

Peripherin Expression in Hippocampal Neurons Induced by Muscle Soluble Factor(s)

Karima Djabali, Afroditi Zissopoulou, Meltsje J. de Hoop, Spyros D. Georgatos, and Carlos G. Dotti

Cell Biology Program, European Molecular Biology Laboratory, D-69012 Heidelberg, Germany

Abstract. Previous studies have shown that neuronal cells in culture can switch neurotransmitters when grown in the presence of different target cells. To examine whether this plasticity extends to structural proteins, we cocultured hippocampal neurons and pituitary-derived neuroendocrine (AtT20) cells with astrocytes, kidney epithelial cells, or skeletal muscle cells. As a marker of phenotypic change we used the cytoskeletal protein peripherin, a type III intermediate filament (IF) subunit which is not expressed in hippocampal neurons and AtT20 cells. We show here that

soluble factor(s) secreted specifically from skeletal muscle cells can induce the expression and de novo assembly of peripherin in a subset of post-mitotic neurons. We further demonstrate that one of these factors is the Leukemia Inhibitory Factor/Cholinergic Neuronal Differentiation Factor. The environmentally regulated expression of peripherin implies a remarkable degree of plasticity in the cytoskeletal organization of postmitotic CNS cells and provides a noninvasive model system to examine the de novo assembly of IF proteins under in vivo conditions.

DURING development, extracellular signals play crucial roles in all aspects of neuronal differentiation. Several types of chemotrophic factors and extracellular matrix components can influence neuronal cytoarchitecture (Harrelson and Goodman, 1988; Dodd and Jessell, 1988; Sanes, 1989; Perris and Bronner-Fraser, 1989; Reichardt and Tomaselli, 1991). Environmental cues are not only important in determining the fate of a developing neuron, but can also induce a change in an otherwise fixed phenotype. The latter is most evident in the choice of neurotransmitter. Postmitotic sympathetic neurons in culture can be induced to synthesize acetylcholine (ACh) instead of catecholamines (CA) when grown in the presence of different target cells or in media conditioned by them (Patterson and Chun, 1977; Fukada, 1980; Schotzinger and Landis, 1988; Nawa and Sah, 1990; Nawa et al., 1990; Rao et al., 1990). This phenotypic plasticity, originally thought to be an exclusive property of peripheral nervous system (PNS) neurons, was also demonstrated for postmitotic neurons of the central nervous system (CNS) (Iacovitti et al., 1987; Iacovitti et al., 1989). Since neurotransmitter switching may be part of the

normal development of the cells in situ, it remains to be established if other traits of the neuronal phenotype are also susceptible to change by environmental cues.

The elaboration of axonal and dendritic processes is a hallmark of neuronal cells. Underlying the extension of such processes is a cytoskeletal framework made of microtubules, microfilaments, and intermediate filaments. From their abundance during the different stages of development, it has been suggested that microtubules and microfilaments play a crucial role in the initial stages of axonal elongation, whereas intermediate filaments have a role at later times during axonal specialization. However, more recent findings show that various IF proteins are sequentially expressed at early times of neuronal differentiation (reviewed in Nixon and Shea, 1992). One of these proteins, peripherin, is of special interest because it is expressed in neurons of the PNS, or in neurons of the CNS with axons projecting outside the brain, but not in other CNS neurons (Portier et al., 1984; Leonard et al., 1988; Parysek et al., 1988; Brody et al., 1989; Escurat et al., 1990). The presence of peripherin in a subset of neurons has been correlated with the microenvironment in which these axons grow and establish contacts.

To investigate whether the expression of peripherin could be induced when neurons of central origin are exposed to a "PNS-like" environment, we devised a simple experimental system. This system involved coculture of hippocampal neurons or neuroendocrine (AtT20) cells with glial, epithelial, or muscle cells. The choice of hippocampal neurons and AtT20 cells appeared suitable because these cells do not express peripherin in situ. We show here that peripherin is specifically induced after coculture with skeletal muscle

Address all correspondence to Dr. C. G. Dotti, Cell Biology Program, European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69012 Heidelberg, Germany.

1. Abbreviations used in this paper: ACh, acetylcholine; CA, catecholamines; CDF, cholinergic neuronal differentiation factor; CNS, central nervous system; LIF, leukemia inhibitory factor; NFL, neurofilament light; PCR, polymerase chain reaction; PNS, peripheral nervous system; RT, reverse transcription.

cells, or after culturing the neurons in medium conditioned by muscle cells. These results provide evidence that postmitotic neurons can modify their cytoskeletal organization when exposed to appropriate environmental cues.

Materials and Methods

Cells in Culture

The hippocampal culture was prepared following the detailed protocols of Banker and Goslin (1991). Briefly, day 18 pregnant Sprague Dawley rats were killed by an overdose of anesthetic, the fetuses removed and decapitated, and the hippocampi dissected and dissociated by chemical and mechanical procedures. Approximately 10^3 cells/cm² were plated onto poly-D-lysine-coated coverslips and maintained for 4 h in E-MEM supplemented with 10% FCS. The cells were then transferred to dishes with E-MEM with the N2 mixture of Bottenstein and Sato (1979) in which semiconfluent monolayers of astrocytes, MDCK cells, or myotubes were present. The neurons were facing the monolayers of the other cells but were physically separated from them by a space of \sim 1–2 mm.

Astrocytes were prepared from neonatal rat brain as described (Booher and Sensenbrenner, 1972). The mouse muscle cell line C₂C₁₂ used in all experiments was obtained from American Type Culture Collection (ATCC; Rockville, MD) and fused myotubes were obtained as described (Morgan et al., 1992). Where indicated, a primary culture of muscle cells was pre-

pared as in Daniels (1990). AtT20 cells (clone D-16U) were grown and differentiated by a slight modification of the protocol described by Tooze et al. (1989). Briefly, cells were plated onto poly-D-lysine-coated coverslips and allowed to reach semi-confluency in MEM-10% horse serum. Elongation of cell processes was accomplished by replacing the medium with MEM with the N2 supplement. In this medium, the cells were maintained for 4 d. Alternatively, after allowing confluency, the cells were transferred for 4 d to the myotubes in MEM-N2 as described for the hippocampal cells. Purified LIF (GIBCO-BRL, Bethesda, MD) was added every 48 h to hippocampal or to AtT20 cells growing in neuronal medium in the absence of glial cells. Cells were fixed and processed for indirect immunofluorescence microscopy after 4 d of treatment.

Indirect Immunofluorescence Microscopy

The cells were fixed with methanol (-20°C , 10 min), air-dried, gradually rehydrated, and the nonspecific-binding sites blocked by 10% BSA. The following primary antibodies were used: anti-peripherin polyclonal (an antibody raised against intact peripherin, at a dilution of 1:100; see Escurat et al., 1990); α PII (an anti-peptide antibody recognizing the tail domain of peripherin, at a dilution of 1:100; see Djabali et al., 1991); anti-vimentin mAb 7A3 (at a dilution of 1:20; see Paparmacaki et al., 1991); anti- α -internexin mAb (used undiluted; see Fliegner et al., 1990); anti-NFL mAb (used at a dilution of 1:10 and obtained through Boehringer Mannheim GmbH, Mannheim, Germany); anti-NFL polyclonal HZ97 (diluted at 1:100; see Shaw et al., 1984); anti-MAP 2 mAb (at a dilution of 1:50, obtained from Boehringer Mannheim GmbH); anti-GFAP mAb (used at a dilution of 1:200

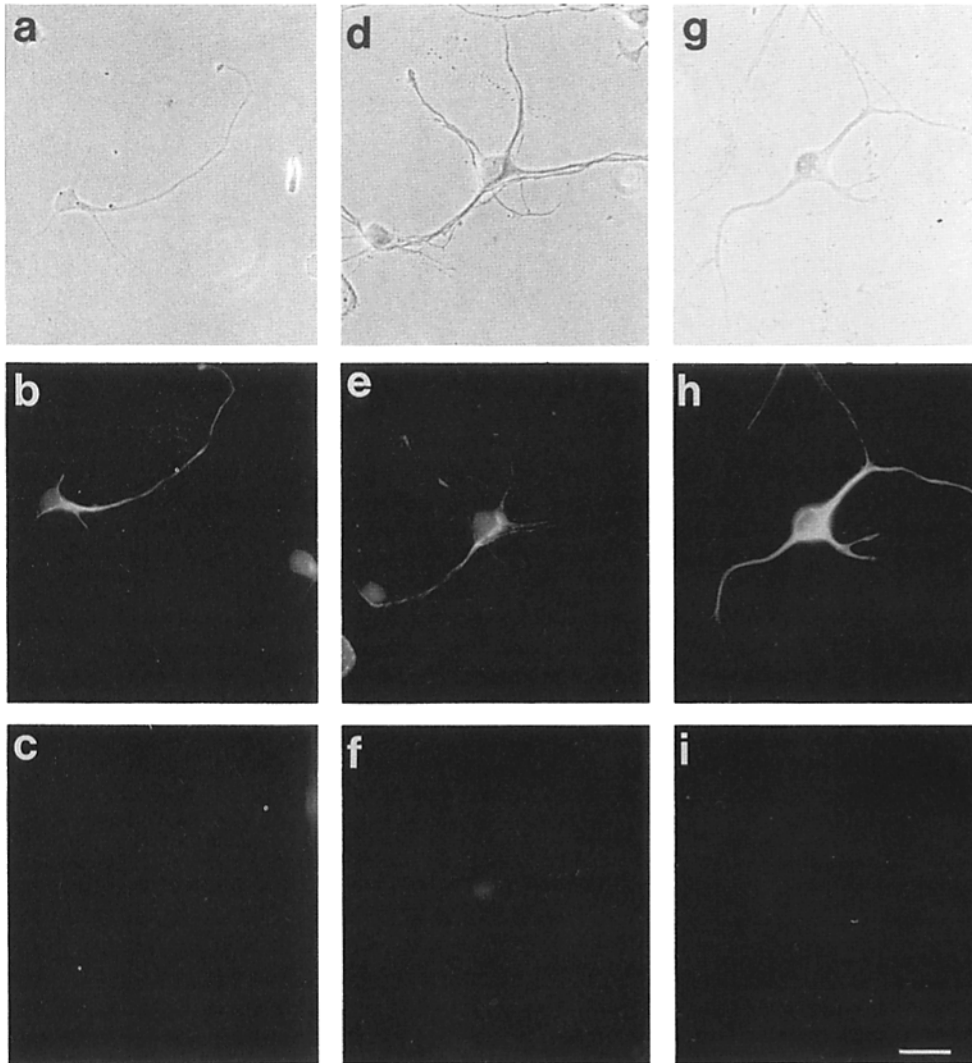


Figure 1. Hippocampal neurons co-cultured with glial cells express vimentin, α -internexin, and NFL, but not peripherin. Double-immunofluorescence microscopy of hippocampal cells fixed after 4 d of co-culture with astrocytes reveal the expression of vimentin (*b*), α -internexin (*e*), and NFL (*h*). Note that the processes evident in the corresponding phase contrast photographs (*a*, *d*, and *g*), are labeled. The same cells are negative for peripherin (*c*, *f*, and *i*). Bar, 10 μm .

and obtained from Boehringer Mannheim GmbH). After an incubation with the primary antibody, the coverslips were thoroughly washed with PBS and incubated with fluorochrome-conjugated species-specific secondary antibodies (diluted 1:100, Jackson Immunochemicals, PA). Microscopy was performed with a Zeiss Axiophot (Zeiss, Germany). Film exposure times and printing were the same in all cases.

Autoradiography

[³H]-thymidine was injected intraperitoneally at day 17.5 of pregnancy (5 μ Ci/g body weight; 20 Ci/mmol; New England Nuclear, Boston, MA). On day 18 the rat was sacrificed and hippocampal cells cultured as described above and cocultured for 48–96 h with glial or C₂C₁₂ muscle cells. At the end of the coculture, the cells were processed first for the detection of peripherin by the alkaline phosphatase immunocytochemistry and then for autoradiography. For this, the coverslips were washed, ethanol-dehydrated, dried, and mounted facing up close to the edge of glass slides. After solidification of the mounting medium, the coverslips were dipped in NTB2 photographic emulsion (1:1; Eastman Kodak Co., Rochester, NY), air-dried, and stored in a light safe box at 4°C, as described by Fletcher and Banker (1989). After exposure for 2 wk, the autoradiographs were developed in Dektol (1:2; Eastman Kodak Co.), washed, dried, and covered with a glass coverslip. Peripherin labeling and silver autoradiographic grains were detected and photographed using bright field microscopy. To correlate the labeling with morphological traits, the same cells were photographed in phase contrast and in bright field mode.

Reverse Transcription/Polymerase Chain Reaction

Hippocampal neurons or AtT20 cells were cultured in the presence of muscle-conditioned medium or leukemia inhibitory factor/cholinergic neuronal differentiation factor (LIF/CDF) for 4 d to induce peripherin expression. Control cells were cultured in glial-conditioned medium (hippocampal neurons) or in LIF-free medium (AtT20 cells). Total RNA was extracted as described by Chomczynski and Sacchi (1987) in the presence of 20 μ g *Escherichia coli* 5S RNA (Boehringer Mannheim GmbH). RNA was reverse transcribed by 20 U AMV-RT (Promega, Madison, WI, USA) and 0.5 mg oligo-dT as primer for 4 h at 42°C, and heated for 5 min at 95°C to stop the reaction. The resulting cDNA was amplified in a total volume of 50 μ l with 2.5 U Taq DNA polymerase (Boehringer Mannheim GmbH) in the presence of 0.25 mM dNTP and 50 pmole peripherin- or rab8-specific primers which had the following sequences: 5' TCA GTG CCC GTT CAT TCC TTT G (peripherin upstream) 5' GCC TCA GTA GCT GTG AAT AGA AG (peripherin downstream), 5' CCT TGT CCT CAC AGG AGA CTG (rab8 upstream), and 5' GTG AAC GAC AAG AGG CAG GTG (rab8 downstream). The sequence of the oligonucleotides was based on data published by Thomson et al. (1989) and Landon et al. (1989) (peripherin), and Elferink et al. (1992) (rab8). The peripherin primers anneal to different exons, thus allowing discrimination between amplification of mRNA or genomic DNA. In pilot experiments, conditions were established in which the amount of polymerase chain reaction (PCR) product increased with increasing amounts of cDNA. Reaction mixtures were amplified for 25, 30, and 35 cycles, each cycle consisting of 30-s denaturation at 94°C, 90-s annealing

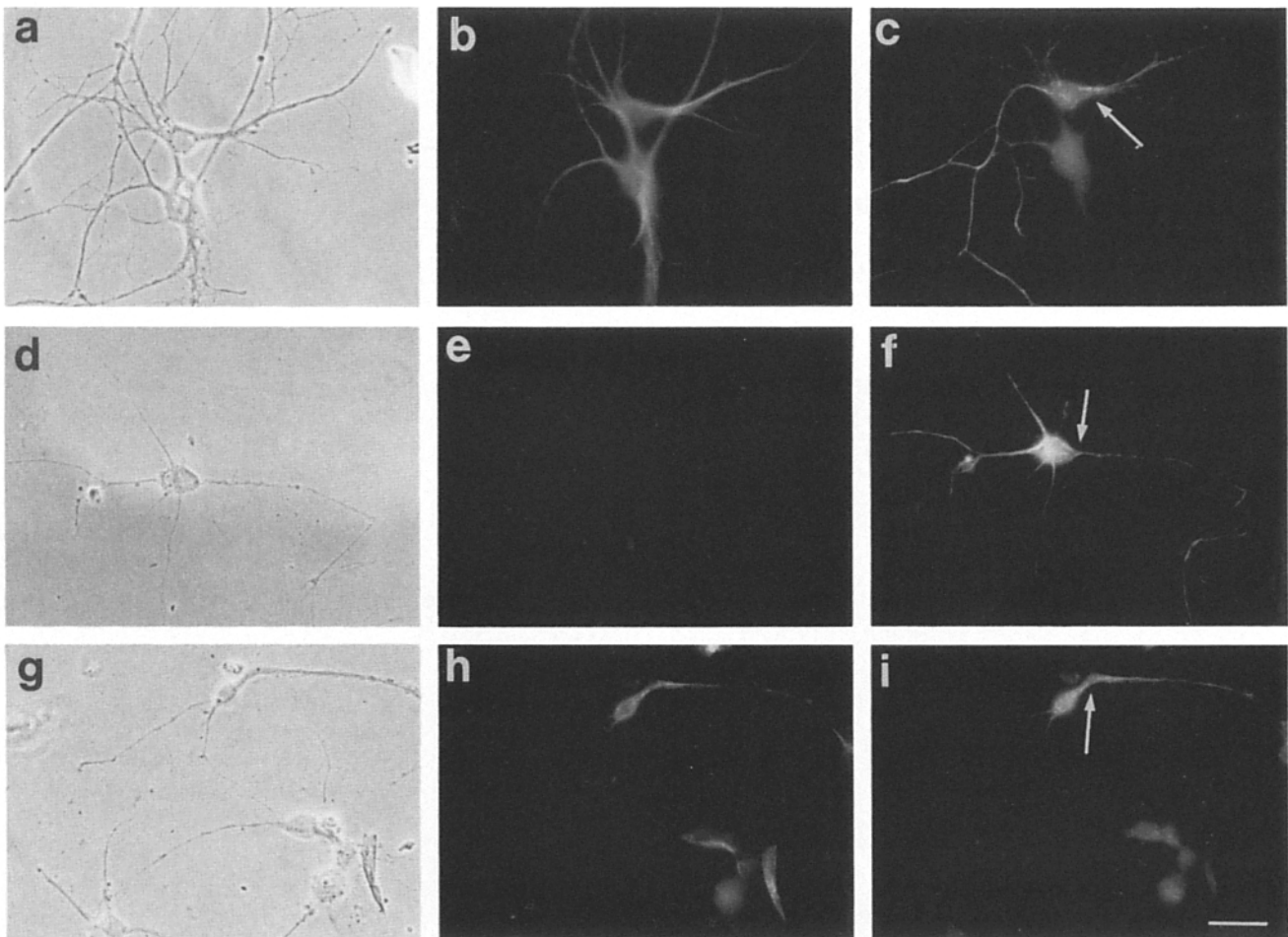


Figure 2. Hippocampal neurons cocultured with muscle cells express peripherin. Hippocampal neurons growing in the presence of C₂C₁₂ cells for 4 d were fixed and processed for double immunofluorescence microscopy. Peripherin expression is evident in *c*, *f*, and *i* (arrows). The neuronal nature of the labeled cells is confirmed by the presence of MAP2 (*b*) and α -internexin (*h*) and by the lack of labeling with the glial-specific marker GFAP (*e*). The phase contrast photographs in *a* and *g* show that peripherin is expressed in a subset of neurons (arrows). Bar, 10 μ m.

at 60°C, and 30-s polymerization at 72°C. 15 μ l of the reaction mixture was analyzed on a 2.5% agarose gel and visualized with UV after ethidium bromide staining.

Results

Peripherin Is Not Expressed in Hippocampal Neurons Co-cultured with Glial or Epithelial Cells

Hippocampal cells were transferred to dishes containing a semiconfluent monolayer of glial cells (predominantly astrocytes) 4 h after plating. After 2–5 d in this environment, the cells were fixed and the presence of various IF proteins was assessed by immunofluorescence microscopy (Fig. 1). Using specific mABs, we could demonstrate that stage 2 and stage 3 hippocampal cells (Dotti et al., 1988) contained vimentin, α -internexin, and the neurofilament light (NFL) subunit. The pattern of the immunostaining was filamentous and extended throughout the cell bodies, the axons and the dendrites. In contrast to this, screening with two specific anti-peripherin antibodies (Djabali et al., 1991; Escurat et al., 1990) revealed the complete absence of peripherin in the same cells, independent of their state of differentiation. Similar results were obtained upon coculture of hippocampal neurons with epithelial (MDCK) cells. Consistently, immunohistochemistry on sections of embryonic (E18) tissue showed that peripherin is not expressed in hippocampal neurons in situ (data not shown). The lack of immunostaining was not due to the fixation method used, since paraformaldehyde, methanol, and ethanol-acetone fixation of the cells yielded the same results. Furthermore, peripherin mRNA could not be detected in hippocampal neurons after reverse transcription (RT) of total RNA and amplification with the polymerase chain reaction (RT-PCR) (see below).

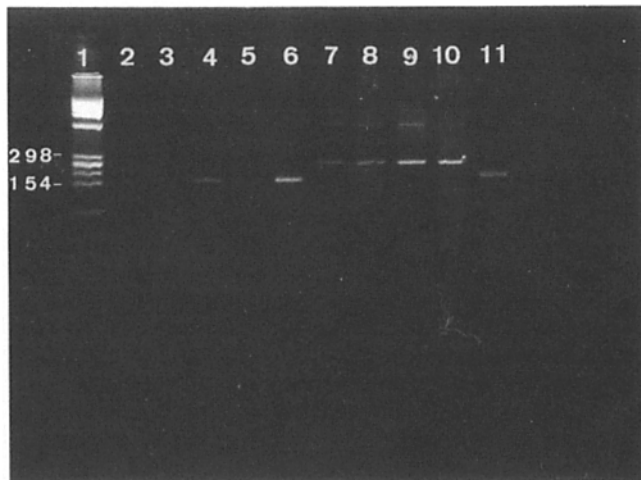


Figure 3. Detection of peripherin (lanes 3–6) and rab8 (lanes 7–10) transcripts in muscle-exposed (lanes 4, 6, 8, and 10) and untreated (lanes 3, 5, 7, and 9) hippocampal neurons. cDNA was amplified 30 (lanes 3 and 4 and 7 and 8) and 35 (lanes 5 and 6 and 9 and 10) cycles in the presence of specific oligonucleotides (see Materials and Methods). Lane 1 shows the DNA molecular-weight markers. Lane 11 corresponds to amplified peripherin mRNA from neuroblastoma cells.

Peripherin Accumulates and Assembles *de novo* in Cultured Hippocampal Cells Growing in Skeletal Muscle-conditioned Medium

The effect of soluble factors derived from muscle cells on the expression of intermediate filament proteins in hippocampal neurons was examined next. After 4 h of plating, hippocampal

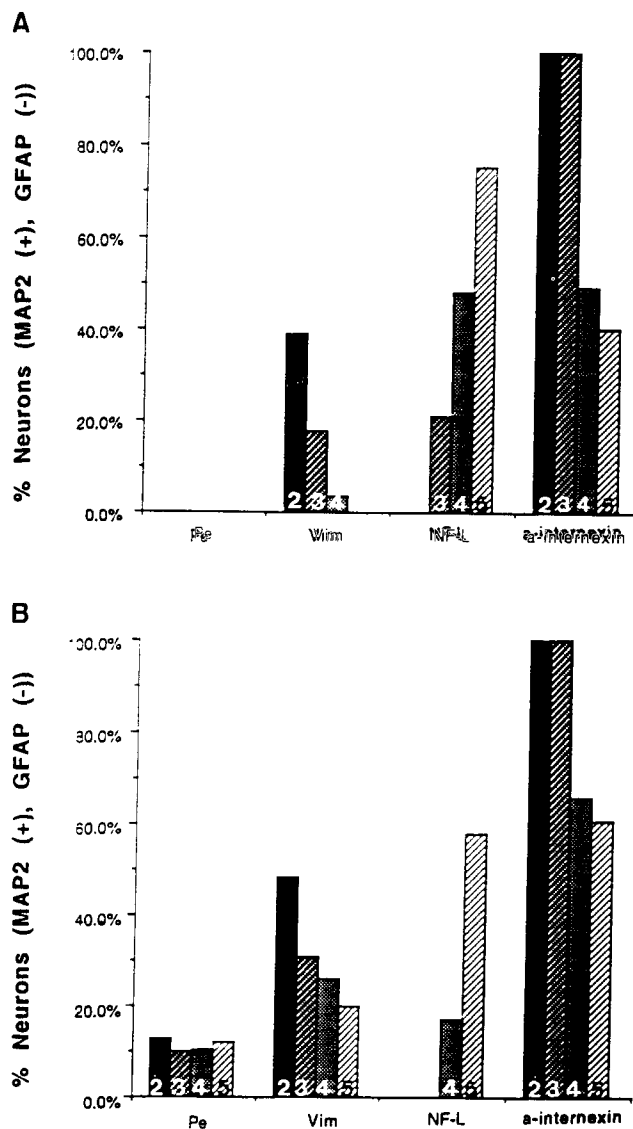


Figure 4. Quantitative analysis of intermediate filament expression in hippocampal neurons growing in the presence of astrocytes (A) or muscle (B) cells. All percentages refer to numbers of MAP2 (specific neuronal marker) positive cells. (A) Hippocampal neurons fixed between days 1–5 in glia co-culture. Peripherin is absent at all times. Vimentin is maximally expressed in the first 2 d, and then slowly disappears. NFL is absent during the first 48 h in culture and then gradually appears. α -internexin is present in all cells immediately after plating but then, decreases. (B) Hippocampal neurons fixed after 1–5 d in C₂C₁₂ co-culture. Peripherin is already evident after 48 h of co-culture in ~10% of the neurons. This level of expression does not change with time. Vimentin expression decreases more slowly than in astrocytes conditioned medium. The expression of NFL and α -internexin are not modified by co-culture with the muscle cells.

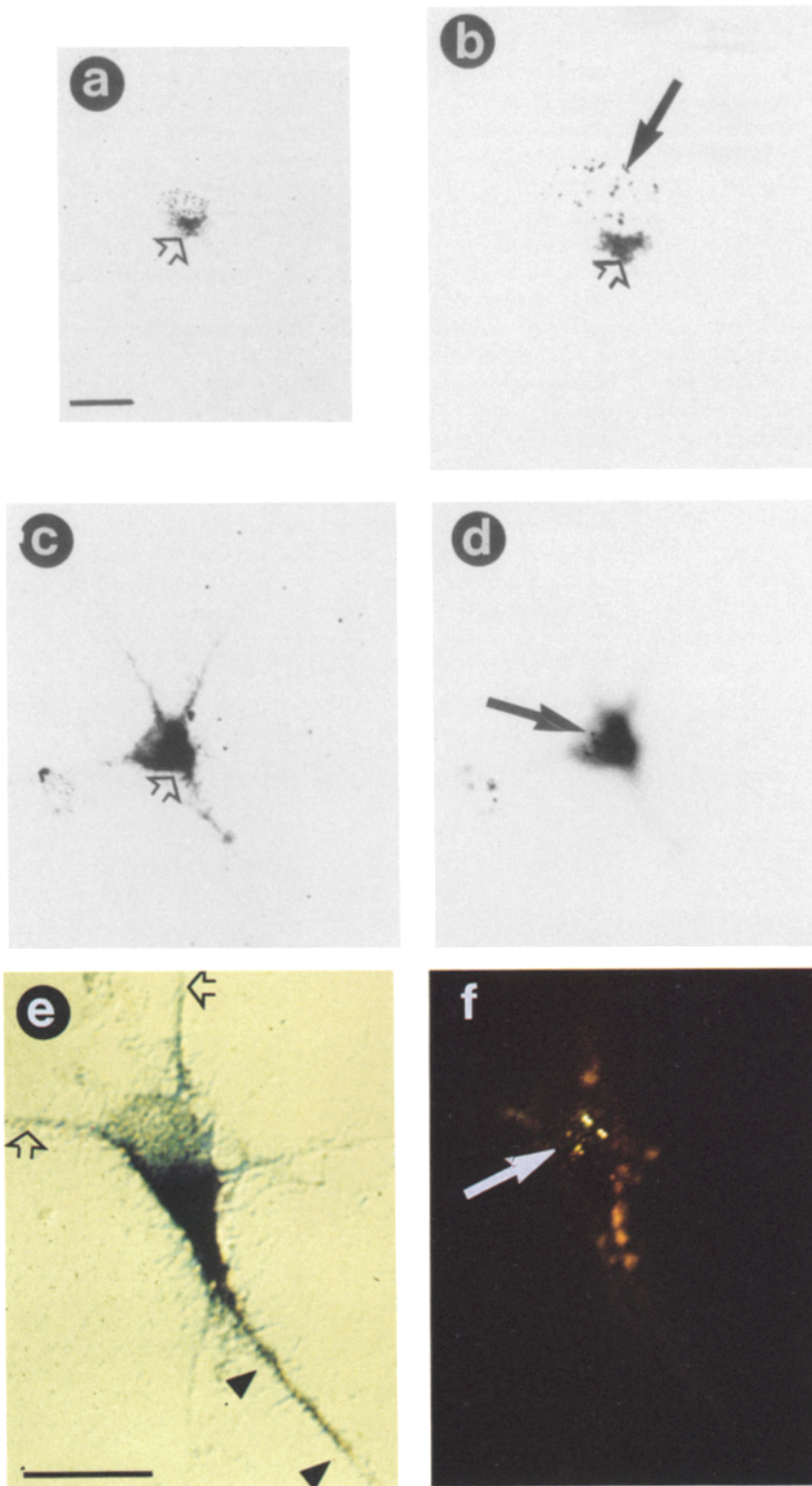


Figure 5. Peripherin expression occurs in early postmitotic hippocampal neurons. [^3H]Thymidine was injected to day 17.5 pregnant rats to label mitotic cells. Hippocampi were dissected on day 18 and the dissociated cells cocultured with muscle cells 4 h after plating. Cells were fixed after 4 d in culture. The presence of radioactive grains in the nuclei reveals the neurons which underwent mitosis in vivo before culture (*b, d, and f, arrows*). Such labeled cells show peripherin immunoreactivity (*a-c, open arrowheads*). Each pair of photograph represents a different developmental stage of the hippocampal neurons: stage 1 (*a and b*), stage 2 (*c and d*), and stage 3 (*e and f*). The black arrowheads in *e* point to the only significantly labeled process of this cell. The minor processes are not labeled (*empty arrowheads*). Bars, 10 μm .

pal cells were transferred to dishes containing a monolayer of skeletal muscle myotubes from the cell line C_2C_{12} (Yaffe and Saxel, 1977) in serum-free medium and fixed 2–5-d later. Under these conditions, a significant number of hippocampal neurons showed peripherin labeling after 48 h of coculture (Fig. 2, *a-c*). The labeling appeared filamentous and it remained even after detergent extraction before fixa-

tion, suggesting that the expressed peripherin had assembled into filaments. The pattern of peripherin labeling was not exactly the same in all neurons, even in cells with similar degree of morphological differentiation. Thus, in some cells peripherin labeling appeared restricted to the perinuclear region and in other cells the labeling filled the cell body and processes (not shown). Although not thoroughly inves-

tigated, this asymmetric distribution of peripherin suggests vectorial assembly, as previously shown for some IF proteins (Albers and Fuchs, 1987; Plancha et al., 1989; Dent et al., 1992; see however Georgatos, 1993).

The specificity of the peripherin-positive immunostaining could be confirmed by showing that the labeling is completely abolished when the anti-peripherin antibodies are pre-absorbed against purified peripherin (not shown). The induction of peripherin mRNA could also be confirmed by RT-PCR of total RNA isolated from neurons cocultured with muscle cells (Fig. 3). Co-culture with primary cultures of myotubes (Daniels, 1990) or with the skeletal muscle cell line L6 (Iacovitti et al., 1989) gave similar results. The induction and accumulation of peripherin was specific to hippocampal neurons, as demonstrated by double staining with antibodies against peripherin and the neuron-specific marker MAP 2 (Fig. 2). Corroborating this, the vast majority of peripherin-expressing neurons were stained with anti α -internexin antibodies (Fig. 2). However, the distribution of peripherin did not coincide with the distribution of α -internexin: in certain cells where peripherin showed a predominantly perinuclear distribution, α -internexin was preferentially axonal; in other cells α -internexin filaments appeared evenly distributed, whereas peripherin was more concentrated around the nuclei or in some of the minor processes (data not shown). All of the peripherin positive cells were free of the glial fibrillary acidic protein (GFAP) (Fig. 2, *d-f*). Finally, non-neuronal cells (3T3 fibroblasts) co-cultured with myotubes were peripherin negative (not shown).

A quantitative analysis of the influence of glial or muscular factors on the pattern of expression of various IF proteins in hippocampal neurons is shown in Fig. 4. Virtually none of the hippocampal cells showed peripherin expression after co-culture with astrocytes cells. However, \sim 8-13% of the neurons were positive in the hippocampal-muscle cell co-culture. All of these cells were vimentin negative. Although not as dramatic, vimentin expression was also affected by the environment. Whereas vimentin disappeared from all neurons after 5 d of co-culture with glial cells, 20% of the neurons were vimentin-positive in the muscle-neuron co-culture. The level of expression of NFL was not changed by culture conditions. NFL was first detected in 20% of the neurons after 3 d in culture and maximum expression occurred after 5-7 d. Finally, α -internexin did not appear to be environmentally regulated. This protein was present in all neurons immediately after plating and disappeared after 4 d in culture.

Early Postmitotic Neurons Are More Responsive to Environmental Cues

The wave of hippocampal neurogenesis begins on E16 and extends into early postnatal life (Angevine, 1965; Altman and Das, 1965; Schlessinger, 1978; Bayer, 1980). Thus, at the time the hippocampi are removed from the fetal brains (E18) to prepare the cultures, cells which originated at different times are present. To analyze the relationship between the peripherin-expressing neurons and the time of neurogenesis, a pregnant rat was injected intraperitoneally with [3 H]thymidine on day 17.5 and hippocampal cells were prepared on day 18. After allowing attachment for 4 h, the cells were transferred to dishes containing muscle cells for 48-

96 h, and then fixed and processed for immunohistochemistry and autoradiography. Analysis of the cells by phase-contrast and bright-field microscopy showed that, from a total of 701 neurons, 8.8% ($n = 62$) contained [3 H]thymidine and 10.6% ($n = 75$) were peripherin positive. All 62 [3 H]thymidine-labeled neurons were peripherin positive (examples of such cells are shown in Fig. 5). The interpretation we offer for these results is as follows. First, judging from the morphological profile of the [3 H]thymidine-labeled neurons, we conclude that these cells must have had their last cycle of division in the 12-h period before culturing. If the cells had divided during the culturing, they would not have differentiated to this extent and would certainly have occurred in pairs of daughter cells as shown previously (Fletcher and Banker, 1989). Second, the small percentage of peripherin-expressing cells that did not contain [3 H]thymidine had probably completed their last division shortly before day 17.5 but were still "plastic" enough to be induced by the muscle cells. Finally, the remaining of the cells that did not express peripherin may constitute a population irreversibly committed to the true, peripherin-free, hippocampal phenotype before the co-culture.

Peripherin Induction in AtT20 Cells

Under appropriate culture conditions, the neuroendocrine

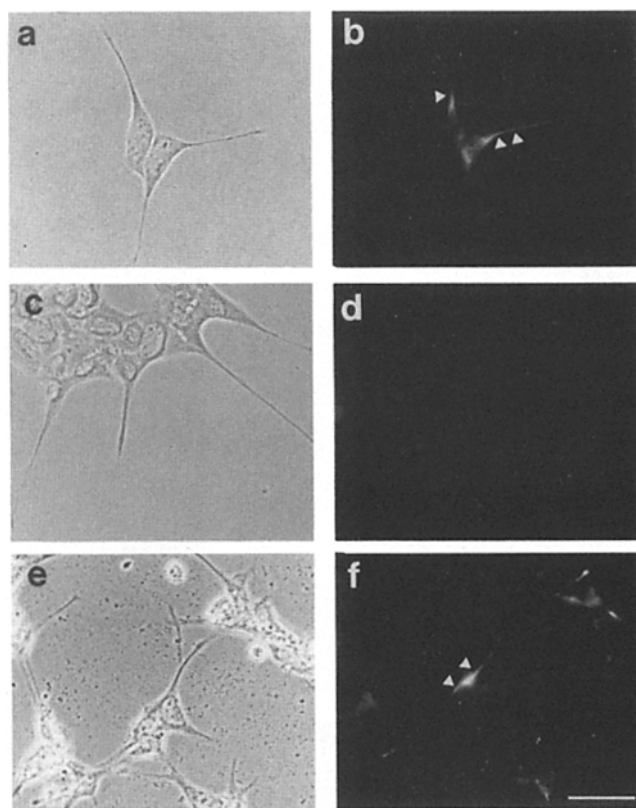


Figure 6. Peripherin is expressed in AtT20 cells co-cultured with muscle cells. AtT20 cells induced to develop neuritelike processes in serum-free, AtT20-conditioned medium for 4 d (*a-d*) express NFL (*b*, arrowheads) but not peripherin (*d*). After 4 d in the presence of C₂C₁₂ myotubes (*e* and *f*) peripherin expression is induced (*f*, arrowheads). Note in *f* that only some cells are peripherin positive.

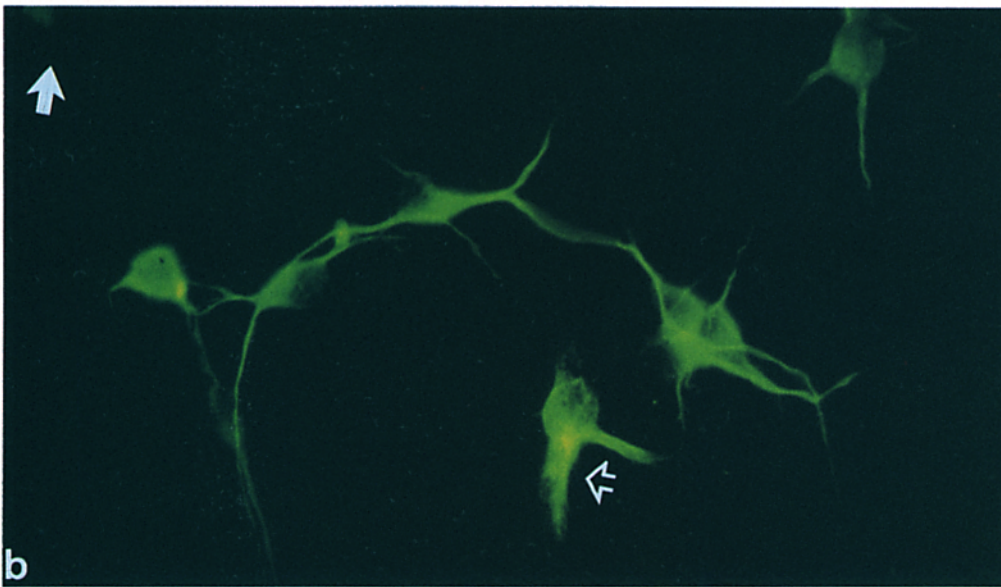
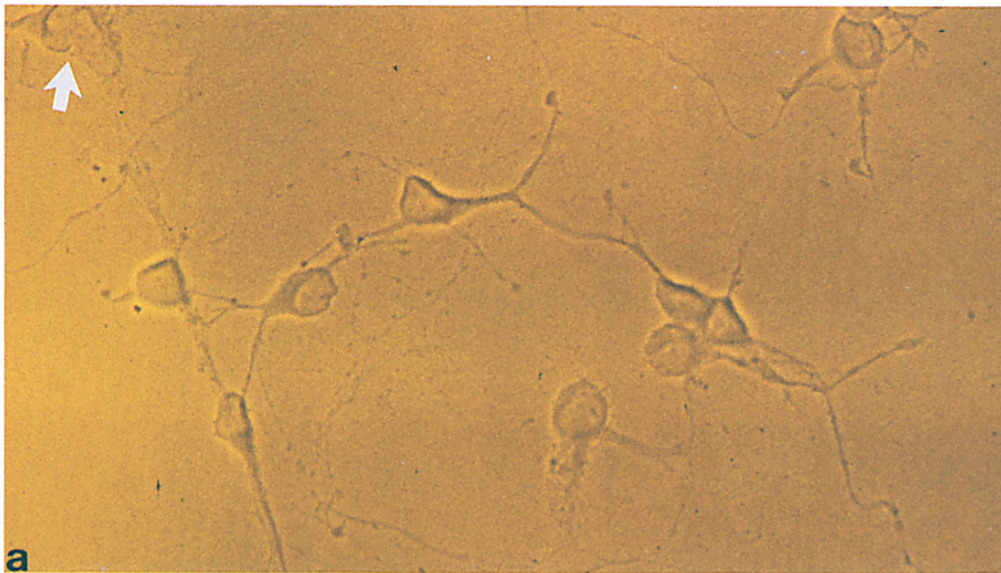


Figure 7. LIF induces peripherin expression in hippocampal neurons in culture. Cells were grown in neuronal medium supplemented with LIF for 4 d. (a) Phase-contrast photograph showing several stage 2 and 3 neurons as well as one glial cell (*arrow*). (b) The anti-MAP 2 antibody labels all the neurons but not the glial cell (*arrow*). (c) The anti-peripherin antibody labels only one neuron (*b* and *c*, *arrowheads*). Bar, 10 μm .

AtT20 cells develop some morphological and molecular characteristics of neurons (Tooze et al., 1989). Since these cells can be induced to acquire neuronal properties, we analyzed whether the muscle cells could induce the expression of peripherin. AtT20 cells were allowed to develop neurites by culturing them in serum-free, AtT20-conditioned medium for 2–4 d, and then fixed and processed for the immunofluorescence microscopy. The result is shown in Fig. 6. Fixation of differentiated AtT20 cells grown for 4 d in AtT20 medium revealed strong immunoreactivity for NFL but not for peripherin (Fig. 6, *a–d*). However, when AtT20 cells were induced to differentiate by coculturing with myotubes and fixed after 4 d, 20% of the cells showed positive peripherin immunoreactivity (Fig. 6, *e–f*). The staining had a filamentous appearance. The expression of peripherin mRNA in the AtT20 cells was also confirmed by RT-PCR (not shown). Since AtT20 is an immortalized cell line, the higher percentage of peripherin-positive cells compared to that of hippocampal neurons can be explained by their higher mitotic rate. Alternatively, the AtT20 cultures may contain cells which respond to a different degree to various environmental factors. We have made no attempts to differentiate between these possibilities in the present study.

Cholinergic Neuronal Differentiation Factor/Leukemia Inhibitory Factor Induces Expression of Peripherin in Neurons and AtT20 Cells

Numerous muscle-derived factors induce the expression and/or switching of neurotransmitters in different neuronal populations (Yamamori et al., 1989; McManaman et al., 1988; Saadat et al., 1989). One of these factors, the CDF or LIF (Yamamori et al., 1989), appeared to be a likely candidate in mediating the induction of peripherin because it fulfills several criteria. Firstly, it has a clear effect in changing neuronal phenotype in sympathetic neurons (Yamamori et al., 1989). Second, it is produced and secreted by skeletal muscle cells (Patterson and Fann, 1992). Third, it binds and undergoes retrograde transport in neuronal cells (Hendry et al., 1992). Thus, in the last series of experiments we tested whether CDF/LIF could induce peripherin expression in hippocampal and AtT20 cells. Addition of purified LIF to hippocampal cells, growing in neuronal medium alone or cocultured with glial cells, induced peripherin expression (Fig. 7). Quantitatively, the effect of LIF on hippocampal cells was comparable to the effect of the neuron muscle cell co-culture and showed a concentration dependence. Doses of 5 nM induced peripherin expression in few cells, while maximum expression occurred at doses of 50–100 nM. In AtT20 cells, addition of 50–100 nM LIF induced peripherin expression in more than 20% of the cells (Fig. 8). The effect of LIF/CDF on AtT20 cells was confirmed at the mRNA level by using RT/PCR (Fig. 9).

Discussion

Hippocampal neurons differentiate, *in vivo* and *in vitro*, without expressing the intermediate filament protein peripherin. We changed this phenotype by exposing cultured hippocampal neurons to muscle cells or their soluble factors. This condition induces peripherin expression. Although muscle-derived factors are known to change neurotransmit-

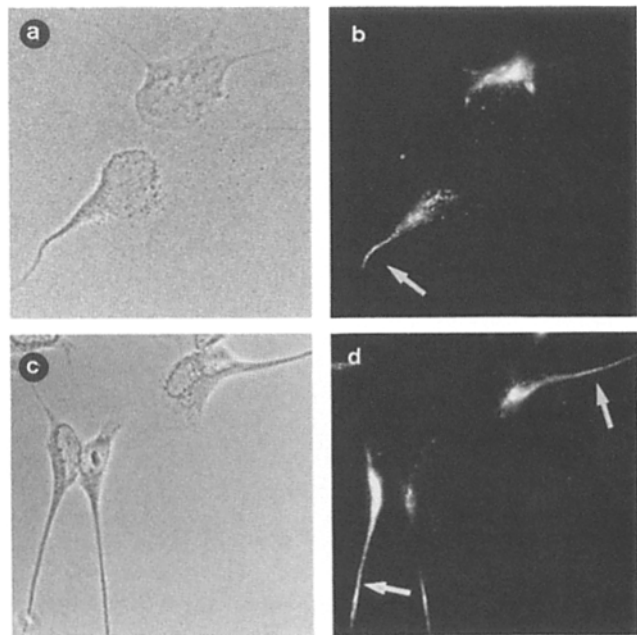


Figure 8. LIF induces peripherin expression in AtT20 cells. Two sets of AtT20 cells treated with 5 nM LIF (*top*) and 100 nM (*bottom*) for 4 d, fixed, and immunoreacted with anti-peripherin antibodies. Note in *b* and *d* the filamentous appearance of the labeling (*arrows*).

ter phenotype, our results show for the first time that neuronal plasticity also extends to cytoskeletal proteins.

Only a subset (8–13%) of the hippocampal neurons can be induced to express peripherin. The differential response to extracellular stimuli may be related to the developmental stage of the neurons. Indeed, our data suggest that early post-

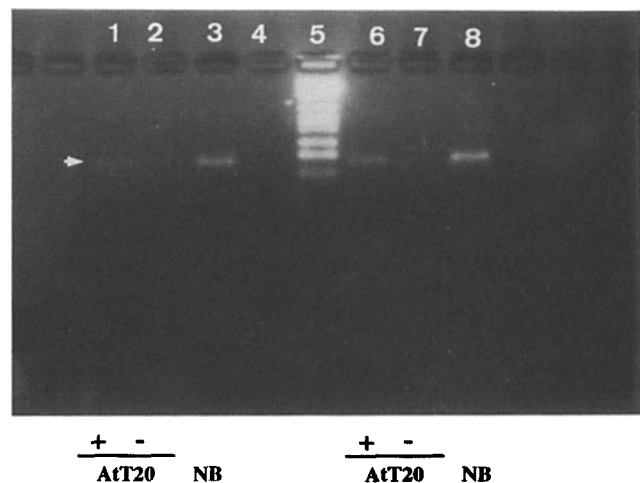


Figure 9. Detection of peripherin transcripts in total RNA from AtT20 cells grown in LIF/CDF (lanes 1 and 6) or in control medium (lanes 2 and 7). cDNA made from 20 ng of total RNA was amplified for 26 (lanes 1–3) and 30 (lanes 6–8) cycles. Lanes 3 and 8 show amplification with peripherin-specific primers of cDNA from neuroblastoma cells. In lane 4 no DNA was loaded. Arrowhead: signal derived from the peripherin mRNA.

mitotic neurons were the most susceptible to environmental influences since all cells which had undergone their last cycle of mitosis in the 12-h period before culturing (as indicated by the presence of [³H]thymidine in their nuclei) were induced to express peripherin. That the earliest postmitotic neurons were more likely to change their phenotype was also suggested by other experiments showing that a 24-h delay in transferring the neurons to myotube-containing dishes failed to induce peripherin expression (K. Djabali, S. D. Georgatos, and C. Dotti, unpublished observations). It should be mentioned, however, that some of the peripherin-positive cells did not contain any [³H]thymidine. These cells may have been in their last mitotic cycle shortly before the injection of [³H]thymidine, being therefore more committed than the thymidine-positive population, but still susceptible to the influences of the environment.

It may be argued that a small number of peripherin-expressing cells already exist in embryonal hippocampal tissue but their survival depends on factors elaborated by target cells. Thus, the coculturing of neurons with muscle cells may reflect specific survival of peripherin-expressing neurons rather than induction of nonexpressing cells. Such a specific survival has been documented in several cases including neuronal (Oppenheim et al., 1991), myeloid (Williams et al., 1990), and endocrine (Wyllie et al., 1980) cells. We find this possibility highly unlikely, since peripherin could not be detected by any method, including the most sensitive techniques currently available (as for example RT-PCR) in the starting cultures and in hippocampal tissue. Furthermore, the percentage of neurons that seem to be inducible exactly corresponds to the percentage of cells which undergo their last division between days 17.5 and 18 in situ (Fletcher et al., 1989). Nevertheless, since we could not precisely measure the degree of induction (due to the limited amount of material), we do not yet know whether trace quantities of peripherin, undetectable by the set of techniques available and therefore much lower than its critical concentration, already occur in noninduced neurons. In our opinion, even this remote scenario does not change the fact that peripherin accumulates in a level higher than its critical concentration and assembles in a filamentous network only after appropriate stimulation by the target cells.

Several important questions remain to be investigated in future work, in particular the nature of the inductive factor(s) and the role of peripherin during the development of central nervous system neurons. Regarding the trophic factor(s) involved, it is known that different substances can determine the expression of different neurotransmitters and neuropeptides (Adler et al., 1989; Nawa and Patterson, 1990; Nawa et al., 1991). We show that one of the best-characterized factors, cholinergic differentiation factor/leukemia inhibitory factor, is able to mimic the inductive effect of muscle-derived factors. Furthermore, how peripherin expression in hippocampal neurons affects their development has to be further analyzed. We and others have previously established that under standard culture conditions hippocampal neurons follow a stereotyped sequence of developmental steps which lead to the acquisition of morphological and molecular polarization (Caceres et al., 1984, 1986; Shaw et al., 1985; Banker and Waxman, 1988; Dotti et al., 1988, Dotti and Banker, 1991; Van Loockeren Campagne et al., 1992). It has been suggested that peripherin expression is important during the ini-

tial stages of axonal growth and during the regeneration that takes place after axotomy (Oblinger et al., 1989; Troy et al., 1990; Wong and Oblinger, 1990). Since the expression of peripherin involves activation of the endogenous gene, very much like in peripheral axons, the muscle-neuron co-culture can be exploited to test if the hippocampal cells benefit from expressing peripherin and whether they elongate or regenerate similarly to peripheral axons.

This work is dedicated to Adamantia and Stavros Politis.

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