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Coexistence of neuropeptides and their possible relation to neuritic regeneration in primary cultures of magnocellular neurons isolated from adult rat supraoptic nuclei

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Summary

The coexistence of vasopressin (VP), oxytocin (OXY), galanin (GAL) and cholecystokinin (CCK) and the synthesis of GAL and CCK during neuritic regeneration was investigated in cultured magnocellular neurons, isolated from adult rat supraoptic nuclei. Double-labelling immunofluorescence was performed after 7 days of culture using primary antibodies for VP, OXY, GAL and CCK (paired in all possible combinations) and secondary antibodies labelled with either fluorescein or rhodamine. Confocal laser scanning microscopy revealed the coexistence of the mentioned peptides in all possible combinations, an unexpected result considering that the only combinations observed in tissue sections are VP-GAL and OXY-CCK. Freshly dispersed cells were devoid of any neuritic processes and showed a very poor immunocytochemical staining reaction for GAL and CCK. In contrast, neurons cultured for 7, 12 and 21 days showed many neurites and a strong immunoreactivity for GAL and CCK indicative of an increased synthesis of both peptides in the regenerating neurons. This increased synthetic activity is consistent with transient upregulation of these peptides observed *in situ* after hypophysectomy by other authors. The results suggest that the upregulation of GAL and CCK is functionally related to the neuronal regeneration processes observed during culture and that the 'uncommon' coexistences as well as the prolonged sythesis of GAL and CCK may be due to the lack of environmental inputs, which normally regulate the expression and up- and downregulation of these peptides *in vivo*.

Introduction

The supraoptic and paraventricular nuclei of the hypothalamus contain the neurosecretory magnocellular system, consisting of separate cellular subpopulations, which are admitted to synthezise either oxytocin (OXY) or vasopressin (VP). Several studies have revealed the coexistence of both hormones with other peptides in these cells. OXY-containing cells have been shown to exhibit immunoreactivity for cholecystokinin (Beinfeld *et al.* 1980, Vanderhaeghen *et al.* 1981, Martin *et al.* 1983, Tsuruo *et al.* 1988, Meister *et al.* 1990), corticotropin-releasing factor (Burlet *et al.* 1983, Sawchenko *et al.* 1984), thyrotropin-releasing factor (Tsuruo *et al.* 1988), Met-enkephalin (Martin & Voigt 1981, Martin *et al.* 1983, Vanderhaeghen *et al.* 1983, Adachi *et al.* 1985, Gaymann & Martin 1987) and renin (Fuxe *et al.* 1982). On the other hand, immunoreactivity for galanin (Melander *et al.* 1986, Rökaeus *et al.* 1988, Gaymann & Martin 1989, Skofitsch *et al.* 1989, Meister *et al.* 1990), dynorphin (Watson *et al.* 1982, Whitnall *et al.* 1983), Leu-enkephalin (Martin & Voigt 1981, Martin *et al.* 1983) and angiotensin II (Kilcoyne *et al.* 1980) has been detected in VP-containing cells. Nitric oxide synthase-like immunoreactivity has been demonstrated in both subpopulations of neurons (see Villar *et al.* 1994).

The functions of these coexisting peptides are, in most cases, unknown. However, some data point to a possible role for galanin (GAL) and cholecystokinin (CCK) in regenerative processes after cell damage (see Villar *et al.* 1989, 1990, 1994). The magnocellular neurons exhibit a surprising capacity for regeneration, even in adult animals (Billenstein & Leveque 1955, Dellman 1973, Raisman 1973, Dellman *et al.* 1986, 1987). This capacity enabled us to obtain primary cultures of dispersed magnocellular neurons isolated from adult rat supraoptic nuclei (Bilinski *et al.* 1996). We have further shown that these cultured neurons were capable of regenerating their projections and that this neuritic outgrowth was paralleled by an enhanced synthetic activity for GAL and CCK (Sánchez *et al.* 1997). This upregulation of GAL and CCK in regenerating neurons *in vitro* is in accordance with some observations *in situ* made by other authors. Villar *et al.*

(1990) have shown that hypophysectomy led to a marked increase in the GAL-like immunoreactivity in VP-ergic and CCK-like immunoreactivity in OXY-ergic neurons of the magnocellular system. Using *in situ* hybridization, the same authors observed that the expression of the corresponding mRNAs was also increased. The increase in expression and storage of both peptides was transient, reaching a maximum in five days, and then gradually declined to basal levels in about two weeks after hypophysectomy (Villar *et al.* 1990). Other studies have confirmed the upregulation of GAL and CCK in the magnocellular system after pituitary stalk transection or hypophysectomy (Meister *et al.* 1990, Young *et al.* 1990). In view of these results, it was of interest to investigate the coexistence of OXY, VP, GAL and CCK in cultured differentiated magnocellular neurons and whether the up- and downregulation of GAL and CCK observed *in situ* after hypophysectomy also occurs in the same manner *in vitro*.

Materials and methods

Magnocellular neurons were isolated and cultured as previously described (Bilinski *et al.* 1996, Sánchez *et al.* 1997). In brief, supraoptic nuclei were obtained from adult male rats of a Holtzman-derived strain and the tissue fragments were dispersed using 0.2% trypsin (1 : 250, Sigma Chemical CO., St. Louis, MO, USA). After a final mechanical dispersion, the dissociated cells were washed several times and resuspended in culture medium (MEM with Earles salts; Sigma Chemical Co., St. Louis, MO, USA) containing 20% fetal calf serum, 10% horse serum (both from GIBCO, Rockville, MD, USA) and antibiotics. The cells were plated on cover slips coated with collagen I (Sigma Chemical Co., St. Louis, MO, USA). The cover slips were placed in 35 mm Falcon dishes, and cultured for 7, 12 or 21 days at 37 °C in a humidified atmosphere with 5% CO_2/95% air. The culture medium was changed first after 20–24 h and thereafter every 3 days of culture. Freshly dispersed cells were placed on culture dishes coated with poly-L-lysine in order to prevent cell loss during fixation and immunocytochemical staining.

The cells were fixed in a formalin–picric acid mixture (Zamboni & De Martino 1967) for 20 min and washed in phosphate-buffered saline (PBS). After a 30 min preincubation with normal goat serum (1 : 25, Vector Laboratories, Burlingame, CA, USA) at room temperature, rabbit antisera to GAL (1 : 10,000) or CCK (1 : 10,000; both from Peninsula Laboratories, Belmont, CA, USA) were used for routine immunocytochemical staining. The incubations and staining procedures, using the ABC Elite kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine, were performed as described elsewhere (see Bilinski *et al.* 1996, Sánchez *et al.* 1997) and following the recommendations of the manufacturer. The cells were examined in a Nikon Diaphot inverted microscope and photographed using Agfapan APX 25 film (Agfa Gevaert, Leverkusen, Germany).

Indirect double excitation immunofluorescence was performed after 7 days of culture using mixtures of primary antisera raised in rabbit (anti-VP 1 : 5000, anti-OXY 1 : 10000 and anti-GAL 1 : 5000; all from Peninsula Laboratories, Belmont, CA, USA), guinea pig (anti-VP 1 : 5000; Advanced Chemtec, Louisville, KY, USA) or mouse (anti-OXY 1 : 5000 and anti-CCK 1 : 5000; kindly provided by T. Hökfelt, Karolinska Institutet, Stockholm, Sweden). Fluorescein isothiocyanate (FITC)-conjugated anti-guinea pig or anti-mouse IgG (1 : 300; Vector Laboratories, Burlingame, CA, USA) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG (1 : 500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used as second antibodies. The cells were preincubated for 30 min at room temperature with normal goat serum (1 : 25; Vector Laboratories, Burlingame, CA, USA) and then incubated overnight at 4 °C with either of the six possible mixtures of two primary antibodies. After a rinse in PBS, the cells were incubated with the corresponding FITC- and TRITC-conjugated second antibodies at room temperature for 30 min. After three final washes with PBS, the cells on the cover slips were placed on microscope slides and mounted with a mixture of glycerol and PBS (3 : 1 v/v) containing N,N-dimethyl-*p*-phenylenediamine (Sigma Chemical Co., St. Louis, MO, USA; see Platt & Michael 1983). FITC and TRITC-labelling was established using a Bio-Rad MRC-600 laser scanning confocal imaging system, equiped with a mixed krypton/argon gas laser and a Nikon Optiphot II microscope. A standard BHS single-channel filter block was used with the excitation filters 488 DF 10 and 568 DF 10 for FITC- and TRITC-induced fluorescence respectively. In order to exclude possible nuclear staining, we checked the position of the nucleus by phase contrast microscopy. The images were printed on a Sony Mavigraph Video Printer.

Preabsorption controls were routinely performed by incubating the different antisera with their corresponding peptides at concentrations of 1 μM for 6 h prior to incubation with the cells (see Orezzoli *et al.* 1995). Other controls were performed omitting the first antibody and unstained cells intermingled with the stained neurons served as a negative control for the specificity of the staining reactions.

Results

As a consequence of the stressing dispersion procedure, freshly dissociated cells were round-shaped and devoid of any neuritic processes. After routine immunocytochemistry for GAL and CCK, most of them remained unstained (Figure 1A and E). In contrast, when the cells were stained with the same dilutions after 7 days of culture, many neurons immunoreactive for GAL (Figure 1B) and CCK (Figure 1F) could be distinguished. Unlike the populations of freshly dispersed cells, the cultured neurons exhibited a pronounced neuritic outgrowth, forming network-like contacts with neighbouring cells. The morphological characteristics of the neurons and

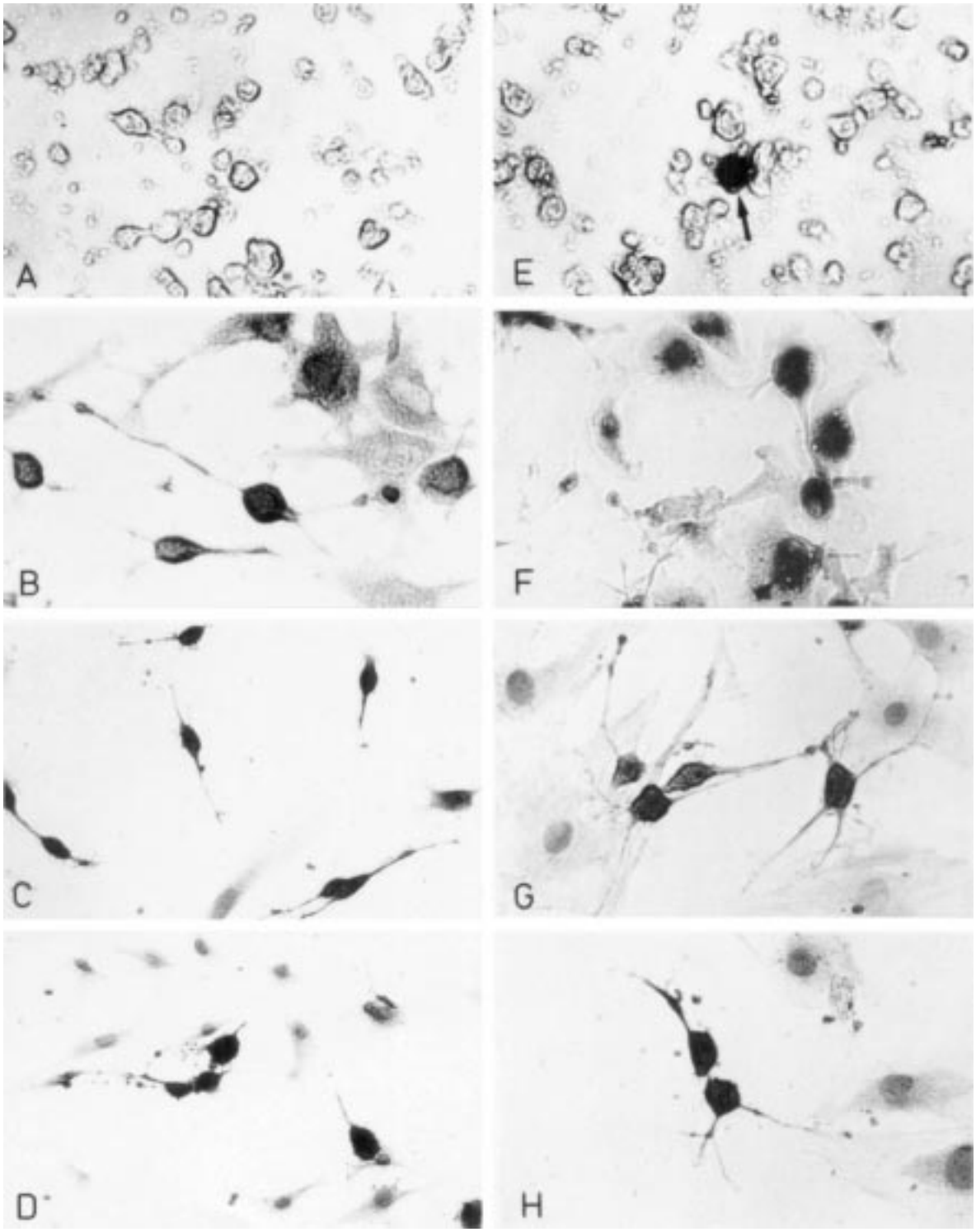

Figure 1. Immunocytochemical localization of GAL (A–D) and CCK (E–H) in freshly dispersed cells (A and E) and in neurons cultured for 7 (B and F), 12 (C and G) and 21 days (D and H). Note the heavy staining in the cultured neurons and the virtual absence of any staining reaction in the freshly dispersed cells; only one cell (arrow in E) shows a positive reaction. 200×.

the staining intensity for GAL and CCK remained unchanged after 12 (Figure 1C and G) and even after 21 days of culture (Figure 1D and H).

The double-labelling experiments performed after 7 days of culture revealed the coexistence and/or colocalization of at least two neuropeptides in several neurons. At first we could establish the expected coexistence of VP and GAL, as well as of OXY and CCK (Figure 2). But further experiments revealed the unexpected coexistence of VP-OXY, VP-CCK, OXY-GAL and GAL-CCK in several neurons (Figure 3).

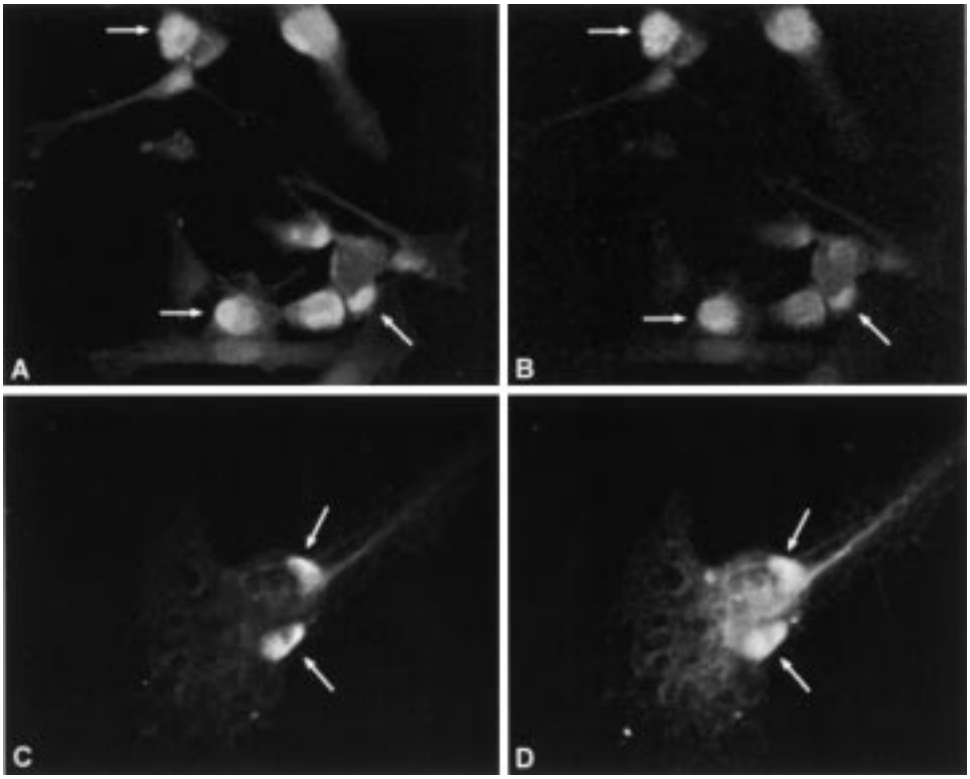

Figure 2. Confocal immunofluorescence images showing the coexistence (arrows) of FITC-labelled VP (A) with TRITC-labelled GAL (B) and of FITC-labelled CCK (C) with TRITC-labelled OXY (D) in magnocellular neurons cultured for 7 days. 200× (A,B); 500× (C,D).

The colocalization was often detected in particular zones of the soma (see Figure 2C and D or Figure 3A–D) and, in some cases, in the neuritic processes (an example is shown in Figure 4).

None of the immunoreactivities described above were observed after incubation with the preabsorbed control sera or after omission of the first antibody.

Discussion

In most cases, the adult mammalian central nervous system provides only a poor environment for neuritic growth and regeneration. This is mainly due to inhibitory factors, which normally predominate and suppress the activity of growth promoting molecules in the adult brain. The neurons may be affected by extrinsic factors, which include glial inhibitors (e.g. myelin-associated inhibitors, neurite-growth inhibiting proteins), cytokines and components of the extracellular matrix (Rogers *et al.* 1983, Wintergerst *et al.* 1997, Sharief 1998, Hirsch & Bahr 1999, Lemons *et al.* 1999, Qiu *et al.* 2000, Streit *et al.* 2000, Venters *et al.* 2000). Other regulatory factors are dependent on the intrinsic state of the neurons, such as cyclic nucleotide levels, semaphorins, several neural cell surface adhesion and recognition molecules, cytoskeletal elements and the more recently described synucleins and the BCL-2 related molecules (Walsh & Doherty 1996, Willamson *et al.* 1996, Schachner 1997, Adams & Cory 1998, Clayton & George 1998, Cotman *et al.* 1998, Hulley *et al.* 1998, Holm & Isakson 1999, Qiu *et al.* 2000). In the magnocellular system the injured neurons may undergo degenerative or regenerative processes depending on the lesion site (Scott & Knigge 1981, Nagy *et al.* 1983, Dellman *et al.* 1987). After hypophysectomy and under certain conditions, the surviving neurons are capable of generating a 'new' neural lobe (Dellman 1973, Raisman 1973). Cultured magnocellular neurons, isolated from adult rat hypothalami and severely damaged during dispersion, revealed a surprising regenerative capacity (Bilinski *et al.* 1996, Sánchez *et al.* 1997), a fact that provides, as shown in the present study, a valuable tool for the study of some aspects related to regeneration processes in differentiated neurons of the central nervous system.

In previous papers we have shown that cultured magnocellular neurons contain VP, OXY, GAL and CCK (Bilinski *et al.* 1996, Sánchez *et al.* 1997). GAL and CCK are normally present at low concentrations in magnocellular hypothalamic neurons (Cunningham & Sawchenko 1991) and this fact is consistent with the poor immunocytochemical staining reaction observed in freshly dispersed cells, using the antibodies at the same dilutions as for cultured cells (see Figure 1A and E). Due to the stressful dispersion procedure, the freshly dispersed cells are round-shaped and devoid of any projections, but after 2 days of culture a visible and progressive neuritic outgrowth takes place (see Bilinski *et al.* 1996). The strong staining reaction observed after 7 days of culture indicates an enhanced synthetic activity for GAL and CCK during culture, which is coincident with the neuritic

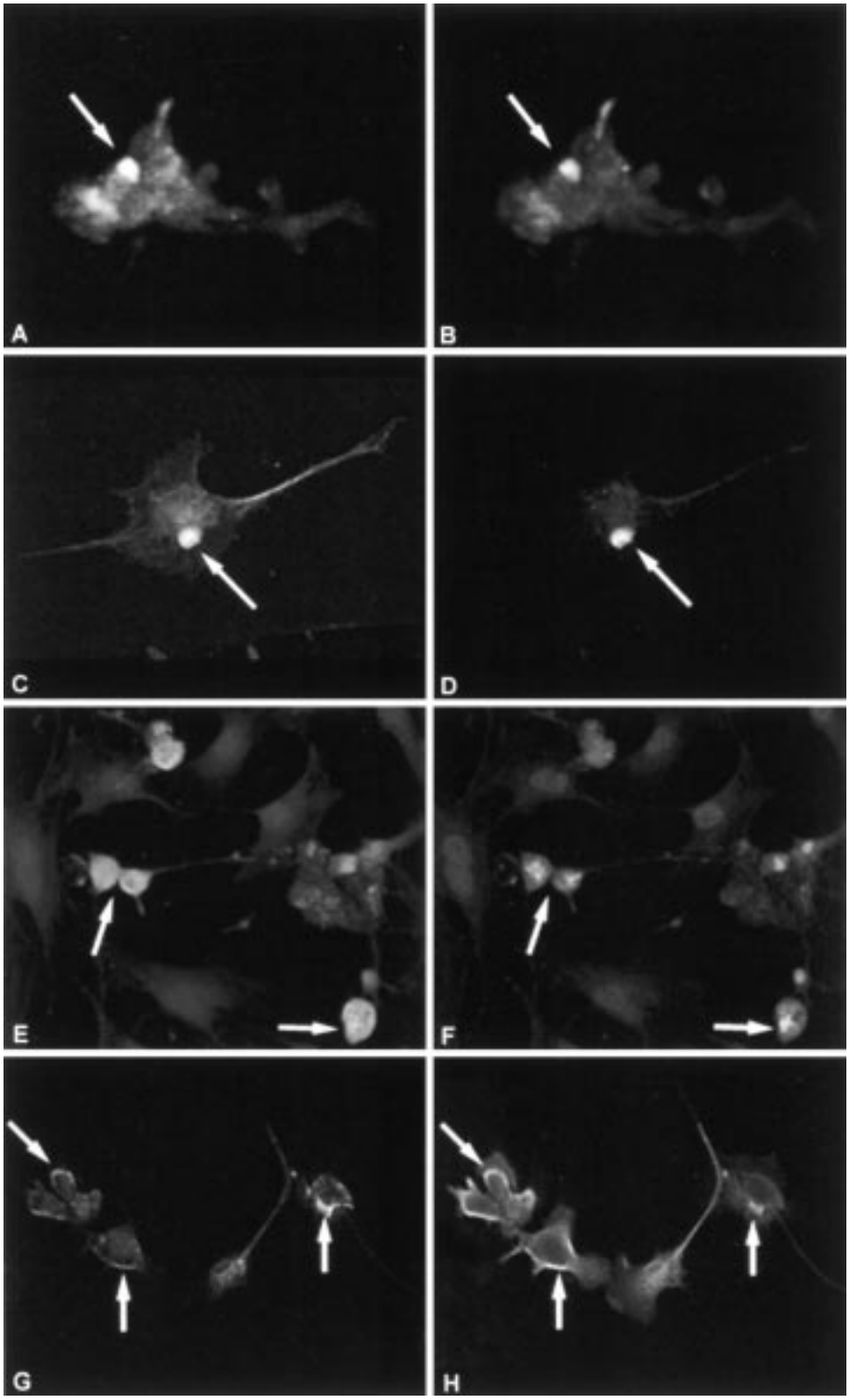

Figure 3. Confocal immunofluorescence images showing the 'uncommon' coexistence (arrows) of FITC-labelled VP (A) with TRITC-labelled OXY (B), of TRITC-labelled VP (C) with with FITC-labelled CCK (D), of FITC-labelled OXY (E) with TRITC-labelled GAL (F) and of TRITC-labelled GAL (G) with FITC-labelled CCK (H) in magnocellular neurons cultured for 7 days. 500× (A–D); 150× (E,F); 200× (G,H).

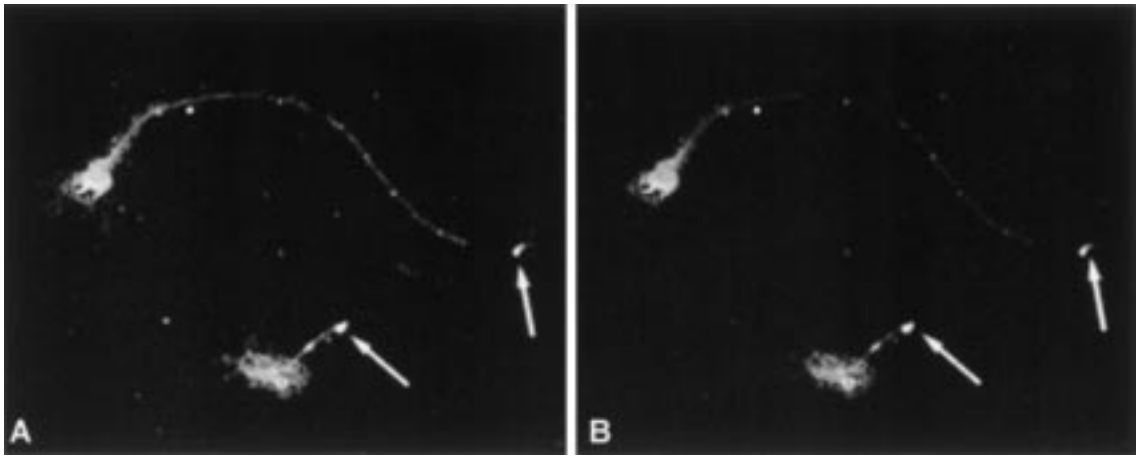

Figure 4. Confocal immunofluorescence images showing the coexistence of TRITC-labelled VP (A) with FITC-labelled CCK (B) in the neural somata, in the neuritic processes and in the terminal buttons (arrows). 150×.

regeneration processes observed in the neurons. As already mentioned in the introduction, a transient upregulation of the synthetic activity for GAL and CCK was also observed in the supraoptic and paraventricular nuclei after pituitary stalk transection or hypophysectomy (Meister *et al.* 1990, Villar *et al.* 1990, Young *et al.* 1990). A similar response for GAL has also been observed in dorsal root ganglion cells and in spinal cord after peripheral nerve injury (Villar *et al.* 1989). Altogether the results strongly suggest that the upregulation of these peptides is related to the regenerative processes taking place after injury, either *in vivo* or *in vitro*, and that this reaction conforms an intrinsic response of the neurons to the new situation. Interestingly, the cultured neurons exhibit the strong staining reaction even after 3 weeks of culture. This result points to a difference with respect to the mentioned observations made by Villar *et al.* (1990) after hypophysectomy. These authors detected a transient increase in the synthesis of GAL and CCK, which reached its maximum after 5 days and then declined, reaching basal levels after 2 weeks. One explanation for the discrepancy may be that the cultured neurons do not contact any target cell (or tissue), which may act as a stop signal for the growing neurites. In other words, the cultured neurons are 'regenerating' over long periods without any restraint.

Several double labelling experiments *in situ* have shown that VP-ergic magnocellular neurons also contain GAL (Melander *et al.* 1986, Rökaeus *et al.* 1988, Gayman & Martin 1989, Skofitsch *et al.* 1989, Meister *et al.* 1990) and that OXY-ergic neurons contain CCK (Beinfeld *et al.* 1980, Vanderhaeghen *et al.* 1983, Martin *et al.* 1983, Tsuruo *et al.* 1988, Meister *et al.* 1990). Other combinations of these peptides (VP-OXY, GAL-CCK, VP-CCK and OXY-GAL) have not been described in the literature. Surprisingly, our double labelling experiments revealed the coexistence of these peptides in all possible combinations. This unexpected result indicates that cultured, differentiated magnocellular neurons are capable of producing any of these peptides and raises the possibility that one neuron may contain more than two of them. All these 'uncommon' combinations appear during culture, presumably due to the lack of tissular and/or humoral regulatory inputs (e.g. inhibitory glial factors, afferent contacts, target cell signalling, etc.).

In summary, we have shown that OXY, VP, GAL and CCK may coexist in all possible combinations in magnocellular neurons cultured for 7 days. This result indicates that the cultured neurons are potentially able to express any of the mentioned peptides, a fact differing from observations made *in situ*. We also have shown that GAL and CCK are synthezised during culture when neuritic regeneration takes place and that the massive presence of both peptides persists in these cells, unlike *in vivo*, for prolonged culture periods (up to 21 days). It is further suggested that GAL and CCK may act without restraints in response to the cell damage produced during dispersion and that the uncommon behaviour of the cultured neurons results from the lack of signals, which normally regulate the synthetic activity and the neuritic growth and/or regeneration in the central nervous system.

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