium is expressed as percentage (%) increase compared to LHRH released from GT_{1-1} neurons grown in DMEM (control). Results are the mean ± SEM of two different experimental manipulations. $^{**}P<0.01$ *vs* hypothalamic glia; $^{\circ\circ}P<0.01$ *vs* cortical and olfactory bulb glia

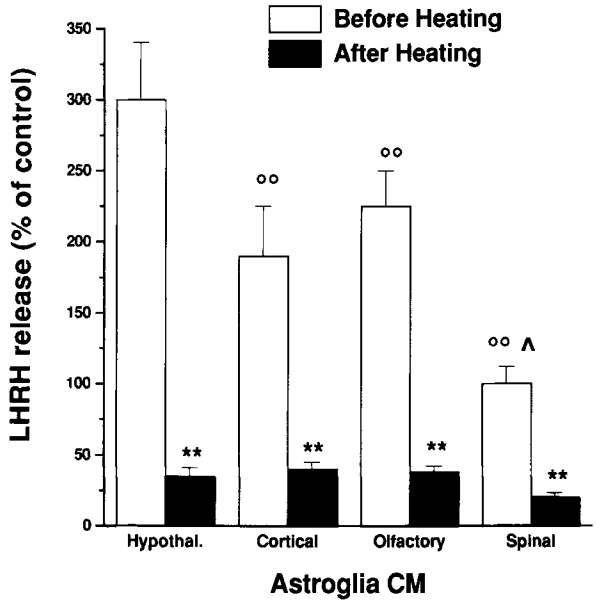


Figure 6 Efect of heat treatment on astroglial conditioned medium (ACM) ability to influence LHRH release from the GT_{1-1} neuronal cell line. To test the protein nature of the glial-derived factors, 12 DIV ACMs from the different; brain regions were heated for 5–10 min at 100° (Avola *et al.*, 1988). GT_{1-1} neurons were grown in the presence of ACMs before and after the boiling procedure. For measurement of LHRH release by RIA, the medium was replaced every 2 days, collected, centrifuged to remove cellular debris, and frozen at −80°C. Results illustrate LHRH release in the medium at 4 DIV. LHRH release is expressed as percent (%) increase of LHRH release from GT_{1-1} neurons grown in DMEM. Results are the mean ± SEM of two different experimental manipulations. $P<0.01$ *vs* before the heating procedure; $^{**}P<0.01$ *vs* before heating $^{\circ\circ}P<0.01$ *vs* hypothalamic glia; $P<0.01$ *vs* cortical and olfactory bulb glia

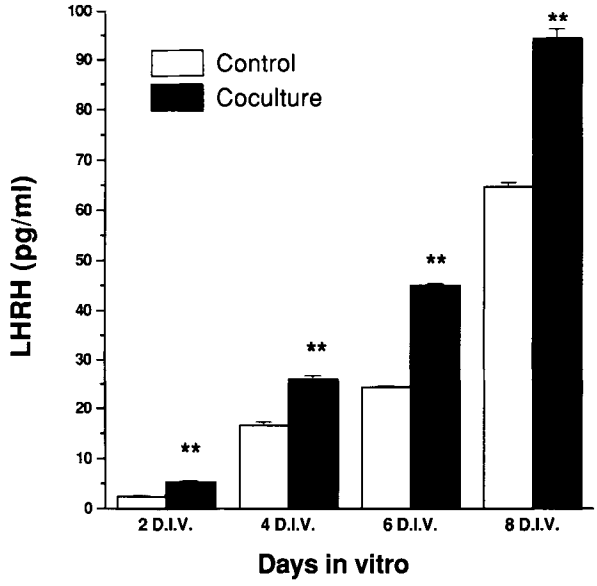


Figure 7 Effect of astroglial (12 DIV)-GT_{1-1} neuron co-culture in the maturation of LHRH secretory potential. The technical procedure is described in details under the Materials and methods and cortical glia was used in this experimental paradigm. LHRH secretion was examined every 2 days for 8 days. For measurement of LHRH release by RIA, the medium was replaced every two days, collected, centrifuged to remove cellular debris, and frozen at −80°C. Results are the mean ± SEM of 2–3 different experimental manipulations. $^{**}P<0.01$ *vs* control

different nature of the glial factors acting at a particular stage of GT1 neuron differentiation *in vitro*, and/or the saturation of some intracellular transducing mechanisms responsible for LHRH production (see next sections). The specificity of astroglial conditioned medium is further corroborated by a series of informations. Firstly, addition of a peptidase inhibitor in the different ACMs did not alter the observed effects, thus excluding nonspecific effects due to different degrees of LHRH degradation in the culture medium. Secondly, ACM from five different regions exhibited significantly different degrees of stimulatory activity in both LHRH morphologic appearance and LHRH secretion, while oligodendrocyte CM was unable to modify LHRH output, then implying a region-specificity of the glial-derived factors in the modulation LHRH neuron morphology and peptide release from the GT_{1-1} cell line. Regional differences in glial-derived factor ability to support axon and dendrite growth, has been also reported by different investigators (Quian *et al.*, 1992; Le Roux & Reh, 1994). The peptide nature of glial-derived factors was suggested by the fact that boiling ACM for 10 min at 100°C completely abolished its activity on both GT_{1-1} neuron and peptide release.

The co-culture experiments revealed another facet of LHRH-astroglia interactions. In these conditions, where the two cell-compartments were allowed to communicate with each other, but in the absence of cell contacts, a significant stimulation of basal LHRH release was observed, although GT_{1-1} proliferative potential was almost doubled, then resulting in a net decrease of neuronal secretory capacity. This experimental paradigm then revealed for the first time the presence of a bidirectional flow of informational molecules between the two cell populations, as observed by a two-times increase of the proliferative potential of each cell population, then suggesting that the LHRH decapeptide and Gfs released by glia, participate in GT_{1-1} neuron-astroglia crosstalk.

In mixed cultures, both spontaneous LHRH release and GT_{1-1}-astroglial cell proliferation were significantly increased. The inability to further stimulate LHRH release in the face of the presence of such mitogenic effect on the GT_{1-1} neurons may have different explanations, depending on both (1) the

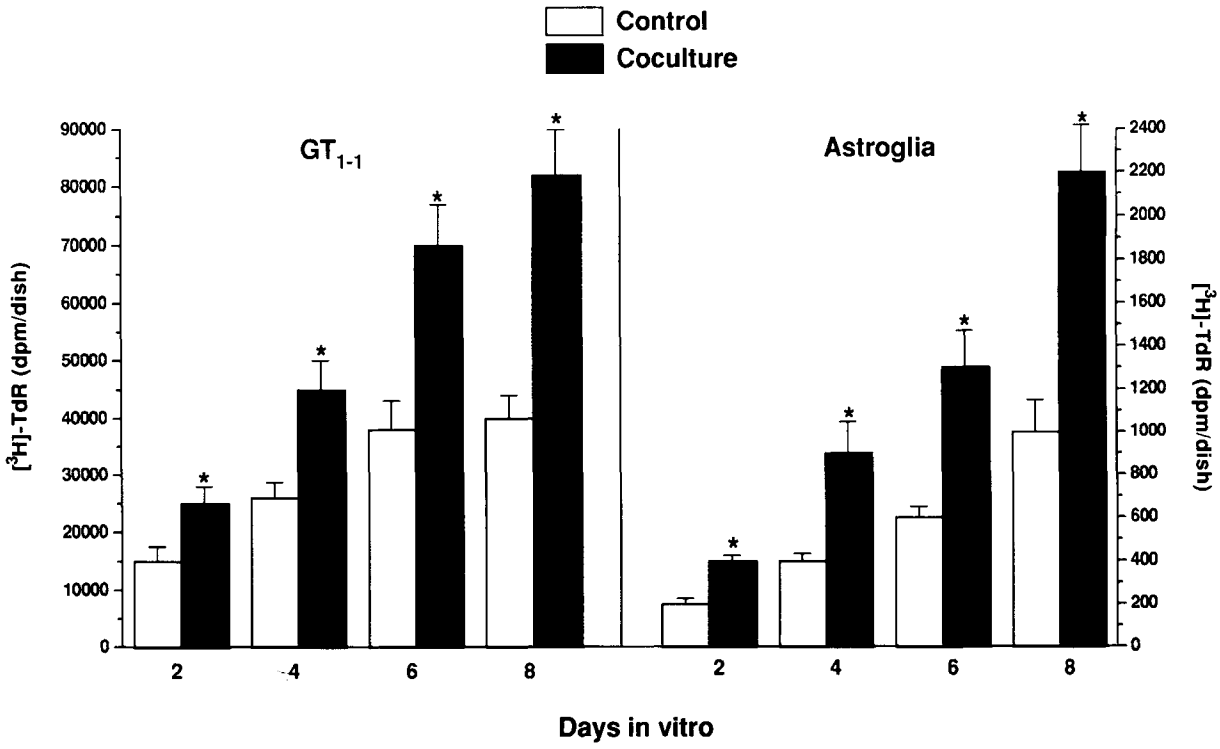


Figure 8 Proliferative capacity of GT_{1-1} neurons and astroglial cells in coculture conditions. The technical procedure is described in details under the Material and methods. At 2, 4, 6 and 8 days and for each respective cell type, GT_{1-1} or cortical astroglial cell proliferation were tested in triplicate by incubation of Methyl-^{3}H]Thymidine (1 μCi/ml of culture medium) for 2 h at 37°C. Labeled DNA was collected and radioactivity was determined by liquid scintillation spectrophotometry, as described. Results are the mean ± SEM of 2–3 different experimental manipulations. * $P<0.01$ *vs* control

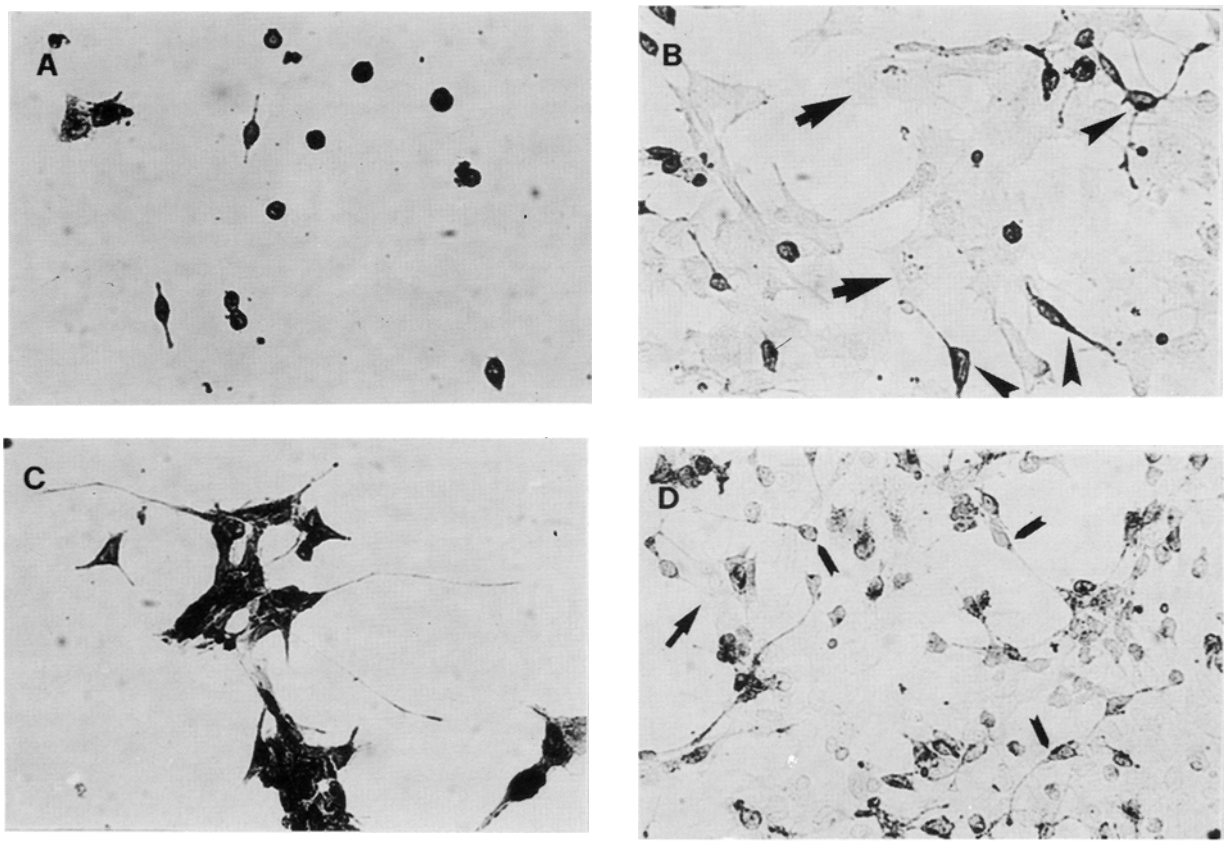


Figure 9 Immunocytochemistry of LHRH neurons grown in the absence or the presence of astroglial cells in a mixed culture preparation. GT_{1-1} neurons were grown in DMEM (A,C) or in mixed culture with astroglial cells (B,D) for 1 (A,B) and 4 (C,D) DIV, as described under Materials and methods. GT_{1-1} cell cultures were fixed and labeled with anti-LHRH (LR-1) antibody. LHRH immunoreactive neurons are rounded and few cells extend neurite after 1 DIV (A), while at 4 DIV clusters of LHRH neurons show neurite extension (C). In LHRH-astroglia mixed culture GT_{1-1} neurons (arrowhead) extend neurite, and establish contacts with glial cells (arrows) already at 1 DIV (B). Note the extensive neurite outgrowth and contacts of LHRH immunoreactive neurons (arrowheads) with astroglial cells (arrows) at 4 DIV (D). (Magnification: × 300)

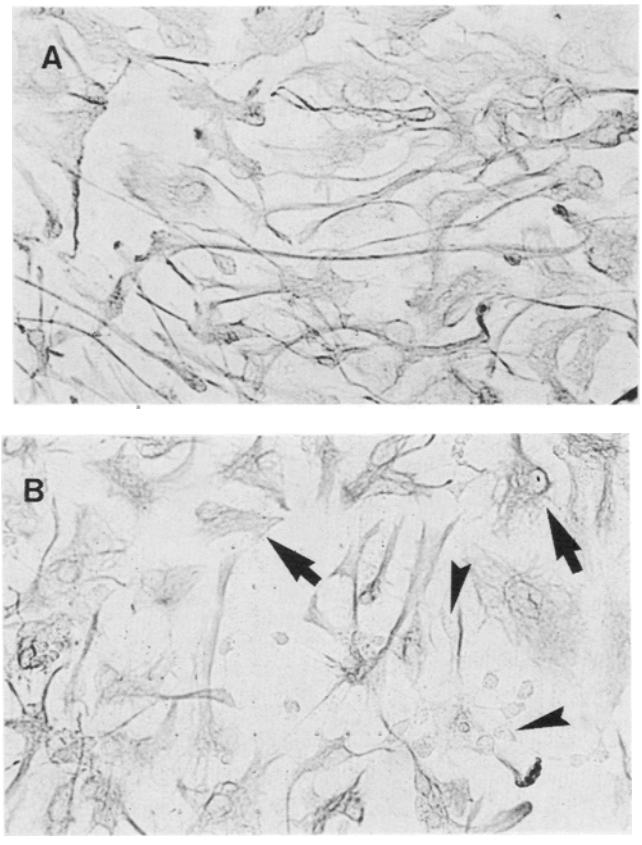


Figure 10 Immunohistochemistry of glial fibrillary acid protein (GFAP). (A) Primary astroglial cell cultures (12 DIV); (B) astroglial cells were cultured for 8 DIV and GT_{1-1} neurons were added on the top and grown for 4 DIV. Astroglia was labeled with anti-glial fibrillary acid protein (GFAP) antibody. Note that astroglia morphology changes from process-bearing (A) to polygonal and flat shapes (B), if 4 DIV GT_{1-1} cells were added in the preparation. In B, glial cells (arrows) are stained, while GT_{1-1} cells (arrowheads) are not. (Magnification: × 300)

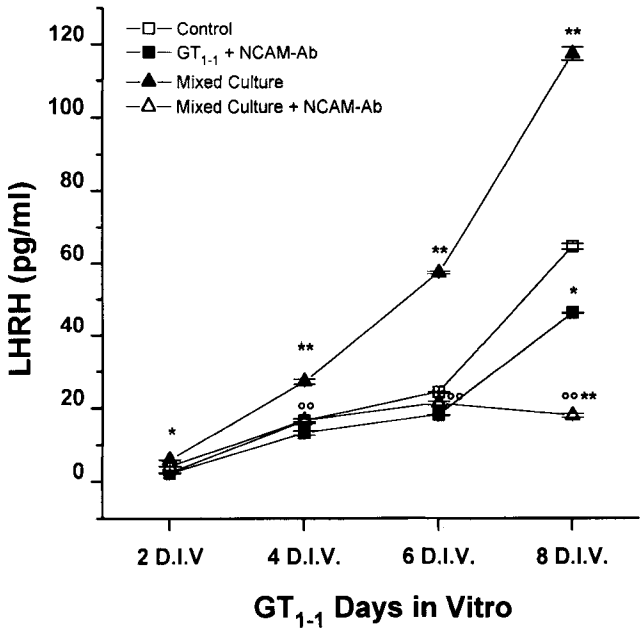


Figure 11 Effect of astroglia (12 DIV)-GT_{1-1} neuron mixed-culture in the absence or the presence of neuronal cell adhesion molecule (N-CAM) antibody on the maturation of GT_{1-1} neuron secretory potential. The technical procedure is described in details under the Materials and methods section. N-CAM antibody (1 μg/ml) was added from the beginning of the experiment (T = 0), and every 2 days, the medium was replaced with fresh medium containing the Ab. Results are the mean ± SEM of 2–3 different experimental manipulations. $*P<0.05$, $**P<0.01$ *vs* control GT_{1-1} $^{oo}P<0.01$ *vs* Mixed Culture

Table 1 Effect of neuronal cell adhesion molecule (N-CAM) antibody on GT_{1-1} neuron, astroglial cell and mixed culture proliferation

	[^{3}H]Thymidine incorporation (dpm/dish)		
Condition	*Astroglia*	*GT_{1-1} neurons*	*Mixed culture*
Control	420 ± 16.97**	43 644 ± 1230**	102 904 ± 17 355
N-CAM-Ab	130 ± 25.45†	31 371 ± 980*	7718 ± 230†

Astroglial cells were cultured for 12 days in 6-well plates, and GT_{1-1} cells were added on top and triplicate wells of cells were incubated in the absence or the presence of N-CAM antibody (Ab,500–10 000 ng/ml), as described under Materials and methods. The effect of N-CAM Ab was also tested on GT_{1-1} neurons and estroglial cells grown in control medium containing a nonsense antibody. Medium was replaced every two days with fresh medium containing or not 0.5–10 μg/ml N-CAM Ab. Results represent the mean ± SEM of 2–3 different experimental manipulations. $**P<0.01$ *vs* mixed culture; $*P<0.05$ and $\dagger P<0.01$ *vs* control within each group, respectively.

autoregulatory actions of LHRH on its own secretion (Krsmanovic *et al.*, 1993); (2) the nature of the GFs released by astroglial cells and their coupling to specific intracellular transducing pathways (see Gallo *et al.*, 1994; Alarid & Mellon 1995); and (3) the presence of cell-cell contacts interfering with LHRH inter/intracellular dynamics. Other possibilities to take into consideration include that LHRH released in the medium could influence the further production of astroglial-derived factors, via receptor-mediated events and/or through second messenger-activated systems.

When GT_{1-1} neurons are grown in the presence of astroglial cells, glial tracks begin to build-up diffuse pathways along which LHRH immunoreactive neurons concentrate. The quantification of the morphometric features of LHRH-astroglia interactions for process length and branching revealed a 3- to 4-time increase in the number of LHRH processes per cell, as well as a significant increase in the length and branches of individual LHRH processes (Gallo *et al.*, 1995). *In vitro*, astroglia possess neuronal-growth promoting properties, including cell adhesion receptor systems that support neurite extension (see Noble *et al.*, 1984; Fallon *et al.*, 1985; Fallon, 1985; Tomaselli *et al.*, 1986; Smith *et al.*, 1990; Hatten, 1993; Le Roux & Reh, 1994; Wang *et al.*, 1994). Indeed, neuron-astroglia interactions are believed to be mediated by 'adhesion molecules', a heterogeneous group of glycoproteins found either in extracellular matrix, or anchored to the cell membrane. Besides other molecules, neural cell adhesion molecule (N-CAM), promotes neurite outgrowth and participate in both kinds of neuron-glia interactions (Doherty *et al.*, 1991; Hemperley *et al.*, 1986; Katsuhiko *et al.*, 1992; Doherty & Walsh, 1992; Kljavin *et al.*, 1994). When moderately high doses of N-CAM were added to GT_{1-1} neurons an approximately 35% reduction of LHRH secretion was measured. In neuron-astroglial cell cultures however, the addition of N-CAM Ab resulted in dramatic effects on LHRH morphology, and a shap (almost 95%) inhibition of both LHRH release and cell proliferation. Polyclonal as well as monoclonal N-CAM Abs have already been shown to inhibit cell aggregation and neurite outgrowth depending on the neuronal cell type and the developmental period (see Klajvin *et al.*, 1994). In PC12 cells and some other neurons, N-CAM appears to stimulate neurite growth through a pertussis toxin-sensitive G protein and activation of Ca^{2+} channels (Doherty *et al.*, 1991). While the present data provide the first documentation that N-CAM Ab exerts

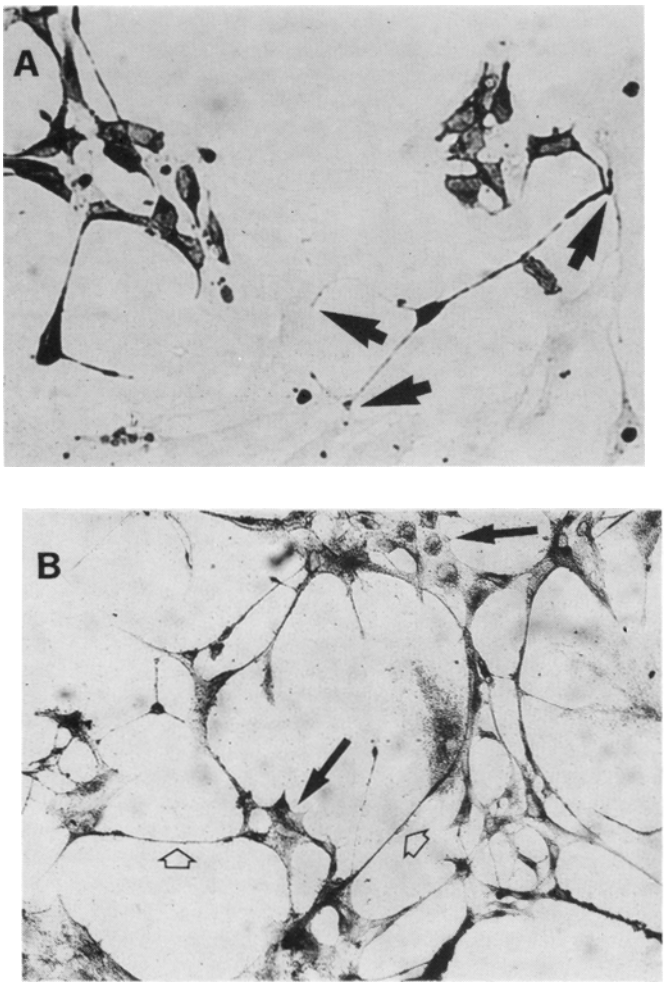


Figure 12 Immunocytochemistry of GT_{1-1} neuron-astroglial mixed cultures in the absence or presence of the neural cell adhesion molecule (N-CAM) antibody (Ab). After 9 days of astroglial cell culture in 6-well plates, GT_{1-1} cells were added on top and triplicate wells of cells were incubated in the presence of a non sense antibody (control) or the presence of a monoclonal N-CAM Ab (1 μg/ml, Boehringer Mannheim, Mannheim, Germany), as described under Materials and methods. (A); control cultures at 4 DIV showing an intense reaction of the LHRH cell bodies sending axons that contact either neighboring LHRH cell bodies (axo-somatic)/or axons (aco-axonic); or astroglial cell (see arrows). The glial cells are often surrounded by LHRH neurons and send prolongations to LHRH cells. B: A general atrophy and degeneration of neurons is observed with a sharp reduction of the immunocytochemical reaction, nuclear vacuolization and chromatolyses (tigrolysis) (see arrows). The axons are longer and thinner, seeking to contact other neurons (arrows). The astroglia compartment is not clearly defined and no clear contacts between the GT-1 neurons and glial cells are visible. (Magnification × 300)

a potent inhibition of GT_{1-1} neuron functional capacity, further studies are required to clarify the mechanisms involved in this phenomenon. The present results may indicate that diffusible factors regulate glia-LHRH interactions in collaboration with molecules associated with the cell surface matrix.

It should be mentioned, that during the processing of the present manuscript, the ability of a thermostable astrocyte-derived factor to stimulate in some instances LHRH release from the GT1 cell line has appeared (Melcangi *et al.*, 1995). The present work, however, for a number of differences in the experimental approach and procedure, technical manipulations, and paradigms analysed, adds important new informations. Indeed, it would appear that astroglia may produce different factors endowed with neurotrophic-differentiating properties, the nature and/or the concentration of which may vary according to the CNS region and the degree of astroglia differentiation. More importantly glial-dervied factors exert different effects according to the degree of GT_{1-1} neuron differentiation.

In the present study we have not directly addressed the question related to the chemical nature of the factors released by the astroglial compartment, nor to the intracellular mechanisms involved in bidirectional communication between the two cell compartments. On the other hand, it seems important to underline that astroglial cells are known to express an array of receptors for signal molecules (for review see Murphy & Pearce, 1987). Then, the possible presence of LHRH receptors on the atroglial cell compartment cannot be discounted 'a priori'. Interestingly enough, the ability of cortical ACM to stimulate lymphocyte proliferation has been recently demonstrated (Gallo *et al.*, 1993; Marchetti *et al.*, 1995c), supporting the concept that astroglia participate in the interaction between the nervous and immune systems (Patterson & Nawa, 1993; Mizuno *et al.*, 1994). Given (a) the parallelism between the astrocyte and the macrophage; (b) the stimulatory effect exerted by LHRH in astroglia proliferation, and (c) the key role of LHRH in the neuroimmune communication network (see Marchetti *et al.*, 1989a,b, 1990; Batticane *et al.*, 1991; Morale *et al.*, 1991; Maier *et al.*, 1992; Silverman *et al.*, 1994; Rettori *et al.*, 1994; Silverman *et al.*, 1994; Wilson *et al.*, 1995; Marchetti *et al.*, 1995a–c), it seems reasonable to hypothesize a functional integration between the LHRH neuronal system and the astroglial cell compartment. In summary, the present work for the first time demonstrates the presence of a bidirectional informative network between the GT_{1-1} neuronal cell line and developing glia. From the present results it seems tempting to speculate that such crosstalk between astroglia and LHRH neurons is susceptible to play a major role in the integration of the multiplicity of brain signals participating in the physiopathological control of LHRH function.

Materials and methods

Animals

Pregnant Sprague-Dawley (Cr:CD (SD) Br) rats, obtained from Charles River Laboratories (Calco, Como, Italy), were housed in a temperature-(22 ± 2°C) and light (14'h light, 10 h dark cycle, light on at 1600 h)-controlled room, and received food and water ad libitum. The guidelines on ethical standards for animal studies were followed. Primary astroglial cell cultures were obtained from different CNS regions of newborn (1–3 days of age) rats, as described.

Neuronal cell culture

The GT_{1-1} hypothalamic neuronal cell line was kindly provided by Dr R.I. Weiner (University of California, San Francisco). Immortalization of hypothalamic LHRH neurons by genetically targeted tumorigenesis was described in full details by Mellon *et al.* (1990). The GT_{1-1} cell lines were manipulated as described (De La Escalera *et al.*, 1992c), with slight modifications. Briefly, GT_{1-1} cells were cultured in DMEM with 10% fetal calf serum (FCS, Gibco, Grand Island, NY) and penicillin-streptomycin directly on 100-mm plastic petri dishes (Corning, New York, NY), at 37°C in a water-saturated atmosphere of 95% O_2 and 5% CO_2. Cells were cultured until they reached 80–90% confluence, at which time cells were replated at a density of 3×10^5 cells in 35 mm pertri dish, in a 6-well plate (Costar, Cambridge, MA) or in a chamber insert (for co-culture), and fed with either DMEM or with ACM. Morphological appearance, growth rates and LHRH secretion were examined every 2 days for 8 days. For measurement of LHRH release the medium was replaced every 2 days, collected, centrifuged to remove cellular debris, and frozen at −80°C.

Astroglial cell culture

Primary astroglial cell cultures from different CNS regions were obtained from newborn rats as described according to Booher & Sensenbrenner (1972) with slight modifications, as described in full details by Avola & coworkers (1988, 1991, 1993). Briefly, after careful removal of the meninges in aseptic conditions, pieces of frontal cerebral cortex, hypothalamus, olfactory bulb, striatum and spinal cord were passed through a sterile nylon sieve (82 μm pore size) in nutrient medium. Dissociated cells were seeded into petri dishes at an initial plating density of $0.5-1 \times 10^6$ cells/cm^2. This low seeding density of brain cells at 1 day post-natal life confirms the astroglial nature of our culture. The low initial plating density of dissociated cells also excludes oligodendroglial and microglial contaminations. To further eliminate possible contaminations with the loosely adhered cells, such as oligodendrocytes, neurons or microglia that might still be present during the first week of culture, glial cell cultures were gently shaken during the first change of the culture medium. Cells were seeded into 35 mm plastic petri dishes (Corning, New York, NY) or 6-well plates (Costar, Cambridge, MA) at the indicated plating density. The cultures were incubated at 37°C in a humidified 5% CO_2/95% O_2 air atmosphere. Oligodendrocytes cultures were prepared as described (Avola *et al.*, 1988) and maintained in basal nutrient medium (DMEM containing 10% heat-inactivated fetal calf serum, FCS, 2 mM glutamine, 5 U/ml penicillin and 5 mg/ml streptomycin).

Astroglial conditioned medium preparation

It is well recognised that hormones and growth factors regulate the expression of the cytoskeletal protein, glial fibrillary acidic protein (GFAP, see Avola *et al.*, 1988). Growing of astroglia in chemically defined media (CDM) in the absence of serum abolishes EGF-induced incorporation of labeled aminoacid precursors into cytoskeletal proteins (Avola *et al.*, 1988, 1991). Moreover, it has been clearly shown that maintenance of astroglia in CDM significantly decreases GFAP mRNA levels, whereas serum readdition for 24–48 h induces a high degree of GFAP expression (Avola *et al.*, 1991). Two different astroglial CM were then prepared to be used in our study. In a first instance, in order to proceed in the more physiological way and since the GT_{1-1} neurons were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS, GIBCO, Grand Island, NY), astroglial cells were maintained in the presence of the basal nutrient medium (DMEM containing 10% heat-inactivated FCS, 2 mM glutamine, 5 U/ml penicillin and 5 mg/ml streptomycin). Two days before CM collection new fresh basal medium containing serum was added. The culture medium was changed after 8 days as a first change and then every 4 days until 40 days. At different 'ages', that is, after different times in culture ranging from 8 to 12 days for 'young' glia, and from 16 to 40 days for 'aged' glia, astroglial conditioned medium (ACM) was collected, centrifuged and stored at −80°C to test the effects on neuronal cells.

In a second instance, astroglial cells were cultured in serum supplemented medium (SSM) for different days and then switched in DMEM without serum for a starvation period of 24 h. Successively, new fresh DMEM without serum was added to the cell cultures and collected after 24 h. This collected medium was considered serum free ACM (SF-ACM) and was stored at −80°C. Both CMs proved to be effective in influencing LHRH neurons, the ACM, however was selected for all the successive experiments since it mimics more the physiological conditions, for growing the LHRH neurons. Both ACMs were however tested to assess the peptidergic nature of the factors released by astroglia. For this end, ACMs were boiled at 100°C for 10 min (see Condorelli *et al.*, 1988). To test for nonspecific effects secondary to different degrees of peptidase activities in the different CMs, the effect of peptidase inhibitor (bacitracin, Sigma, 2×10^{-2} M) was tested in some experiments, as described in the text. For the co-culture and mixed-culture experiments, cortical astroglial cells of 12 DIV were selected, when cellular confluence reached approximately 40–50%.

GT_{1-1}-astroglial cell co-cultures

To test for the effects of secreted factors from astroglia and GT_{1-1} neurons, astroglial cells of 10–12 DIV (cellular confluence < 50%), and GT_{1-1} neurons were maintained in the same well, but were physically separated by a porous membrane (0.4 μm pore size). Molecules in solution are allowed to pass the membrane but direct cell to cell contacts are prevented. Astroglial cells were seeded on 6-well plates (Costar, Cambridge, MA), after 10–12 days neuronal cells were added to the plate in a cell culture chamber insert (Costar, Cambridge, MA), and the co-culture examined every 2 days, for 8 days. For measurement of LHRH release the medium was replaced every 2 days, collected, centrifuged to remove cellular debris, and frozen at −80°C.

GT_{1-1}-astroglial cell mixed cultures

The role of cell-cell contact and adhesion molecules in GT_{1-1}-astroglial cell interactions was assessed in mixed cultures, and the neuronal adhesion molecule (N-CAM) selected for its pivotal role in neuron-neuron/neuron-glia interactions in the developing CNS. After 9 days of astroglial cell culture in 6-well plates, GT_{1-1} cells were added on top and triplicate wells of cells were incubated in the absence or the presence of N-CAM Ab. Two antibody reagents that react with N-CAM were used in the present study. A monoclonal N-CAM antibody (Boehringer Mannheim, Mannheim, Germany), from rat-mouse hybrid cells (clone H28-123-16). The antibody belongs to the IgG2a class and was produced in ascites of nude mice and purified by ion exchange chromatography using a DEAE Tris-Acryl column. The antibody is > 95% pure as determined by FPLC and specifically binds to N-CAM, an integral membrane glycoprotein with three polypeptide chains (180 kD, 140 kD and 120 kD) (Sadoul, 1981; Rougon, 1986). The second antibody reagent used was a monoclonal antibody (N-CAM-Ab, MAB310, from CHEMICON Int. Inc., Temecula, CA 92590). MAB 310 binds to neurons and astrocytes *in vivo*. This antibody recognizes at the neural cell surface a triplet of glycoproteins with molecular weights of 180, 140 and 120 kD (identical to NCAM). (Kruse, 1982; Vincent, 1993). Purification was carried out by affinity chromatography (Purity > 95%) on DEAE trisacryl, buffered in bovine serum albumin (1 mg/ml) and phosphate buffer saline. A preliminary experiment was carried out to test the two Abs and a dose-response (0.1–10 μg/ml) study showed comparable effects of both reagents, and the presented results refer to anti-N-CAM from Boeringer Mannheim. The presence of a nonsense antibody included in the control medium (indicated as Control), did induce any significant effects. The effects of N-CAM Ab was tested on GT_{1-1} neurons grown in DMEM and in GT_{1-1}-astroglial cell mixed cultures. Medium was replaced every two days with fresh medium containing or not 0.1–10 μg/ml N-CAM Ab. Collected media was centrifuged and stored at −80°C for LHRH RIA.

Immunohistochemical identification of cells in culture

GT_{1-1} and glial cells were distinguished using respectively the LHRH-1 polyclonal antibody rabbit anti-mouse LHRH 1:2000 (generously provided by Dr R. Benoit, Montreal, Canada) and the monoclonal antibody anti-cow glial fibrillary acid protein (GFAP) 1:3000 (DAKO A/S, Denmark). The cells were plated onto 13 mm glass coverslip and placed in individual wells of 24-well plate, and allowed to form a

confluent monolayer. Prior to immunostaining, coverslip were fixed in 4% paraphormaldehyde (0.1 M Tris Saline Buffer, pH 7.4, 20 min, 20°C) and then washed three times in 0.1 M Tris Saline Buffer (TBS). In order to visualize these intracellular antigens, cells were permeabilized with 0.1% Triton X-100. Primary antibodies were diluted in TBS and incubated overnight at 4°C, After the primary incubation, the cells were washed three times in TBS. Detection of antigen-antibody binding sites was accomplished with a biotinylated secondary mouse anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and an avidin-biotin HRP complex (Vector Laboratories, Burlingame, CA). 3,3' Diaminobenzidine (Sigma) was the chromogen. Immunostained cultures were washed in TBS, dehydrated in ethanol, cleared in xylene and mounted onto glass slide with permount.

Microscopy

Photomicrographs of growing cells were taken with a Zeiss inverted microscope (Oberkochen, West Germany) on phase contrast with a × 16 objective. The immunohistological preparations were photographed on a Leitz Aristoplan microscope (with × 10 and × 20 objectives).

LHRH RIA

LHRH in the supernatants was determined by RIA (Martinez De La Escalera *et al.*, 1992c) in duplicate, using the rabbit polyclonal antibody R1245 (kindly obtained from Dr T Nett), which is specific for the decapeptide (Nett *et al.*, 1973). All samples from an experiment were analysed in the same assay. The limit of detection was 1.4 pg/ml, and the intra-assay coefficient of variation was 4.2%.

[^{3}H]-Thymidine incorporation in GT_{1-1} and astroglial cells

At 2, 4, 6 and 8 days and for each respective cell culture system GT_{1-1}, or astroglial cell proliferation were tested in triplicate by incubation of [Methyl-^{3}H]Thymidine (Amersham, Arlington Heights, IL) (1 μCi/ml of culture medium) for 2 h at 37°C. After this time dishes were rinsed three times with ice-cold isotonic PBS, pH 7.4, and cells were extracted with 1 N perchloric acid, once with ethanol, and solubilized in 0.3 N NaOH at 37°C for 30 min (Avola *et al.*, 1993). Labeled DNA was collected and radioactivity was determined by liquid scintillation spectrophotometry.

Statistics

The presented results are the mean ± SEM of at least three different experimental manipulations. The age-dependent effects of ACM (8–40 days, *in vitro* DIV), on GT_{1-1} neurons, and the time-dependency of GT_{1-1} neurons in culture (2, 4, 6 and 8 days *in vitro*, DIV), on both LHRH release, or GT_{1-1} neuron proliferation, as well as comparisons between co-culture, mixed-culture preparations and ACM, were analysed by two-way analysis of variance, with group and time as independent variables. Comparisons a posteriori between different experiments were made by Newman-Kreuls tests (Winer, 1971).

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