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A neuropeptide precursor in cerebellum: proenkephalin exists in subpopulations of both neurons and astrocytes

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The adult rat cerebellum has minimal enkephalin immunoreactivity and is devoid of opiate-binding activity. Using novel monoclonal antibodies to the mammalian enkephalin precursor, we describe the immunofluorescent detection of proenkephalin, in the absence of mature enkephalin peptides, in subpopulations of rat cerebellar neurons and astrocytes. In cryostat sections, neurons that express proenkephalin include Golgi cells, macroneurons within deep cerebellar nuclei and a subpopulation of Purkinje cells. Proenkephalin messenger RNA and protein are present in subpopulations of both grey and white matter astrocytes, but not Bergmann glia. In dissociated glial culture, proenkephalin is expressed in process-bearing astrocytes, apparently in association with a subset of intermediate filaments. Proenkephalin within astrocytes is not seen until the second postnatal week and increases through to adulthood. Neuropeptide gene expression adds to the growing range of neuronal-type properties glial cells can display.

Key words: glia/*in situ* hybridization/intermediate filaments/monoclonal antibodies/opioid gene

Introduction

The biological significance of the mammalian enkephalin precursor, proenkephalin A, has remained elusive owing to a lack of antibodies that would enable its reliable identification. Complete proteolytic processing of proenkephalin proceeds via a succession of intermediates to liberate the small enkephalin peptides Met- and Leu-enkephalin, together with Met-enkephalin-Arg^6-Gly^7-Leu^8 and Met-enkephalin-Arg^6-Phe^7. In contrast, incomplete processing at selected cleavage sites generates an array of extended opioid and non-opioid products, for which biological roles have also yet to be established (for review see Eiden, 1987). Antibodies to the small enkephalin peptides exhibit minimal or no cross-reactivity with the full-length precursor, presumably due to the conformation of the intact molecule before proteolytic cleavage. Immunohistochemical studies in particular have been restricted therefore to a search for small enkephalin peptides, and assays for the high mol. wt forms have in general required chromatographic separation followed by trypsinization to liberate the smaller peptides.

The availability of cloned cDNAs from different mammalian species (Comb *et al.*, 1982; Legon *et al.*, 1982; Noda *et al.*, 1982; Yoshikawa *et al.*, 1984) has revealed a high degree of sequence homology in both opioid and non-opioid regions of the molecule, indicating evolutionary conservation, and thereby possible biological significance, of the intact precursor. Furthermore, regional differences in the proteolytic cleavage of proenkephalin within brain (Birch and Christie, 1986) may indicate that high mol. wt proenkephalin-derived peptides, previously regarded as mere processing intermediates, may be acting as neuropeptides in their own right.

We have generated a novel series of monoclonal antibodies (Mabs) to human proenkephalin-β-galactosidase fusion proteins, synthesized in *Escherichia coli* (Spruce *et al.*, 1988; this manuscript). We are now using these Mabs to examine the distribution of enkephalin precursor peptides within brain. We were interested to examine cerebellum, since data included in a study from Pittius *et al.* (1985) indicated a discrepancy between significant levels of proenkephalin mRNA but negligible amounts of immunoreactive mature enkephalin peptides in bovine cerebellum. One could interpret this as evidence for reduced proteolytic processing leading to a predominance of enkephalin precursor peptides. We were also interested to determine whether glial cells might express the proenkephalin molecule since two previous studies have described preproenkephalin mRNA in cultured astrocytes (Vilijn *et al.*, 1988; Shinoda *et al.*, 1989). In this work we describe the detection of proenkephalin mRNA and protein, in the absence of mature enkephalin peptides, in cerebellar neuronal and astroglial cells of young adult rat.

Results

Monoclonal antibodies to proenkephalin

We have previously described two Mabs, PE-1 and PE-2, which we generated to a central segment of proenkephalin (Spruce *et al.*, 1988). We have now obtained a new series of Mabs, all of which display strong reactivity against the enkephalin precursor, by immunization with a β-galactosidase fusion protein incorporating the entire sequence of human preproenkephalin [PPE-(1-267); Figure 1]. Hybridoma supernatants were screened by immunoblotting against another chimeric peptide comprised of preproenkephalin fused to 11 amino acids of the T7 phage capsid protein gene 10 (see Materials and methods and Spruce *et al.*, 1988). Anti-β-galactosidase antibodies could therefore be disregarded. We wished then to determine if the antibodies would recognize intact proenkephalin. We had recently generated a stable line of rat phaeochromocytoma PC12 cells transformed with human proenkephalin cDNA (PC12::hPE) (B.A.Spruce, D.Cutler and D.M.Glover, submitted). These cells express high levels of a 36 kd protein corresponding

to the uncleaved human enkephalin precursor (lane 2 in Figure 2A), which is not present in untransformed PC12 cells.

Six of our newly generated Mabs elicit an identical immunofluorescent staining pattern of rat cerebellum; in this paper we show immunofluorescence data using two representative Mabs, PE-18 and PE-25, which belong to subclasses IgG2a and IgG1 respectively. Preliminary epitope mapping of PE-18 and PE-25 has been carried out by immunoblotting against a series of β-galactosidase fusion proteins incorporating overlapping regions of proenkephalin primary sequence, including those shown in Figure 1 [PPE-(69−207); PPE-(1−131); PPE-(130−267)]. This experiment indicates that both Mabs recognize a central region of the molecule between amino acids 130 and 207, common to three of the four fusion proteins in this blot. Figure 2(A) illustrates the capacity of PE-18 and PE-25 to recognize intact proenkephalin. This contrasts with one of our earlier antibodies, PE-1, which displays considerably weaker recognition of the intact prohormone, despite its ability to recognize a subset of high mol. wt cleavage products in bovine adrenomedullary chromaffin granules (shown in lanes 2 and 1 respectively). At this stage, we cannot comment with certainty on the relative ability of PE-18 and PE-25 to recognize primary cleavage products since they do not cross-react with the bovine protein (lanes 3, Figure 2B) and cannot therefore be tested against bovine chromaffin granules. (Rat adrenal is, by contrast, a poor source of proenkephalin-derived peptides.) What we can say, however, is that PE-18 and PE-25 appear significantly better able to recognize the intact enkephalin precursor than PE-1, which is none the less able to recognize high mol. wt cleavage products. We therefore provisionally interpret the presence of PE-1 immunoreactivity, indicating the existence of high mol. wt cleavage products, as suggestive of limited proteolytic processing. In contrast, the absence of PE-1 immunoreactivity but presence of immunoreactivity with PE-18 and PE-25, we interpret as suggestive of a predominance of uncleaved precursor.

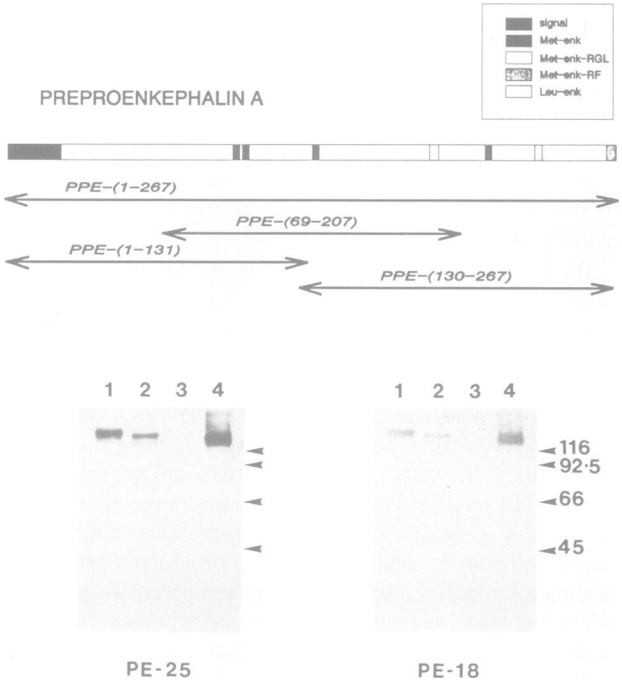

Fig. 1. Human preproenkephalin−β-galactosidase fusion proteins used for immunization and epitope mapping. The primary sequence of mammalian preproenkephalin A is illustrated in the upper panel. The fusion protein β-galactosidase−PPE-(1−267), incorporating the entire 267 amino acid sequence of preproenkephalin, was used for immunization. Preliminary epitope mapping of the Mabs PE-18 and PE-25 by immunoblot analysis of the purified fusion proteins β-galactosidase−PPE-(1−267), −PPE-(69−207), −PPE-(1−131) and −PPE-(130−267) is shown in the lower panel (**lanes 1−4** respectively).

Proenkephalin in cerebellum

In this study we chose to look at the possible existence of proenkephalin in cerebellum because of a previously reported discrepancy between preproenkephalin mRNA levels and levels of mature enkephalin peptides in this region of brain (Pittius *et al.*, 1985). We subjected cryostat sections of cerebella from 25 and 40 day old rats to indirect immunofluorescence using our PE Mabs and conventional Met- and Leu-enkephalin polyclonal antisera. The latter antisera would be expected to cross-react with mature enkephalin peptides only. We observed identical staining of subsets of cerebellar neuronal and astroglial cells with a total of six of our monoclonal antibodies. We could see no significant staining of any of these cells using the Met- and Leu-enkephalin antisera, indicating an absence of small enkephalin peptides.

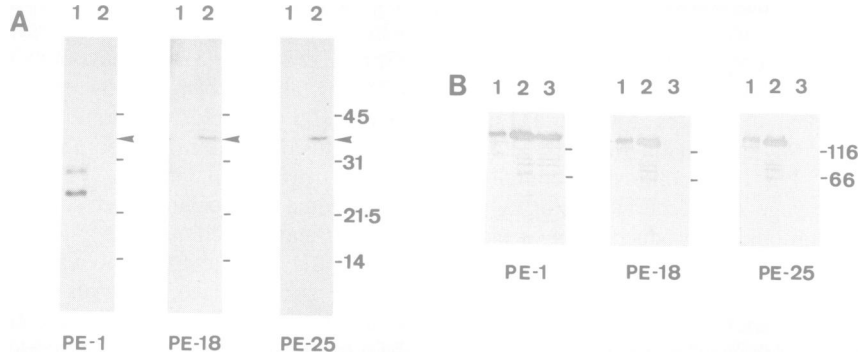

Fig. 2. (**A**) Differential recognition of intact enkephalin precursor. Bovine adrenomedullary chromaffin granule lysates (**lanes 1**) and whole-cell lysates of PC12::hPE transformants (**lanes 2**) were immunoblotted against the PE Mabs. PE-18 and PE-25 exhibit much stronger recognition of intact proenkephalin (migrating as a 36 kd band in lanes 2) than PE-1 which does, however, recognize high mol. wt proenkephalin-derived peptides in bovine chromaffin granules (lane 1, first strip). (**B**) Cross-species reactivity of the PE Mabs. Human, rat and bovine proenkephalin expressed as β-galactosidase fusion proteins (**lanes 1−3** respectively) were immunoblotted against the PE Mabs. PE-1 recognizes all three fusion peptides whereas PE-18 and PE-25 cross-react with rat and not bovine proenkephalin.

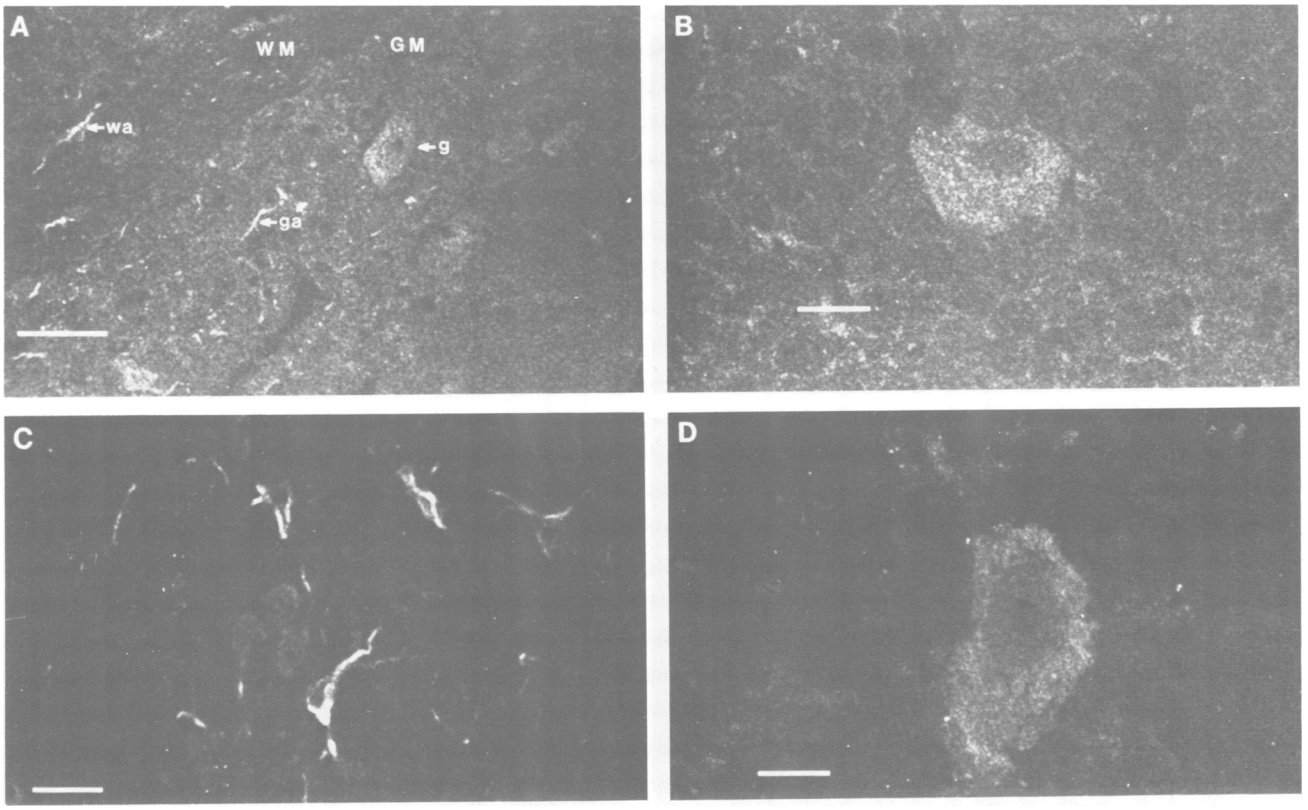

Fig. 3. Proenkephalin-like immunofluorescence in cerebellar neuronal and astroglial cells (confocal micrographs). Cerebellar cryostat sections from 25 day old (**A**,**B**) and 40 day old (**C**,**D**) rats were incubated with PE-25, followed by FITC conjugated goat anti-mouse immunoglobulin. (**A**) White (wa) and grey (ga) matter astrocytes in a folia white matter tract (WM) and the granule cell layer (GM) are indicated. A brightly labelled Golgi cell (g) is also shown. (**B**) Another Golgi cell under higher magnification. (**C**) Central white matter astrocytes showing proenkephalin-like immunofluorescence. (**D**) A macroneuron in a deep cerebellar nucleus labelled with PE Mab. Scale bars are 25 μm (A) and 10 μm (B–D).

The enkephalin antisera did, however, elicit strong staining of the caudate putamen and globus pallidus, regions rich in mature enkephalins, thereby proving their immunohistochemical capability (data not shown). With the Mab PE-1 we observed staining of neuronal cells, indicating the presence of high mol. wt cleavage products and thereby suggesting that at least limited proteolytic processing is occurring in these cells. However, PE-1 did not stain astrocytes, suggesting that the uncleaved precursor predominates in these cells and that there is little or no processing. We incubated control sections with PE Mab which had been preabsorbed with purified proenkephalin–β-galactosidase fusion protein, under which conditions no astrocytic or neuronal staining was observed.

Figure 3(A) and (B) shows bright punctate staining of a cell type resembling Golgi cells, inhibitory interneurons, with PE Mab. From the numbers of stained cells observed we estimate that the entire Golgi cell population may be expressing proenkephalin. Figure 3(D) shows proenkephalin staining of a macroneuron within a deep cerebellar nucleus, a region where Purkinje cell axons terminate. A subset of the Purkinje cells themselves also show staining. These are seen at the boundary between the molecular and granule cell layers (Figure 4A). A brightly stained cell, 'p', is flanked by non-labelled cells, showing that only a subpopulation of Purkinje cells express proenkephalin.

Six of our anti-proenkephalin Mabs stain astrocytes in both grey and white matter, as in Figure 3(A) (ga and wa respectively). White matter astrocytes showing proenkephalin-like immunofluorescence are present throughout the cerebellar white matter tracts, although they are more numerous in the central white matter (Figure 3C). All these cells co-express glial fibrillary acidic protein (GFAP), a major component of astrocytic intermediate filaments, confirming their identity as astrocytes (Figure 4). This figure demonstrates that the proenkephalin-expressing glia comprise only a subpopulation of both the grey (Figure 4A and B) and white (Figure 4C) matter astrocytes. At low magnification the quality and distribution of the proenkephalin stain is very similar to the intermediate filament stain as revealed by the GFAP antibody. However, at higher magnification differential staining can be observed. Figure 4(D) shows such a cell where there is an apparently different distribution of PE and GFAP, suggesting localization with subsets of filaments which may be less rich in GFAP. This is a confocal micrograph, and therefore one is comparing staining at an identical optical plane. Preliminary data from other brain regions indicates that proenkephalin-expressing astrocytes are not unique to the cerebellum, since we find them in hypothalamus and striatum also.

In order to confirm proenkephalin gene expression in the cerebellum, we performed *in situ* hybridization upon sections from 25 day old animals using a ^{35}S-labelled rat proenkephalin antisense riboprobe, combined with a GFAP immunostain. We were unable to perform simultaneous immunostaining for proenkephalin which did not withstand the rigorous hybridization treatments. A sense riboprobe was used as a negative control. After 8 days exposure we observed clusters of grains in locations consistent with neuronal expression. Clusters of grains in white matter

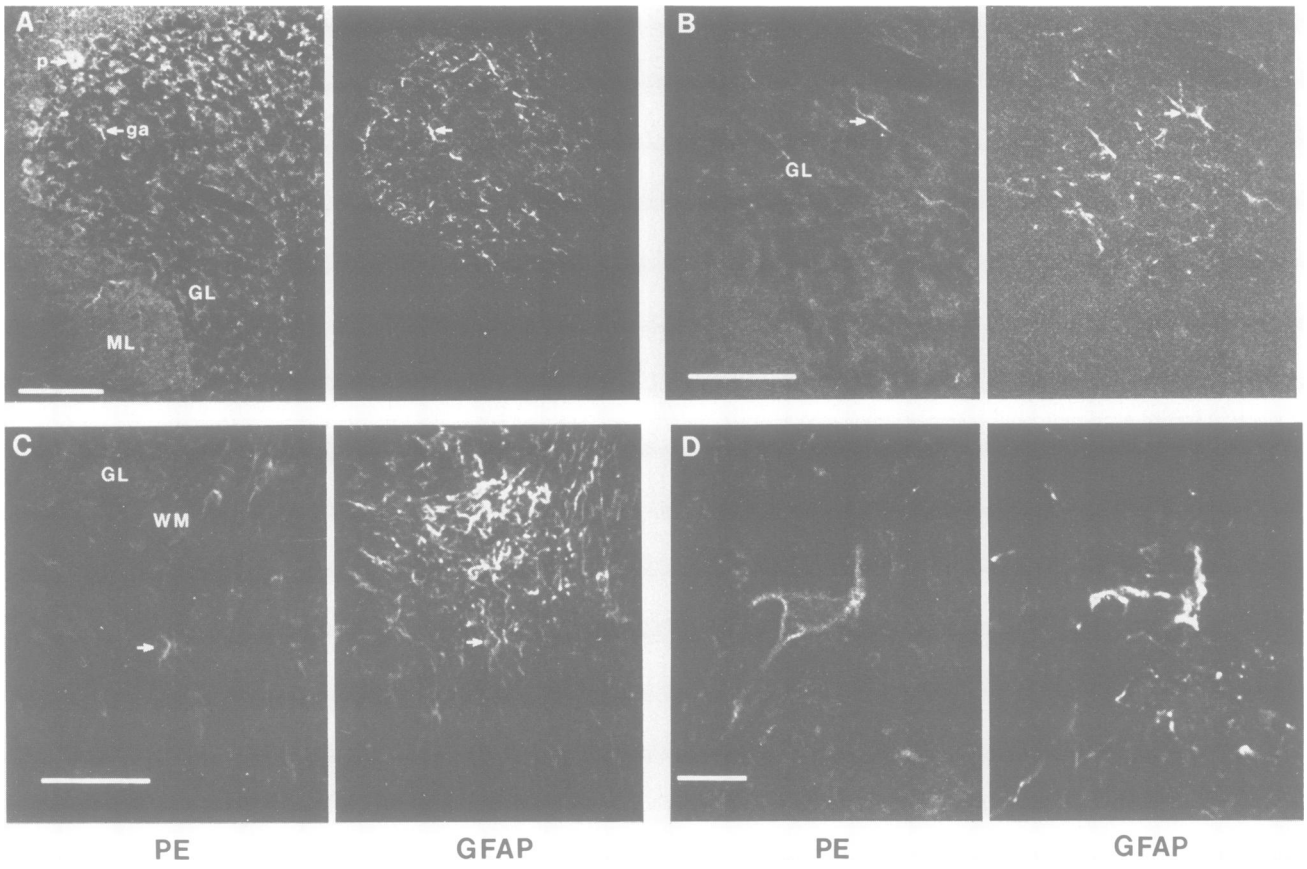

Fig. 4. Proenkephalin expression in subpopulations of grey and white matter astrocytes (confocal micrographs). Cerebellar cryostat sections from 25 day old (**A**–**C**) and 40 day old (**D**) rats were dual labelled with either PE-18 (**A**,**B**) or PE-25 (**C**,**D**), and anti-GFAP antiserum, followed by FITC-conjugated goat anti-mouse immunoglobulin and Texas Red-conjugated goat anti-rabbit immunoglobulin. Proenkephalin immunofluorescence (fluorescein) and GFAP fluorescence (Texas red) are shown in the left- and right-hand panels respectively, each pair representing an identical optical plane. (**A**) A brightly labelled Purkinje cell (p) is indicated at the boundary between the molecular (ML) and granule cell (GL) layers. A grey matter astrocyte showing PE immunofluorescence is also shown (ga), with the corresponding cell visible in the GFAP fluorescence field. (**B**) Another grey matter astrocyte labelled with PE Mab (arrow), under higher magnification. (**C**) An astrocyte showing PE immunofluorescence in central white matter is indicated by the arrow. (**D**) A white matter astrocyte at higher magnification, showing differential PE and GFAP immunofluorescence. (A) and (C) illustrate that glia expressing proenkephalin comprise only a subpopulation of grey and white matter astrocytes. Scale bars are 100 μm (A), 50 μm (B,C) and 10 μm (D).

locations did not become apparent until between 2 and 5 weeks of exposure (Figure 5). Each panel of Figure 5 depicts GFAP immunofluorescence alone (left) alongside a combined bright field/fluorescence exposure. In Figure 5(A) a rim of grain clusters is seen at the boundary between the molecular (ML) and granule cell layers (GL), compatible with expression of proenkephalin by Purkinje cells. Clusters of grains in the granule cell layer (Figure 5A and B) may be over either Golgi cells or grey matter astrocytes. The presence of proenkephalin mRNA in astrocytes is confirmed by clusters of grains clearly situated in folial white matter tracts, where only glial cells exist, as in Figure 5(B). The grain clusters obscure the penetration of the fluorescence signal and so give an apparent negative image on the GFAP fluorescence field. However, where the grains are less dense, it is possible to discern more clearly a signal over astrocytes. Figure 5(C) shows such an astrocyte in cerebellar white matter. Proenkephalin mRNA in astrocytes in other brain regions is also observed, as in the hypothalamic astrocyte shown in Figure 5(D). However, clear dual labelling cannot be achieved due to impedance of the fluorescence signal by the silver grains. Instead, confirmation of expression is provided by the location of grain clusters.

Proenkephalin expression by cerebellar astrocytes in culture

In order to determine whether the subpopulation of astrocytes expressing proenkephalin correspond to a particular type, we established primary cultures of cerebellar astrocytes from 8 day old rats. Cerebellar astrocytes in culture have previously been demonstrated to exhibit essentially two morphologies—irregularly epithelioid (flat) and stellate (process-bearing) (Wilkin *et al.*, 1983). The relationship of these morphological types to the type 1 and type 2 astrocyte lineage cells of optic nerve (Raff *et al.*, 1983a,b) is not wholly clear.

We observed proenkephalin-like immunofluorescence in a subpopulation of astrocytes from 3 days in culture which became maximal between 9 and 12 days. Dual-labelling with anti-GFAP showed that all the PE-positive cells are also GFAP positive, confirming their identity as astrocytes and not oligodendrocytes or neurons. In culture conditions where the cells are well separated, astrocytes expressing proenkephalin are stellate (process-bearing) in appearance (as in Figure 6). However, when the cells are more confluent, the PE-positive cells are flatter in appearance and not so typically stellate (as in Figure 7). This tendency of

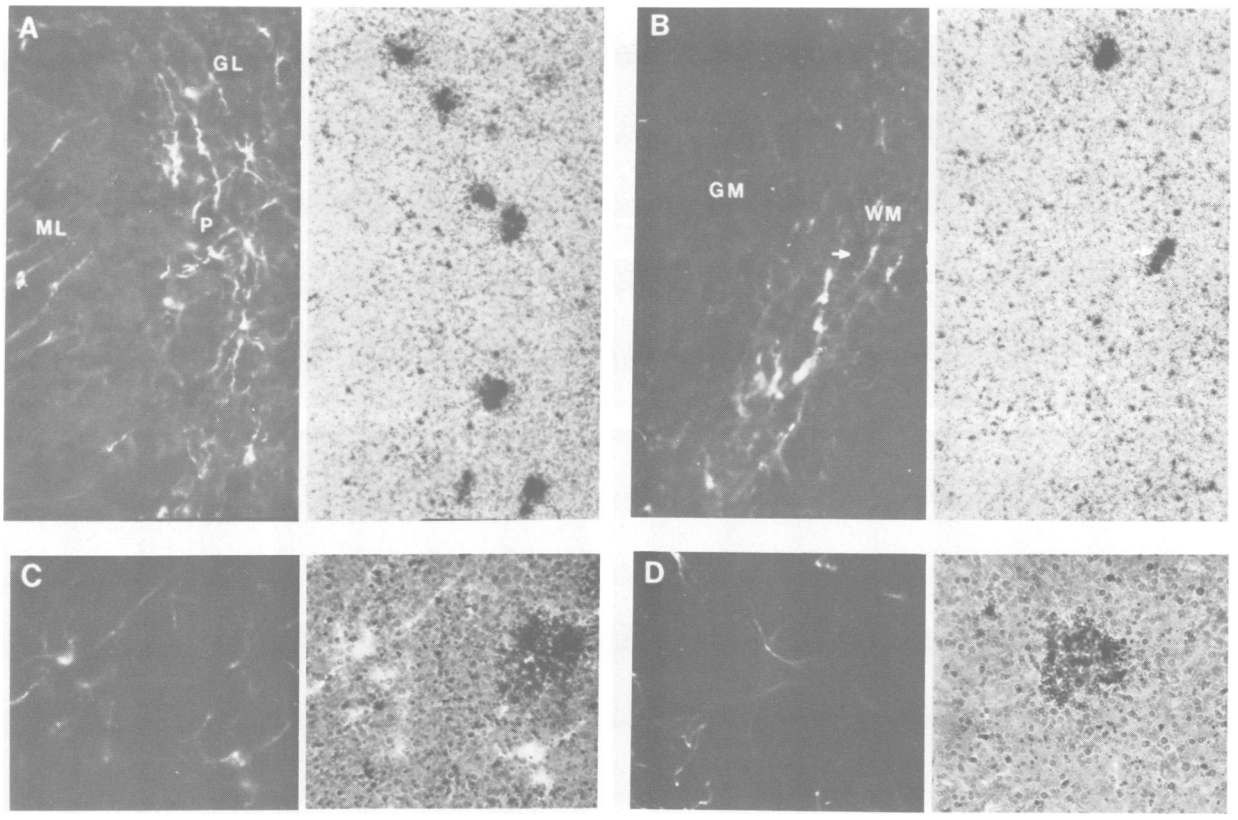

Fig. 5. Confirmation of proenkephalin synthesis by *in situ* hybridization. Cryostat sections from 25 day old rats were incubated with a ^{35}S-labelled rat proenkephalin anti-sense riboprobe, followed by a GFAP immunostain and exposed for 5 weeks. Left-hand panels show GFAP fluorescence; right-hand panels show combined bright-field and GFAP fluorescence. (**A**) A rim of grain clusters in the Purkinje cell layer (P) at the boundary between the molecular (ML) and granule cell (GL) layers. A more deeply situated cluster may be over a Golgi cell or a grey matter astrocyte. (**B**) A cluster of grains in a folia white matter tract (WM) is indicated by the arrow. (**C**,**D**) At higher magnification, clusters of grains over a cerebellar white matter astrocytes (C) and a hypothalamic astrocyte (D) are shown.

stellate astrocytes in cerebellar culture to assume a more epithelioid appearance in confluent conditions, possibly due to cell−cell contact, has been documented previously (Wilkin *et al.*, 1983). More than 95% of the proenkephalin-expressing cells co-express the surface ganglioside GD_3, as detected with the Mab LB_1 (Figure 6E and F), which has previously been demonstrated on a subset of astrocytes (Levi *et al.*, 1986).

The proportion of astrocytes which express proenkephalin, and the magnitude of expression, increase with time. Proenkephalin expression is first seen in occasional cells after 3 days *in vitro* (from postnatal day 8 animals); between 6 days and 8 days in culture, the proportion of GD_3-positive process-bearing cells that express proenkephalin rises from 10−20% to 30−40%. At all stages examined there are some stellate astrocytes that do not express proenkephalin (as in Figure 7D).

Generally speaking, the proenkephalin immunostain in astrocytes is fibrous in nature and is frequently stronger in some processes than in others (Figure 6A−C). In addition, a coarse punctate stain can sometimes be seen over the nucleus, together with a bright rim of perinuclear staining (as Figure 6A inset; same cell as in the main picture in a different optical plane). Figure 6(C) and (D) shows a cell dual labelled with PE Mab (C) and anti-Met-enkephalin (D), confirming the absence of Met-enkephalin immunoreactivity.

Many of the proenkephalin-expressing cells display a striking axial concentration of the molecule (as in Figure 6B). This is also apparent in cells dual labelled with PE Mab and anti-GFAP (Figure 7A−C). Overall, our impression from the quality and distribution of the stain is that proenkephalin appears to be co-localizing with subsets of intermediate filaments. We have also looked for microtubule association using a rat monoclonal anti-tubulin antibody (YL1/2) (not shown). We find that microtubules are diffusely scattered throughout the cell and there does not appear to be any enrichment coincident with the proenkephalin stain. However, it remains possible that proenkephalin is associated with a subset of microtubules, which may in turn be co-tracking with a subset of intermediate filaments.

Discussion

We were surprised at the extent and strength of the proenkephalin immunostaining in cerebellar neuronal and astroglial cells, given that previous workers have found little enkephalin immunostaining in this brain region, although enkephalin immunofluorescence of germinative cells in 10 day old rat cerebellum has been observed (Zagon *et al.*, 1985). This would be in keeping with the detection of opiate-binding activity only in the developing rat cerebellum (Tsang *et al.*, 1982). These previous studies suggest a role for small enkephalin peptides in cerebellar development. On the other hand, our results raise the interesting possibility that, in the

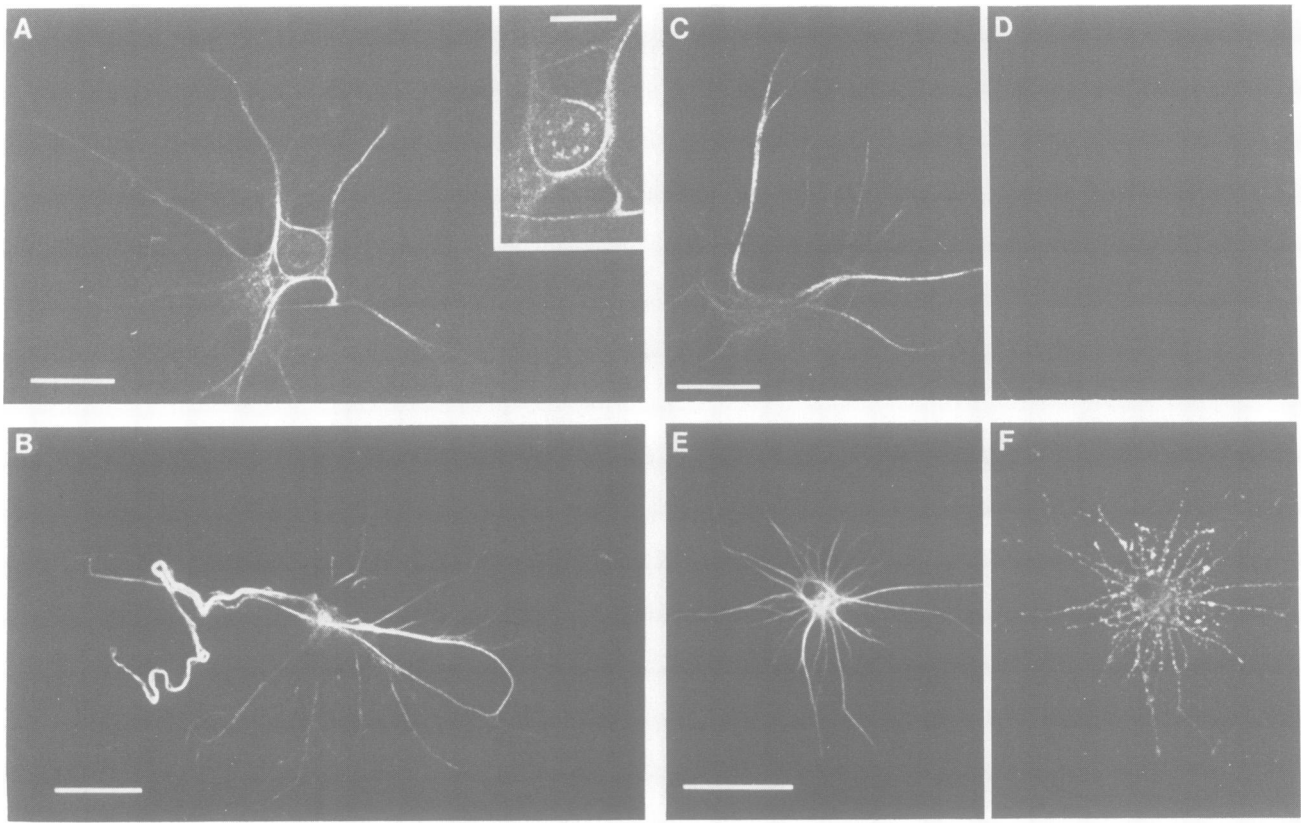

Fig. 6. Proenkephalin expression by process-bearing astrocytes in primary cerebellar glial cultures (confocal micrographs). Astrocyte cultures were established from postnatal day 8 rat cerebella. Cells were fixed and immunostained as described in Materials and methods, with conditions appropriate for the particular antigen. Cells shown were immunostained after either 6 or 9 days in culture. (**A**) A process-bearing cell single labelled with PE-25 followed by FITC conjugated goat anti-mouse immunoglobulin. The inset shows the nuclear region of the same cell at a different optical plane, under higher magnification. (**B**) Another process-bearing cell immunostained as above shows a striking axial concentration of proenkephalin. (**C**,**D**) A cell dual labelled with PE-18 (fluorescein) (**C**) and anti-Metenkephalin (Texas Red) (**D**) shows proenkephalin immunofluorescence only. (**E**,**F**) A cell dual labelled with PE-25 (fluorescein) (**C**) and LB_1 (Texas Red) (**F**) shows co-expression of proenkephalin and GD_3. Scale bars are 25, 12.5 μm (A, A inset), 25 μm (B–D) and 50 μm (E,F).

adult rat cerebellum, enkephalin precursor forms may have a non-opiate role, distinct from that of the enkephalin peptides themselves.

Enkephalin precursor forms in cerebellar neurons

We see Golgi cell fluorescence with several of our proenkephalin Mabs. Met-enkephalin-Arg^6-Gly^7-Leu^8 immunofluorescence in Golgi cells has been reported in association with γ-aminobutyric acid (GABA) (Ibuki *et al.*, 1988). Antibodies to Met-enkephalin-Arg^6-Gly^7-Leu^8 quite often cross-react with high mol. wt proenkephalin-derived peptides that terminate with this sequence. Therefore, this result is consistent with our own finding of PE-1 immunofluorescence, which together suggest the presence of high mol. wt cleavage products—indicating limited proteolytic processing—in Golgi cells. We also observed strong proenkephalin-like immunofluorescence in the somata of a subpopulation of Purkinje cells. Proenkephalin gene expression in these cells is confirmed by our *in situ* hybridization study (Figure 5). A previous *in situ* hybridization study on cerebellum did not detect proenkephalin mRNA within Purkinje cells (Shivers *et al.*, 1986). However, this discrepancy is now explained by our finding that only a subpopulation of Purkinje cells express the molecule. It is interesting that we also detect fluorescence in macroneurons within deep cerebellar nuclei upon which Purkinje cell axons terminate. This might suggest participation of proenkephalin and its high mol. wt cleavage products in the efferent arm of the cerebellar neuronal circuitry.

Proenkephalin in a subpopulation of astrocytes

In cerebellar sections, a subpopulation of grey and white matter astrocytes show strong proenkephalin-like immunofluorescence. We have performed a preliminary developmental profile which indicates that the staining first appears between postnatal days 5 and 10, becomes maximal between 3 and 4 weeks, and is maintained at 6 weeks. The proportion of glia expressing proenkephalin also increases with time. By 6 weeks, the majority of astrocytes in central white matter do express proenkephalin, although in folial white matter and in grey matter a smaller proportion of the total astrocyte population show expression. The temporal profile in dissociated culture (see Results) is consistent with that *in vivo*. Proenkephalin mRNA has been reported previously in cultures of astrocytes from different brain regions, including cerebellum (Vilijn *et al.*, 1988; Shinoda *et al.*, 1989), and also in the rat C6 glioma cell line (Yoshikawa and Sabol, 1986a). Our findings of proenkephalin in astrocytes, both in cryostat sections and in culture, confirm that these previous reports of proenkephalin gene expression in astrocytes are not due to a tissue culture artefact. One

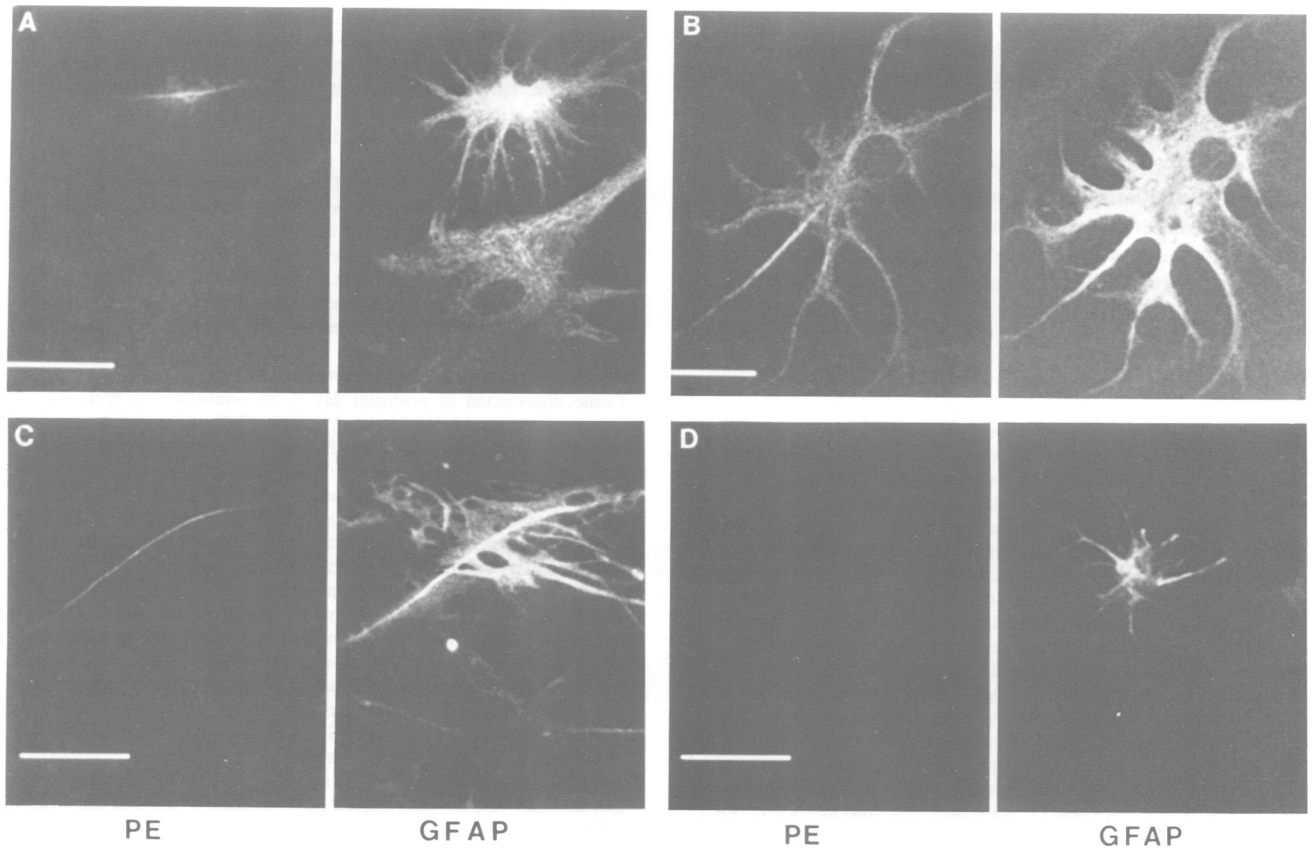

Fig. 7. Co-staining of astrocytes in primary culture with anti-proenkephalin monoclonal antibodies and anti-GFAP (confocal micrographs). Astrocytes in primary culture as in Figure 6 were dual labelled with PE-25 and anti-GFAP followed by FITC-conjugated goat anti-mouse and Texas Red-conjugated anti-rabbit immunoglobulins (fluorescein optics, left-hand panels; Texas Red, right-hand panels). These cells were at a higher density than in Figure 6 which can influence the morphological appearance (see text). (**A**) GFAP field shows examples of astrocytes with stellate-like (upper) and epithelioid-like (lower) morphology. Proenkephalin fluorescence is restricted to the cell with the stellate-like morphology. (**A**), (**B**) and (**C**) all show a striking concentration of proenkephalin stain along a longitudinal axis. (**D**) A process-bearing cell, displaying strong GFAP fluorescence but absent (left hand panel) proenkephalin fluorescence. Scale bars are 50 μm (A,C,D) and 25 μm (B).

apparent difference, however, is that we find that astrocytes express proenkephalin relatively late in development. Nevertheless, although these previous studies analysed cultures prepared from embryonic and early postnatal rats, in both cases analysis of mRNA was not carried out until after 3–4 weeks in culture. Since astrocytes have been shown to be capable of developing *in vitro* on the same schedule as *in vivo* (Williams *et al.*, 1985), the effective developmental stage of these cultures will have been much later.

Proenkephalin-positive glia in subconfluent culture exhibit a stellate phenotype, all express GFAP and >95% co-express the surface ganglioside GD_3 as detected with the Mab LB_1. Thus, they appear to comprise a distinct subpopulation of astrocytes. However, astrocyte classification is controversial, and reliable, universally accepted surface antigen markers for the respective lineages are not available.

The distribution and nature of the proenkephalin stain in the cultured astrocytes strongly suggests co-localization with a subset of intermediate filaments. We were somewhat surprised at the diffuse, fibrous nature of the stain. However, since this is a cell type not known to possess classical storage granules (for a review of the ultrastructure of fibrous astrocytes, see Peters *et al.*, 1976), there are no immediately obvious candidate organelles for the packaging and transport of such a molecule. Nevertheless, the apparently diffuse distribution of proenkephalin along intermediate filaments at the light microscopic level does not exclude an intravesicular localization. The only previously described intermediate filament associated protein in astrocytes, a 48 kd molecule of unknown identity, also has an apparently continuous distribution along the filaments at the light microscope level. However, at the ultrastructural level the molecule is concentrated alongside the filaments in pockets in electron-dense regions, which is in contrast to the uniform distribution of the integral filament protein, GFAP (Abd-el-Basset *et al.*, 1989). Unlike proenkephalin, however, all filaments are stained rather than a subset. A particularly unusual feature of the intracellular distribution of proenkephalin is the sometimes striking axial concentration of the molecule, possibly generated by association with subsets of filaments. The purpose of this longitudinal localization is at this stage very difficult to predict.

Concluding remarks

There has been accumulating evidence that astrocytes may possess neuronal-type properties. For example, they can bind tetanus toxin (Raff *et al.*, 1983a), and they exhibit 'neuron-like' GABA transport (Johnstone *et al.*, 1986). Furthermore, they display a complex ion channel phenotype (Barres *et al.*, 1988), and there has been recent demonstration of glutamate-

activated ion channels in type 2 cerebellar astrocytes in culture (Usowicz *et al.*, 1989). Neuropeptide gene expression therefore provides a further dimension to the remarkable hybrid mixture of neuronal and glial properties displayed in this cell type.

Although neuropeptide genes were among the first to be cloned, the relevance of the encoded propeptide precursor molecules has remained unclear. The elucidation of the role of proenkephalin within astrocytes, together with an ultrastructural analysis of its subcellular distribution, will pose an interesting challenge.

Materials and methods

Production and screening of monoclonal antibodies

All the human proenkephalin−β-galactosidase fusion proteins used for immunization and epitope mapping have been described previously (Figure 1 and Spruce *et al.*, 1988). BALB/c mice were immunized with 10−20 μg each of electroeluted β-galactosidase−PPE-(1−267) at 4−8 weekly intervals. Animals required a total of 6−8 injections before a clear serum response was observed, when a successful fusion ensued.

The method for hybridoma fusion was essentially as described by Mole and Lane (1987). Screening of hybridoma supernatants was performed by immunoblotting against a chimeric construct consisting of the full-length preproenkephalin sequence fused to 11 amino acids of the bacteriophage T7 capsid protein gene 10. *Bam*HI linkers were added to the *Hin*dII PPE-(1−267) fragment, excess linkers were removed by *Bam*HI digestion, then the fragment cloned into *Bam*HI cleaved pAR 3039, a kind gift from Dr Studier (Studier and Moffat, 1986). Preparation of immunoblotted material for use in screening of supernatants has been described previously (Spruce *et al.*, 1988). A total of 13 new PE Mabs were generated in this way, PE-13 to PE-25.

Generation of rat and bovine proenkephalin fusion proteins

Constructs analogous to the previously described β-galactosidase−human PPE-(69−207) (Spruce *et al.*, 1988) were made. This fragment binds all of our new Mabs. Central *Pst*I fragments from rat and bovine proenkephalin cDNAs were cloned into the vector pUR 291. Rat preproenkephalin cDNA in pRPE2 was a generous gift from Dr S.Sabol (Yoshikawa *et al.*, 1984). Bovine preproenkephalin cDNA was kindly provided by Dr U.Guebler (Hoffman la Roche Inc.). These constructs were used to check cross-species reactivities of our Mabs.

Indirect immunofluorescence of cerebellar cryostat sections

Animals underwent transcardiac perfusion with 4% paraformaldehyde in PBS. Cerebella were dissected, placed in the same fixative for 3 h at room temperature, then transferred to 30% sucrose in PBS and left overnight at 4°C. Tissue was frozen in isopentane pre-chilled on dry ice. Sections of 5−10 μm were cut on a cryostat at −25°C. Aldehyde quenching was achieved by two 5 min soaks in sodium borohydride (0.5 mg/ml in PBS), followed by a blocking step with 10% FCS in PBS. Primary antibodies were as follows: PE-1, PE-18 and PE-25 (ascites fluid diluted 1/500 in 10% FCS in PBS); Met- and Leu-enkephalin polyclonal antisera (Immunonuclear Corporation) diluted 1/100 as above; polyclonal anti-GFAP antiserum (DAKO Ltd) diluted 1/500 as above. Secondary antibodies were FITC-conjugated F(ab')2 fragment goat anti-mouse IgG (heavy and light chain), with or without Texas Red conjugated F(ab')2 fragment goat anti-rabbit (heavy and light chain), both diluted 1/500 in 10% FCS in PBS. All second antibodies used throughout the study were from Jackson Immunoresearch. Primary antibody incubations were performed overnight, secondary incubations for 1−2 h at room temperature. After PBS washes, sections were mounted under coverslips in medium consisting of 2.5% DABCO (Sigma Chemical Corporation) in glycerol/PBS (9:1).

In situ hybridization

All procedures were carried out under strict RNase-free conditions. The vectors pYSEA 1 and pYSEC 1, which were further kind gifts from Dr S.Sabol (Yoshikawa *et al.*, 1986b), were used to generate anti-sense and sense RNA probes respectively. Template DNA was linearized with either *Sma*I (pYSEC) or *Sac*I (pYSEA). *In vitro* transcription reactions were performed in the presence of ^{35}S-labelled uridine triphosphate using SP6 polymerase. Limited hydrolysis to 50−200 bases was carried out, then the probe resuspended in 50% formamide, aiming for 5×10^5 counts per min/μl, and stored at −20°C until use.

Cryostat sections from paraformaldehyde-perfused animals were cut under RNase-free conditions onto poly-L-lysine-coated slides. The sections were subjected to limited permeabilization using 0.2 M HCl for 20 min at room temperature, but protease treatment had to be omitted to preserve GFAP antigenicity. After refixation in 4% paraformaldehyde, an acetylation step was performed (0.5% acetic anhydride in 0.1 M triethanolamine, pH 8), to reduce non-specific binding of the probe. Subsequent steps were performed as described by Ingham *et al.* (1985), with the exception that prior to the final dehydration step we performed a GFAP immunostain. Sections were refixed with 4% paraformaldehyde in PBS for 20 min at room temperature, followed by a sodium borohydride quenching step. After completion of immunostaining, as described in the previous section, the slides were finally dehydrated through an ethanol series in 0.3 M ammonium acetate, air dried, then dipped in Kodak NTB2 emulsion. Exposure time was 8 days−5 weeks.

Primary astrocyte cultures from cerebellum

Tissue dissociation of postnatal day 8 rat cerebella was performed as described previously (Wilkin *et al.*, 1983). Cells were plated on poly-L-lysine-coated coverslips in 24-well dishes at $1-2 \times 10^5$/well. Cultures were grown in DMEM containing 10% FCS. Optimal immunostaining—for PB Mabs, Met- and Leu-enkephalin, and GFAP antisera—was achieved by fixation with methanol then acetone (pre-chilled to −20°C, 4 min each). As a blocking step, cells were incubated in 10% FCS, 0.2% saponin in PBS for 1 h at room temperature. Primary antibody dilutions were identical to those used for cryostat sections. Incubation with primary antibody, diluted in blocking buffer, was carried out overnight at 4°C and followed by several washes in 1% BSA, 0.2% saponin in PBS. FITC-conjugated goat anti-mouse IgG (heavy and light chain), with or without Texas Red-conjugated goat anti-rabbit IgG (heavy and light chain), diluted 1/500 in blocking buffer, were then added for 1−2 h at room temperature, and followed by washes as above. After final rinses in PBS, coverslips were mounted over slides in medium as for cryostat sections.

For labelling of GD_3 on the cell surface, live cells were incubated with LB_1 antibody [ascites fluid diluted 1/500 in Earle's balanced salt solution (EBS) plus 5% normal goat serum] at room temperature for 30 min. Cells were washed with EBS plus 0.3% BSA, then incubated with class-specific second antibody [Texas Red goat anti-mouse IgM (mu chain) 1/500 in EBS plus 0.3% BSA] for 30 min at room temperature. These steps were followed by 4% paraformaldehyde fixation then brief (1 min) methanol permeabilization. Subsequent co-staining with PE Mabs was as described, except in the absence of saponin and using an anti-IgG specific second antibody [goat anti-mouse IgG(Fc)].

For dual labelling with PE and anti-tubulin antibodies (YL1/2 rat Mab supernatant from Seralabs, diluted 1/5), cells were methanol/acetone fixed, as described. Sequential antibody incubations were carried out thus: PE Mab followed by Texas Red-conjugated goat anti-mouse IgG (heavy and light chain); then YL1/2 followed by FITC-conjugated mouse anti-rat IgG (heavy and light chain).

Microscopy

All microscopy was performed using a Biorad/MRC Lasersharp Confocal microscope, apart from combined bright field and fluorescence work where a Nikon Microphot FX was used.

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Note added in proof

Since this manuscript was submitted, Melner *et al.* (*EMBO J.*, 1990 **9**, 791−796) have shown proenkephalin mRNA apparently in type-1-like but not in immature type-2-like astrocytes in culture. These workers also found

from chromatographic data that astrocytes contain significant amounts of unprocessed proenkephalin protein. We have found proenkephalin protein in a subpopulation of mature process-bearing astrocytes. However, until such time as reliable surface antigen markers for the respective astrocyte lineages exist, a firm classification cannot be assigned.

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