British Journal of Pharmacology (1997) 120, 509–515

Identification of 5-hydroxytryptamine receptors positively coupled to adenylyl cyclase in rat cultured astrocytes

[1]Warren D. Hirst, *Gary W. Price, †Marcus Rattray & Graham P. Wilkin

Biochemistry Department, Imperial College, London SW7 2AZ; *Psychiatry Research Department, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW and †UMDS Division of Biochemistry & Molecular Biology, Guy's Hospital, London SE1 9RT

1 5-Hydroxytryptamine (5-HT) elicited a dose-dependent stimulation of intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation in cultured astrocytes derived from neonatal rat (Sprague Dawley) thalamic/hypothalamic area with a potency (pEC_{50}) of 6.68 ± 0.08 (mean ± s.e.mean).

2 In order to characterize the 5-HT receptor responsible for the cyclic AMP accumulation the effects of a variety of compounds were investigated on basal cyclic AMP levels (agonists) and 5-carboxamidotryptamine (5-CT) stimulated cyclic AMP levels (antagonists). The rank order of potency for the agonists investigated was 5-CT ($pEC_{50} = 7.81 \pm 0.09$) > 5-methoxytryptamine (5-MeOT) ($pEC_{50} = 6.86 \pm 0.36$) > 5-HT ($pEC_{50} = 6.68 \pm 0.08$). The following compounds, at concentrations up to 10 μM, did not affect basal cyclic AMP levels 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), cisapride, sumatriptan, DOI and RU 24969. The rank order of potency of antagonists was methiothepin ($pK_i = 7.98 \pm 0.25$) > mesulergine ($pK_i = 7.58 \pm 0.18$) > ritanserin ($pK_i = 7.20 \pm 0.24$) > clozapine ($pK_i = 7.03 \pm 0.19$) > mianserin ($pK_i = 6.41 \pm 0.19$). The following compounds, at concentrations up to 10 μM, were inactive: ketanserin, WAY100635, GR127935. This pharmacological profile is consistent with that of 5-HT_7 receptor subtype-mediated effects.

3 The cultured astrocytes exhibited regional heterogeneity in the magnitude of cyclic AMP accumulation (E_{max}). Cells cultured from the thalamic/hypothalamic area had significantly higher E_{max} values ($588 \pm 75\%$ and $572 \pm 63\%$ of basal levels for 5-CT and 5-HT, respectively) compared to brainstem ($274 \pm 51\%$ and $318 \pm 46\%$, respectively) and colliculus astrocytes ($244 \pm 15\%$ and $301 \pm 24\%$, respectively). No significant differences in pEC_{50} (for either 5-HT or 5-CT) values were observed.

4 Reverse transcriptase-polymerase chain reaction (RT–PCR) with primers specific for the 5-HT_7 receptor confirmed expression of messenger RNA for this receptor subtype by the cultured astrocytes derived from all regions investigated. Primers specific for the 5-HT_6 receptor also amplified a cDNA fragment from the same samples.

5 From these findings, we conclude that astrocytes cultured from a number of brain regions express functional 5-HT receptors positively coupled to adenylyl cyclase and that the level of receptor expression or the efficiency of receptor coupling is regionally-dependent. The pharmacological profile of the receptor on thalamic/hypothalamic astrocytes suggests that the 5-HT_7 receptor is the dominant receptor that is functionally expressed even though astrocyte cultures have the capacity to express both 5-HT_6 and 5-HT_7 receptor messenger RNA.

Keywords: 5-HT_7 receptors; 5-HT_6 receptors; cyclic AMP accumulation; cultured astrocytes; 5-carboxamidotryptamine

Introduction

5-Hydroxytryptamine (5-HT) exerts a wide variety of behavioural and physiological effects through actions on multiple receptor subtypes. These receptors have been classified by operational, transductional and structural criteria into four distinct receptor classes (5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4), comprising ten receptor subtypes. Four additional recombinant receptors (5-HT_{5a}, 5-HT_{5b}, 5-HT_6 and 5-HT_7) provide strong evidence for the existence of three additional receptor classes (Hoyer *et al*., 1994; Hoyer & Martin, 1996). Three of the receptor subtypes, namely: 5-HT_4, 5-HT_6 and 5-HT_7 receptors are coupled to the stimulation of adenylyl cyclase. Despite sharing a common signal transduction mechanism these three receptors have unique and highly divergent amino acid sequences. The pharmacological profiles of these receptors are unique but consistent across species (Boess & Martin, 1994; Eglen *et al*., 1994).

The 5-HT_7 receptor has been cloned from several species including the rat (Lovenberg *et al*., 1993; Meyerhof *et al*., 1993; Ruat *et al*., 1993; Shen *et al*., 1993), mouse (Plassat *et al*., 1993) and man (Bard *et al*., 1993). Functional assays measuring adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation, have revealed the presence of receptors that may correspond to the 5-HT_7 receptor subtype in guinea-pig brain (Shenker *et al*., 1985; Tsou *et al*., 1994) and rat brain (Markstein *et al*., 1986; Fayolle *et al*., 1988). Several pharmacological studies have suggested that functional 5-HT_7 receptors are expressed by both intact peripheral tissues such as guinea-pig ileum (Carter *et al*., 1995), rabbit femoral vein (Martin & Wilson, 1994) and *Cynomolgus* monkey jugular vein (Leung *et al*., 1996) and by cultured cells derived from human vascular smooth muscle (Shoeffter *et al*., 1996).

Primary astrocyte cultures have been shown to express neurotransmitter receptors for amines, purines, amino acids and peptides (Kimelberg, 1995). There is evidence that stimulation of many of these receptors results in activation of second messenger systems affecting cyclic AMP, cyclic GMP, inositol phosphates and diacylglycerol levels (Kimbelberg, 1995). There is a controversey about the subtypes of 5-HT receptor expressed by astrocytes; this is largely due to the early studies on cultured astrocytes (Hertz *et al*., 1979; Tardy *et al*., 1982; Whitaker-Azmitia & Azmitia, 1986), preceding the development of selective 5-HT receptor ligands and the cloning of the multiple 5-HT receptor subtypes.

[1] Author for correspondence

The aim of the present study was to investigate whether primary astrocyte cultures derived from different brain areas express 5-HT receptors which are positively coupled to adenylyl cyclase and to characterize them in terms of their pharmacology and molecular biology.

Some of these data have been presented in abstract form (Hirst *et al*., 1996a,b).

Methods

Primary astrocyte cultures

Type-1 astrocyte enriched cultures were prepared as previously described (Marriott *et al*., 1995). Briefly, the brain areas of interest, namely the thalamic/hypothalamic area, cerebral cortex, brainstem, colliculus and cerebellum, were dissected from 2 day old Sprague-Dawley rat pups of either sex. Following trypsinization, mechanical chopping and trituration, the cells were collected and resuspended in DMEM supplemented with 10% foetal calf serum, 10000 units ml^{-1} penicillin, 10 mg ml^{-1} streptomycin and 25 μg ml^{-1} amphoteracin B. The cells were plated out at a density of 2×10^5 cells ml^{-1} in 24 well tissue culture plates and in 225 cm^2 flasks which had been pre-coated with poly-L-lysine (5 μg ml^{-1}). After 5 days *in vitro* contaminating fibroblasts were removed by the substitution of D-valine for L-valine, O-2A progenitor cells and microglia were removed by shaking. Immunocytochemical analysis has shown these cultures to comprise >95% glial fibrillary acidic protein (GFAP) positive astrocytes (Marriott *et al*., 1995). The primary astrocyte cultures were used for cyclic AMP assays or RNA extraction after 12 days in culture.

Cyclic AMP assays

Confluent cultures were changed to serum-free medium (DMEM and Ham's F-12, 1 : 1 v/v, supplemented with antibiotic/antimycotic, as above) 24 h before the assay, to exclude any serum components, particularly 5-HT, interfering with the assay. Intact cells were washed twice and preincubated in serum-free medium containing 500 μM isobutyl-1-methylxanthine (IBMX), 1 μM paroxetine, 10 μM pargyline and 1 μM ascorbate for 30 min at 37°C. The cells were exposed to various concentrations of agonist for 15 min at 37°C. To investigate the effect of antagonists on intracellular cyclic AMP levels, the astrocytes were first stimulated with 100 nM 5-carboxamidotryptamine (5-CT), a concentration which elicited a sub-maximal cyclic AMP accumulation. The cells were then incubated for 15 min at 37°C with increasing concentrations of each antagonist. The reactions were terminated by addition of 50 μl of 30% perchloric acid. Cells were solubilized and cyclic AMP was extracted into the aqueous phase of a 50 : 50 (v/v) mixture of trichlorofluoroethane and trioctylamine. Cyclic AMP levels were measured by an NEN cyclic AMP [^{125}I]-RIA Flashplate kit.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT–PCR)

Total RNA was extracted as described by Too and Maggio (1995). Briefly, cultured astrocytes were harvested in sterile 0.1 M phosphate buffered saline and pelleted by centrifugation at 3500 *g*. Cell pellets or tissue from adult rat cerebral cortex were homogenized in 4 M guanidium isothiocyanate buffer (10% w/v). Following the addition of an equal volume of chloroform : isobutanol (2 : 1) and vortex mixing, the solution was centrifuged for 5 min at 10,000 *g*. The supernatant was collected and an equal volume of 1% sarkosyl was added followed by an equal volume of phenol (pH 4.0) : chloroform (4 : 1). This was vortexed and centrifuged for 20 min at 14,000 *g*. The supernatant was removed, RNA was precipitated by the addition of 3 volumes of 100% ethanol and again centrifuged for 20 min at 12,000 *g*. Following a wash in 70% ethanol, the RNA pellet was air dried and resuspended in RNase and DNase free water.

Complementary DNA (cDNA) was generated by reverse transcription of 2–4 μg of total RNA. The reaction consisted of 5 mM $MgCl_2$, 80 u RNasin, 1 μg oligo (dT) 15 primers, 1 mM dNTP, 200 u M-MLV RT (Moloney murine leukemia virus reverse transcriptase) in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl and 10 mM dithiothreitol. The reaction mixture was incubated at 42°C for one hour and then at 95°C for 5 min.

Primers for the 5-HT_6 and 5-HT_7 receptors were designed by use of a computer programme (PC/GENE, Intelligenetics) and synthesized by Genosys (Cambridge, U.K.). Primer sequences (5′-3′) were as follows: CTCCTCCCGATCTCTTTGAAATCGC and TGTTCGAGCTTTGCCCAGTTCG corresponding to bases 289-312 and 938-959, respectively of the cloned rat 5-HT_6 receptor sequence (Accession No. L03202) (Monsma *et al*., 1993).

ATCTTCGGCCACTTCTTCTGCAACG and CAGCACAAACTCGGATCTCTCGGG corresponding to bases 569-593 and 1397-1420, respectively of the cloned rat 5-HT_7 receptor sequence (Accession No. L22558) (Lovenberg *et al*., 1993).

PCR was carried out in a reaction volume of 50 μl with a master mix containing 1.5 mM $MgCl_2$, 0.3 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.0% Triton X-100 and 1.25 u *Taq* polymerase. To this mixture the appropriate oligonucleotide primers were added (50 pmol) together with 5 μl of the RT (cDNA) product. The reaction was overlaid with two drops of mineral oil and after a 2 min denaturation at 96°C, 35 cycles of amplification were performed consisting of 1 min denaturation at 95°C, 1 min annealing at 56°C and 1.5 min primer extension at 72°C. This was followed by an extension at 72°C for 10 min. Amplified cDNA fragments were subjected to agarose gel electrophoresis and visualized by uv illumination in the presence of ethidium bromide. Amplified cDNA fragments were ligated into a PCRII cloning vector (Stratagene) and sequenced by an automated process carried out at the Advanced Biotechnology Research Centre (Charing Cross and Westminster Medical School).

Protein assay

Protein concentrations were determined by a BioRad protein assay kit (York, U.K.) with bovine serum albumin as a standard.

Data analysis

Concentration-response curves were analysed with Grafit (Erathicus Software Ltd.), with a four parameter logistic fit. Comparisons of the maximal response to 5-HT and 5-CT stimulation by astrocytes cultured from different brain regions were made by the Kruskall-Wallis test with *post-hoc* Mann-Whitney U-test and statistical significance was taken as $P < 0.05$.

Materials

5-Hydroxytryptamine (5-HT), 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), 5-methoxytryptamine (5-MeOT), isobutyl-1-methylxanthine (IBMX) and pargyline were purchased from Sigma Chemical Co. (Poole, U.K.). (±)-2,5-Dimethoxy-4-iodoamphetamine (DOI hydrochloride), clozapine, ritanserin and ketanserin were supplied by RBI (Natick, MA, U.S.A.). 5-Methoxy-3-(1,2,3,6-tetrahydro-4-pyridin-4-yl)-1H-indole (RU24969) was supplied by Roussel-UCLAF (Romainville, France). Paroxetine HCl, 5-carboxamidotryptamine (5-CT), sumatriptan, cisapride, methiothepin, mesulergine, mianserin, N-[2-[4-(2-[O-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexane trihydrochloride (WAY100635) and N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxidiazol-3-yl) [1,1-biphenyl]-4-carboxamide (GR127935) were synthesized at SmithKline Beecham.

Unless otherwise stated, all the reagents used for the RNA extraction and RT-PCR were obtained from Promega (Southampton, U.K.) or Merck-BDH (Lutterworth, U.K.).

Results

Effects of 5-HT receptor agonists and antagonists on cyclic AMP accumulation in thalamic/hypothalamic astrocytes

Basal levels of cyclic AMP in cultured astrocytes were 21.25 ± 1.34 pmol mg^{-1} protein ($n = 34$). 5-HT elicited a concentration-dependent increase in basal cyclic AMP levels with a pEC_{50} value of 6.68 ± 0.08 ($n = 5$). 5-CT and 5-methoxytryptamine (5-MeOT) were more potent than 5-HT ($pEC_{50} = 7.81 \pm 0.09$, $n = 5$ and 6.86 ± 0.36, $n = 4$, respectively) whereas 8-OH-DPAT, RU 24969, sumatriptan, DOI and cisapride, at concentrations up to 10 μM, did not significantly affect basal cyclic AMP levels in the cultured astrocytes (Figure 1, Table 1). Maximal effects of 5-HT and 5-CT on intracellular cyclic AMP accumulation were 572 ± 63 and 588 ± 75% of basal levels ($n = 8$), respectively.

The effects of 5-HT receptor antagonists were investigated with thalamic/hypothalamic astrocytes that had been exposed to a submaximal concentration of 5-CT (100 nM) (Figure 1, Table 1). Methiothepin, clozapine, ritanserin, mesulergine and mianserin fully reversed the 5-CT stimulated cyclic AMP levels (Figure 2). However, WAY100635, GR127935 and ketanserin were inactive up to 10 μM. The data from these experiments are summarized in Table 1.

Differences in 5-HT and 5-CT stimulated cyclic AMP accumulation by astrocytes cultured from different brain regions

5-HT and 5-CT consistently elicited a concentration-dependent accumulation of cyclic AMP in astrocytes cultured from the thalamic/hypothalamic area ($pEC_{50} = 6.52$ and 7.66, respectively), brainstem ($pEC_{50} = 6.48$ and 7.65, respectively) and colliculus ($pEC_{50} = 6.56$ and 7.23, respectively). There were no significant differences in pEC_{50} values for either 5-HT or 5-CT in the cells cultured from the different regions; 5-CT was consistently more potent than 5-HT (Table 2). However, the cultured astrocytes exhibited regional heterogeneity in the magnitude of cyclic AMP accumulation (E_{max}) (Table 2). Cells cultured from the thalamic/hypothalamic area had significantly higher E_{max} values (588 ± 75% and 572 ± 63% of basal levels for 5-CT and 5-HT, respectively) compared to brainstem (274 ± 51% and 318 ± 46%, respectively) and colliculus astrocytes (244 ± 15% and 301 ± 24%, respectively).

Astrocytes cultured from the cerebral cortex and cerebellum did not respond consistently to 5-HT or 5-CT stimulation. These cells responded to the agonists in two out of four experiments with mean pEC_{50} values of 6.45 and 6.74 for 5-HT and 5-CT respectively (mean $E_{max} = 206$% and 173%, respectively). Astrocytes from the cerebellum responded to 5-HT in

Table 1 Pharmacological profile of the 5-HT receptor positively coupled to adenylyl cyclase expressed by cultured astrocytes derived from the thalamic/hypothalamic area

Compound	*Cyclic AMP accumulation (pEC_{50})*	n
5-HT	6.68 ± 0.08	5
5-CT	7.81 ± 0.09	5
5-MeOT	6.86 ± 0.36	4
8-OH-DPAT	Inactive	5
Cisapride	Inactive	5
Sumatriptan	Inactive	5
DOI	Inactive	2
RU24969	Inactive	2
	Inhibition of 5-CT stimulated cyclic AMP accumulation (pK_i)	
Methiothepin	7.98 ± 0.25	6
Clozapine	7.03 ± 0.19	6
Ritanserin	7.20 ± 0.24	6
Mesulergine	7.58 ± 0.18	4
Mianserin	6.41 ± 0.19	4
WAY100635	Inactive	5
GR127935	Inactive	5
Ketanserin	Inactive	2

Cells were exposed to increasing concentrations of the compounds shown (10^{-10} M – 10^{-5} M), as described in Methods. pEC_{50} values correspond to the concentration of agonists required to obtain half-maximal stimulation of cyclic AMP accumulation. The concentration of antagonists required to obtain a half-maximal inhibition of 5-CT (100 nM) induced cyclic AMP levels (IC_{50}) were determined experimentally and converted to pK_i) values according to the equation:

$$K_i = IC_{50}/(1 + C/K_d),$$

where C is the 5-CT concentration (100 nM) and K_d is the EC_{50} value for 5-CT (15 nM). Data represent the mean ± s.e.mean of n separate experiments.

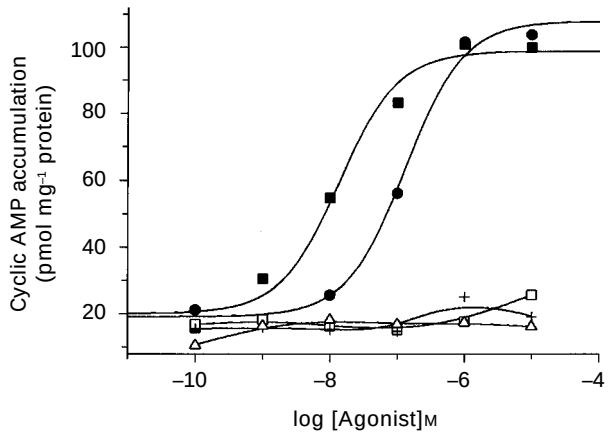

Figure 1 Effect of 5-HT receptor agonists on cyclic AMP accumulation in cultured thalamic/hypothalamic astrocytes: 5-CT (■), 5-HT (●), 8-OH-DPAT (□), RU24969 (+), sumatriptan (△). Data shown are from a representative experiment which was repeated at least twice, results are summarized in Table 1.

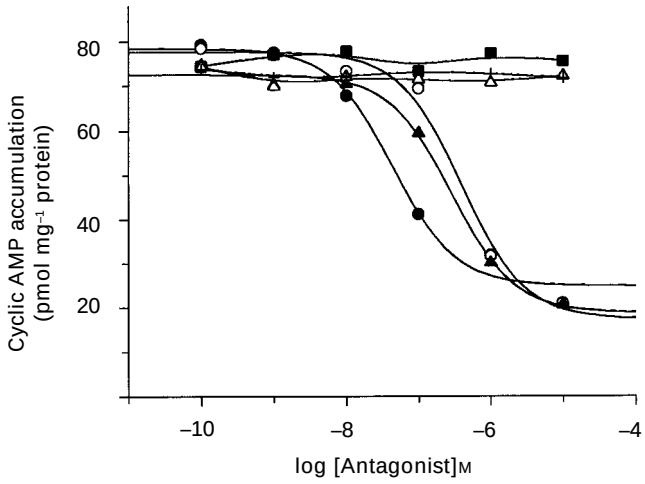

Figure 2 Effect of 5-HT receptor antagonists on 5-CT (100 nM) induced cyclic AMP accumulation in cultured thalamic/hypothalamic astrocytes: methiothepin (●), clozapine (○), ritanserin (▲), ketanserin (+), GR127935 (△), WAY100635 (■). Data shown are from a representative experiment which was repeated at least twice, results are summarized in Table 1.

three out of four experiments ($pEC_{50} = 6.19 \pm 0.12$, $E_{max} = 287 \pm 48\%$). However, 5-CT only elicited an accumulation of cyclic AMP in two of four experiments with a mean pEC_{50} of 6.10 and mean E_{max} of 348%.

Table 2 Effects of 5-HT and 5-CT on basal cyclic AMP levels in astrocytes cultured from different brain regions of neonatal rats

	pEC_{50}		E_{max} (% of basal)		
Brain region	*5-HT*	*5-CT*	*5-HT*	*5-CT*	n
Thal.	6.52±0.09	7.66±0.06	572±63*	588±75*	8
Bs.	6.48±0.22	7.65±0.19	318±46	274±51	4
Coll.	6.56±0.12	7.23±0.21	301±24	244±15	4

Astrocytes cultured from the thalamus/hypothalamus (Thal.), brain stem (Bs.) and colliculus (Coll.) were exposed to increasing concentrations of 5-HT or 5-CT (10^{-10} M–10^{-5} M), as described in Methods. pEC_{50} values and the mean maximal response (E_{max}) were determined for each experiment. E_{max} values are expressed as a percentage of basal cyclic AMP levels (basal levels were 21.25±1.34, 18.37±1.29 and 16.42±1.48 pmol cyclic AMP mg^{-1} protein for astrocytes derived from the thalamic/hypothalamic area, brain stem and colliculus, respectively). Data represent the mean±s.e.mean of *n* separate experiments. $^*P < 0.05$, Kruskall-Wallis test with *post-hoc* Mann Whitney U-test.

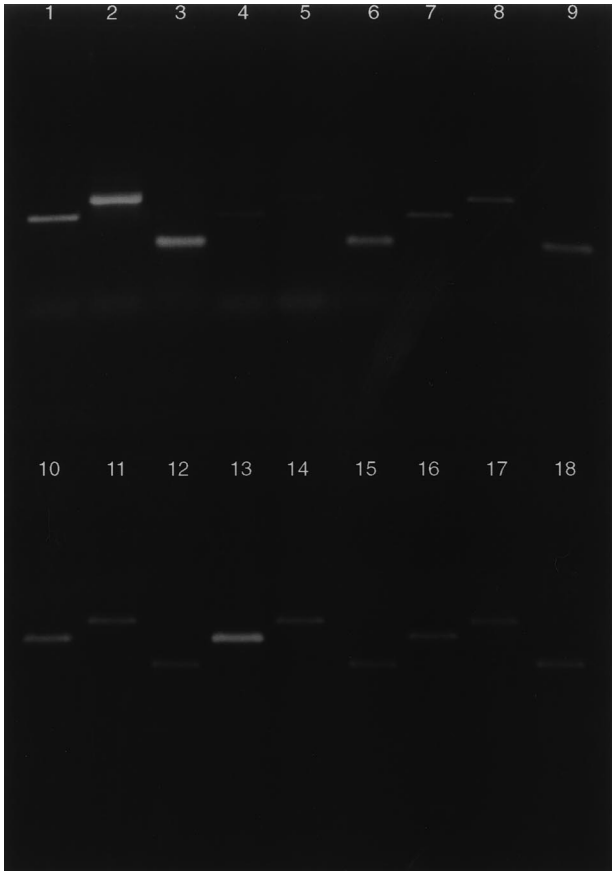

Figure 3 Agarose gel electrophoresis of PCR amplified products from cDNA of adult rat cortex (lanes 1, 2, 3) and astrocytes cultured from colliculus (4, 5, 6), brainstem (7, 8, 9), cortex (10, 11, 12), cerebellum (13, 14, 15) and thalamus/hypothalamus (16, 17, 18). Primers specific for the 5-HT_6 receptor gene (lanes 1, 4, 7, 10, 13, 16), the 5-HT_7 receptor gene (lanes 2, 5, 8, 11, 14, 17) and the constitutively expressed gene for GADPH (glyceraldehyde-3-phosphate dehydrogenase) (lanes 3, 6, 9, 12, 15, 18) were used to amplify DNA fragments of 670, 851 and 445 base pairs, respectively.

RT-PCR on RNA samples extracted from astrocytes cultured from different brain regions

Oligonucleotide primers specific for 5-TH_6 and 5-HT_7 receptors were used to investigate the expression of messenger RNA (mRNA) for these receptors by the astrocytes cultured from the different brain regions. The appropriate controls, namely exclusion of RNA from the reverse transcriptase step, exclusion of the reverse transcriptase and exclusion of the *Taq* enzyme gave no bands (data not shown). Positive controls included the amplification of a constitutively expressed housekeeping gene GADPH (glyceraldehyde-3-phosphate dehydrogenase) and the use of a control tissue (adult rat cerebral cortex) for 5-HT_6 and 5-HT_7 receptor amplification. PCR reactions yielded cDNA fragments of the correct size, corresponding to the 5-HT_6 and 5-HT_7 receptor cDNA (670 and 851 base pairs, respectively). The amplified products were cloned and sequenced to confirm their identity (data not shown). The sequences were analysed by a BLAST search of several databases (Alschtul *et al*., 1990) and were found to correspond to published sequences for the 5-HT_6 and 5-HT_7 receptors (Monsma *et al*, 1993; Lovenberg *et al*., 1993). 5-HT_6 and 5-HT_7 receptor messenger RNAs were observed in the positive control tissue and in astrocytes cultured from each of the different brain regions examined in this study (Figure 3).

Discussion

In the present study the expression of functional 5-HT receptors positively coupled to adenylyl cyclase in cultured astrocytes was investigated. Three 5-HT receptor subtypes have been shown to be positively linked to adenylyl cyclase; 5-HT_4, 5-HT_6 and 5-HT_7 receptors. There is, however, some evidence for other receptor subtypes coupling to G_s and stimulating adenylyl cyclase. For example, cells transfected with 5-HT_{1D} receptors expressed in mammalian cells have been shown to increase intracellular cyclic AMP levels in response to 5-HT (Maenhaut *et al*., 1991; Watson *et al*., 1994). Hence, it is possible that 5-HT receptor subtypes, other than 5-HT_4, 5-HT_6 or 5-HT_7 receptors could elicit the increases in intracellular cyclic AMP levels observed. For these reasons, and because there are no selective agonists or antagonists currently available which distinguish between 5-HT_6 and 5-HT_7 receptors, an extensive characterization of the pharmacology of the receptors on the astrocytes was undertaken.

The classical, potent 5-HT_{1A} receptor agonist 8-OH-DPAT, at concentrations up to 10 μM failed to stimulate cyclic AMP accumulation in cultured astrocytes. In binding studies on cloned rat, mouse, guinea-pig and human 5-HT_7 receptors, 8-OH-DPAT has an affinity (pK_i) of 6.3–7.5 (Shen *et al*., 1993; Ruat *et al*., 1993; Lovenberg *et al*., 1993; Plassat *et al*., 1993; Bard *et al*., 1993; Tsou *et al*., 1994). 8-OH-DPAT has a lower potency at stimulating cyclic AMP accumulation in cells transfected with cloned rat or mouse 5-HT_7 receptors (Lovenberg *et al*., 1993; Plassat *et al*., 1993) or in guinea-pig hippocampal membranes (Tsou *et al*., 1994) (pEC_{50} values of 5.3–6). Interestingly, recent studies have shown that 8-OH-DPAT has either a low potency ($pEC_{50} < 6$) at putative 5-HT_7 receptors expressed in *Cynomolgus* monkey jugular vein (Leung *et al*., 1996) or is completely inactive at stimulating cyclic AMP accumulation in human vascular smooth muscle cells which endogenously express 5-HT_7 receptors (Schoeffter *et al*., 1996). In the present study the selective 5-HT_{1A} receptor antagonist WAY100635 (Fletcher *et al*., 1996) did not affect 5-CT stimulated cyclic AMP levels. Taken together, these results indicate that the 5-HT_{1A} receptor is not involved in the stimulation of adenylyl cyclase in the cultured astrocytes.

Sumatriptan, a 5-HT_{1B} and 5-HT_{1D} receptor agonist and RU 24969, a 5-HT_{1A} and 5-HT_{1B} receptor agonist were inactive in the present study. GR127935, a 5-HT_{1B} and 5-HT_{1D} receptor antagonist (Skingle *et al*., 1996), had no effect on 5-CT stimulated cyclic AMP levels, excluding the involvement of

these receptor subtypes. 5-HT_{1E} and 5-HT_{1F} receptors are not likely to be responsible for the increase in intracellular cyclic AMP observed, since there is no evidence that they are linked to adenylyl cyclase stimulation. Furthermore, 5-CT has low affinity for 5-HT_{1E} and 5-HT_{1F} receptors (pK_i 5.5–6.0) (Boess & Martin, 1994), whereas 5-CT was the most potent agonist in the present study.

Neither 5-HT_{2A} nor 5-HT_{2C} receptors are involved in increasing the cyclic AMP levels in the cultured astrocytes, despite evidence for these cells expressing mRNA for both receptor subtypes and functional 5-HT_{2A} receptors (Deecher *et al*., 1993; Hirst *et al*., 1994). This was confirmed with the selective 5-HT_2 receptor agonist (DOI) which was inactive and the selective 5-HT_2 receptor antagonist (ketanserin) which did not inhibit 5-CT stimulated cyclic AMP accumulation.

The effects observed are unlikely to be due to stimulation of 5-HT_4 receptors as cisapride (10 μM), a 5-HT_4 receptor agonist, was inactive. Also the affinity of 5-CT for 5-HT_4 receptors is approximately 200 fold lower than the values obtained in the present study (Boess & Martin, 1994).

This implies that the receptors most likely to cause the cyclic AMP accumulation are 5-HT_6 and/or 5-HT_7. With the current lack of selective antagonists it is not possible to clearly discriminate between the 5-HT_6 and 5-HT_7 receptors based on the order of antagonist affinity. The antagonist profile observed in this study is characteristic of the 5-HT_6 and 5-HT_7 receptor subtypes, both of which exhibit high affinities towards methiothepin, clozapine, mianserin and ritanserin (Monsma *et al*, 1993; Shen *et al*., 1993; Plassat *et al*., 1993; Lovenberg *et al*., 1993). However, the potency of mesulergine (pK_i 7.58) indicates 5-HT_7 receptors. Cloned rat 5-HT_6 receptors (Monsma *et al*., 1993) and humans 5-HT_6 receptors (Kohen *et al*., 1996) exhibit a lower affinity for mesulergine (pK_i values of 5.76 and 5.42, respectively), as opposed to all data on 5-HT_7 receptors (pK_i 7.6–8.2) (Bard *et al*., 1993; Lovenberg *et al*., 1993; Plassat *et al*., 1993; Shen *et al*., 1993). In addition, these two receptors can be discriminated by the relative order of potency of 5-CT and 5-HT. The higher potency of 5-CT compared to 5-HT (pEC_{50} values of 7.81 and 6.68, respectively) indicates that this effect is likely to be mediated by 5-HT_7 receptors. These values are comparable to those obtained for cyclic AMP accumulation in HeLa cells transfected with the rat 5-HT_7 receptor cDNA (7.89 and 6.81 for 5-CT and 5-HT, respectively) (Lovenberg *et al*., 1993). Thus the rank order of agonist potency, 5-CT > 5-MeOT > 5-HT, obtained in the present study indicates expression of a functional 5-HT_7 receptor.

It is of interest to note that despite the consistent cyclic AMP responses observed, there was no detectable [^{3}H]-5-CT binding to the thalamic/hypothalamic astrocyte cultures (data not shown). This implies that the level of 5-HT_7 receptor expression by the cultured astrocytes is below that detectable by this type of assay i.e. below approximately 20 fmol mg^{-1} protein. This observation is anomalous but not unprecendented; Giles *et al*. (1994) have shown functional 5-HT_{1B} receptor expression in transfected CHO cells which mediated inhibition of forskolin induced cyclic AMP levels but they did not observe any specific binding of [^{3}H]-5-HT or [^{125}I]-cyanopindolol.

The RT-PCR studies (Figure 3) demonstrate the presence of 5-HT_7 receptor messenger RNA confirming that the thalamic/hypothalamic astrocytes have the capacity to express this receptor subtype. 5-HT_7 receptor messenger RNA was detected in RNA extracted from astrocytes cultured from all the regions investigated, but since the RT-PCR data are not quantitative no comments can be made on regional differences in mRNA expression levels. 5-HT_6 receptor specific primers also amplified a cDNA frgament corresponding to this receptor subtype. However, as discussed above, the pharmacological profile of this receptor was not clearly observed in the thalamic/hypothalamic astrocytes. With the lack of subtype selective compounds, the expression of functional 5-HT_6 receptors cannot be ruled out. It is possible that this receptor is expressed at a higher density on astrocytes cultured from other brain regions. However, a full pharmacological profile of the astrocytic 5-HT receptor was only determined for astrocytes derived from the thalamic/hypothalamic area. For example, in the astrocytes derived from the cerebellum the pEC_{50} value for 5-CT was 6.1 compared to 7.7 in the thalamic/hypothalamic astrocytes. This lower figure could be indicative of a different receptor profile in astrocytes cultured from other brain regions.

Astrocytes cultured from the brain area incorporating the thalamus and hypothalamus showed the greatest magnitude of response to 5-HT and 5-CT, this was significantly greater than the response observed in astrocytes derived from the brain stem and colliculus (Table 2). This result is consistent with the 5-HT_7 receptor mRNA and protein distribution in the adult rat brain, where the highest levels are detected in the thalamus (Gustafson *et al*., 1996). Thus, a regional correlation could be proposed between the *in vivo* expression of the receptor and the responses of astrocytes cultured from different regions of the neonatal rat brain.

Such regional variation has been previously documented in astrocyte responses to neurotransmitters and neuropeptides (Wilkin *et al*., 1990). Astrocytes could be analogous to neurones in terms of regionally defined phenotype heterogeneity. Moreover, the regional heterogeneity of astrocytes may reflect the different functional requirements exacted by the different neuronal populations with which they are associated. Thus, local populations of astrocytes may be biochemically specialised to interact with particular neurones and respond selectively to extracellular stimulation. Our results add to the list of neurotransmitter receptors expressed by cultured astrocytes (Kimelberg, 1995).

The data presented here raise the question of whether 5-HT_7 receptor expression by the astrocytes *in vitro* reflects the ability of the cells to express this receptor *in vivo*. Interestingly, the earliest accounts of 5-HT stimulating an accumulation of cyclic AMP were from Fillion and colleagues (1980) with a glial membrane fraction derived from horse striatum. The structural complexity of the mammalian brain has often precluded definitive studies of the expression of neurotransmitter receptors by astrocytes *in vivo*. In spite of this, receptors for several neurotransmitters have been shown including α_1-, α_2-, β_1- and β_2-adrenoceptors, $GABA_A$ receptors, purinoceptors, histamine receptors and tachykinin receptors (Kettenman & Ransom, 1995). The issue of astroglial 5-HT receptor expression *in vivo* requires further study and is currently under investigation in this laboratory.

At the present time physiological roles for astrocytic 5-HT_7 receptors remain speculative although these cells have been suggested to play a role in the development of the 5-hydroxytryptaminergic system within the central nervous system (Whitaker-Azmitia, 1991). At the cellular level, there is accumulating evidence that glia are critical for the establishment, organization and maintenance of neuronal systems (Kettenman & Ransom, 1995). These glial functions may be exerted on the 5-hydroxytryptaminergic system in part through the action of S-100β, a calcium binding protein, which is synthesized by astrocytes *in situ* and has been shown to have neurotrophic activity on 5-hydroxytryptaminergic neurones (Donato, 1991). Transcription of the S-100 gene is regulated through a conserved cyclic AMP response element (Montminy *et al*., 1990), thus 5-HT receptor-mediated increases in intracellular cyclic AMP levels could affect the expression of this protein.

In summary, the present study has shown that cultured astrocytes derived from the thalamic/hypothalamic area express functional 5-HT receptors positively coupled to adenylyl cyclase. The pharmacological profile of this receptor suggested it to be of the 5-HT_7 subtype. Messenger RNA corresponding to this receptor was detected by RT-PCR. However, 5-HT_6 receptor mRNA was also detected and expression of this receptor by the astrocytes cannot be ruled out in the absence of selective compounds. Regional heterogeneity in the magnitude of the cyclic AMP accumulation was observed with the

greatest response in thalamic/hypothalamic astrocytes. To our knowledge, these data provide the first evidence for the presence of 5-HT receptors positively coupled to adenylyl cyclase on cultured astrocytes.

This work was supported by the BBSRC (CASE award to WDH) and by SmithKline Beecham Pharmaceuticals.

References

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W. & LIPMAN, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.*, **215,** 403–410.

BARD, J.A., ZGOMBICK, J., ADHAM, N., VAYSSE, P., BRANCHEK, T.A. & WEINSHANK, R.L. (1993). Cloning of a novel human serotonin receptor (5-HT_7) positively linked to adenylate cyclase. *J. Biol. Chem.*, **268,** 23422–23426.

BOESS, F.G. & MARTIN, I.L. (1994). Review: Molecular biology of 5-HT receptors. *Neuropharmacology*, **33,** 275–317.

CARTER, D., HUANG, B., CHAMPNEY, M. & EGLEN, R.M. (1995). Characterisation of a postjunctional receptor mediating relaxation of guinea-pig isolated ileum. *Eur. J. Pharmacol.*, **246,** 243–248.

DEECHER, D.C., WILCOX, B.D., DAVE, V., ROSSMAN, P.A. & KIMELBERG, H.K. (1993). Detection of 5-hydroxytryptamine2 receptors by radioligand binding, northern blot analysis, and Ca^{2+} response in rat primary astrocyte cultures. *J. Neurosci. Res.*, **35,** 246–256.

DONATO, R. (1991). Perspectives in S-100 protein biology. *Cell Calcium*, **12,** 713–726.

EGLEN, R.M., JAKEMAN, L. & ALVAREZ, R.A. (1994). The 5-hydroxytryptamine (5-HT)$_7$ receptor. *Expert Opin. Invest. Drugs*, **3,** 175–177.

FAYOLLE, C., FILLION, M.P., BARONE, P., OUDAR, P., ROUSSELLE, J.C. & FILLION, G. (1988). 5-Hydroxytryptamine stimulates two distinct adenylate cyclase activities in rat brain: high-affinity activation is related to a 5-HT_1 subtype different from 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1C}. *Fundam. Clin. Pharmacol.*, **2,** 195–214.

FILLION, G., BEAUDOIN, D., ROUSELLE, J.C. & JACOB, J. (1980). [^{3}H]5-HT binding sites and 5-HT-sensitive adenylate cyclase in glial cell membrane fraction. *Brain Res.*, **198,** 361–374.

FLETCHER, A., FORSTER, E.A., BILL, D.J., BROWN, G., CLIFFE, I.A., HARTLEY, J.E., JONES, D.E., MCLENACHAN, A., STANHOPE, K.J., CRITCHLEY, D.J.P., CHILDS, K.J., MIDDLEFELL, V.C., LANFUMEY, L., CORRADETTI, R., LAPORTE, A., GOZLAN, H., HAMON, M. & DOURISH, C.T. (1996). Electrophysiological, biochemical, neurohormonal and behavioural studies with WAY-100635, a potent, selective and silent 5-HT_{1A} receptor antagonist. *Behav. Brain Res.*, **73,** 337–353.

GILES, H., LANSDELL, S.J., FOX, P., LOCKYER, M., HALL, V. & MARTIN, G.R. (1994). Characterisation of a 5-HT_{1B} receptor in CHO cells: Functional responses in the absence of radioligand binding. *Br. J. Pharmacol.*, **112,** 317P.

GUSTAFSON, E.L., DURKIN, M.M., BARD, J.A., ZGOMBICK, J. & BRANCHEK, T.A. (1996). A receptor autoradiograhpic and *in situ* hybridisation analysis of the distribution of the 5-ht_7 receptor in rat brain. *Br. J. Pharmacol.*, **117,** 657–666.

HERTZ, L., BALDWIN, F. & SCHOUSBOE, A. (1979). Serotonin receptors on astrocytes in primary cultures: effects of methysergide and fluoxetine. *Can. J. Physiol. Pharmacol.*, **57,** 223–226.

HIRST, W.D., PRICE, G.W., RATTRAY, M. & WILKIN, G.P. (1996a). Astrocytes *in vitro* express functional 5-ht_7 receptors. *Br. J. Pharmacol.*, **117,** 93P.

HIRST, W.D., PRICE, G.W., RATTRAY, M. & WILKIN, G.P. (1996b). Regional differences in functional 5-ht_7 receptor expression by cultured astrocytes. *Br. J. Pharmacol.*, **117,** 141P.

HIRST, W.D., RATTRAY, M., PRICE, G.W. & WILKIN, G.P. (1994). Expression of 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors by astrocytes. *Soc. Neurosci. Abstracts*, **20,** 1547.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.*, **46,** 157–203.

HOYER, D. & MARTIN, G.R. (1996). Classification and nomenclature of 5-HT receptors: A comment on current issues. *Behav. Brain Res.*, **73,** 263–268.

KETTENMAN, H. & RANSON, B.R. (1995). Neuroglia. Oxford: Oxford University Press.

KIMBELBERG, H.K. (1995). Receptors on astrocytes - what possible functions? *Neurochem. Int.*, **26,** 27–40.

KOHEN, R., METCALF, M.A., KHAN, N., DRUCK, T., HEUBNER, K., LACHOWICZ, J.E., MELTZER, H.Y., SIBLEY, D.R., ROTH, B.L. & HAMBLIN, M.W. (1996). Cloning, characterisation, and chromosomal localisation of a human 5-HT_6 serotonin receptor. *J. Neurochem.*, **66,** 47–56.

LEUNG, E., WALSH, L.K.M., PULIDO-RIOS, M.T. & EGLEN, R.M. (1996). Characterisation of putative 5-ht_7 receptors mediating direct relaxation in Cynomolgus monkey isolated jugular vein. *Br. J. Pharmacol.*, **117,** 926–930.

LOVENBERG, T.W., BARON, B.M., DE LECEA, L., MILLER, J.D., PROSSER, R.A., REA, M.A., FOYE, P.E., RACKE, M., SLONE, A.L., SIEGEL, B.W., DANIELSON, P.E., SUTCLIFFE, J.G. & ERLANDER, M.G. (1993). A novel adenylyl cyclase-activating serotonin receptor (5-HT_7) implicated in the regulation of mammalian circadian rhythms. *Neuron*, **11,** 449–458.

MAENHAUT, C., VAN SANDE, J., MASSART, C., DINSART, C., LIBERT, F., MONFERINI, E., GIRALDO, E., LADINSKY, H., VASSART, G. & DUMONT, J.E. (1991). The orphan receptor cDNA RDC4 encodes a 5-HT_{1D} serotonin receptor. *Biochem. Biophys. Res. Commun.*, **180,** 1460–1468.

MARKSTEIN, R., HOYER, D. & ENGEL, G. (1986). 5-HT_{1A} receptors mediate stimulation of adenylate cyclase in rat hippocampus. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **333,** 335–341.

MARRIOTT, D.R., HIRST, W.D. & LJUNGBERG, M.C. (1995). Astrocytes. In *Neural Cell Culture - A Practical Approach*, ed. Cohen, J. & Wilkin, G.P. pp. 85–96. Oxford: Oxford University Press.

MARTIN, G.R., & WILSON, R. (1994). Operational characteristics of a 5-HT receptor mediating direct vascular relaxation: identity with the 5-HT_7 receptor? *Br. J. Pharmacol.*, **114,** 383P.

MEYERHOF, W., OBERMULLER, F., FEHR, S. & RITCHER, D. (1993). A novel rat serotonin receptor: primary structure, pharmacology, and expression pattern in distinct brain regions. *DNA Cell Biol.*, **12,** 401–409.

MONSMA, F.J., JR., SHEN, Y., WARD, R.P., HAMBLIN, M.W. & SIBLEY, D.R. (1993). Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Mol. Pharmacol.*, **43,** 320–327.

MONTMINY, M.R., GONZALEZ, G.A. & YAMAMOTO, K. (1990). Regulation of cAMP-inducible genes by CREB. *Trends Neurosci.*, **13,** 184–188.

PLASSAT, J.L., AMLAIKY, N. & HEN, R. (1993). Molecular cloning of a mammalian serotonin receptor that activates adenylate cyclase. *Mol. Pharmacol.*, **44,** 229–236.

RUAT, M., TRAIFFORT, E., LEURS, R., TARDIVEL LACOMBE, J., DIAZ, J., ARRANG, J.M. & SCHWARTZ, J.C. (1993). Molecular cloning, characterization, and localization of a high-affinity serotonin receptor (5-HT_7) activating cAMP formation. *Proc. Natl. Acad. Sci. USA*, **90,** 8547–8551.

SCHOEFFTER, P., ULLMER, C., BOBIRNAC, I., GABBIANI, G. & LUBBERT, H. (1996). Functional, endogenously expressed 5-hydroxytryptamine 5-ht_7 receptors in human vascular smooth muscle cells. *Br. J. Pharmacol.*, **117,** 993–994.

SHEN, Y., MONSMA, F.J., JR., METCALF, M.A., JOSE, P.A., HAMBLIN, M.W. & SIBLEY, D.R. (1993). Molecular cloning and expression of a 5-hydroxytryptamine$_7$ serotonin receptor subtype. *J. Biol. Chem.*, **268,** 18200–18204.

SHENKER, A., MAAYANI, S., WEINSTEIN, H. & GREEN, J.P. (1985). Two 5-HT receptors linked to adenylate cyclase in guinea pig hippocampus are discriminated by 5-carboxamidotryptamine and spiperone. *Eur. J. Pharmacol.*, **109,** 427–429.

SKINGLE, M., BEATTIE, D.T., SCOPES, D.I.C., STARKEY, S.J., CONNOR, H.E., FENIUK, W. & TYERS, M.B. (1996). GR127935: a potent and selective 5-HT_{1D} receptor antagonist. *Behav. Brain Res.*, **73,** 157–161.

TARDY, M., COSTA, M.F., FAGES, C., BARDAKDJIAN, J. & GONNARD, P. (1982). Uptake and binding of serotonin by primary cultures of mouse astrocytes. *Dev. Neurosci.*, **5,** 19–26.

TOO, H.P. & MAGGIO, J.E. (1995). Simultaneous extraction of total RNA and peptides from tissues: application to tachykinins. *Peptides*, **16,** 45–53.

TSOU, A.P., KOSAKA, A., BACH, C., ZUPPAN, P., YEE, C., TOM, L., ALVAREZ, R., RAMSEY, S., BONHAUS, D.W., STEFANICH, E., JAKEMAN, L., EGLEN, R.M. & CHAN, H.W. (1994). Cloning and expression of a 5-hydroxytryptamine(7) receptor positively coupled to adenylyl cyclase. *J. Neurochem.*, **63,** 456–464.

WATSON, J., BURTON, M., COLDWELL, M., PRICE, G.W., CRONK, D., WILSON, S. & JONES, B. (1994). The human 5-$HT_{1D\alpha}$ receptor is positively coupled to adenylyl cyclase in transfected CHO cells. *Br. J. Pharmacol.*, **112,** 96P.

WHITAKER-AZMITIA, P.M. (1991). Role of serotonin and other neurotransmitter receptors in brain development: basis for developmental pharmacology. *Pharmacol. Rev.*, **43,** 553–561.

WHITAKER-AZMITIA, P.M. & AZMITIA, E.C. (1986). [^{3}H]5-hydroxytryptamine binding to brain astroglial cells: differences between intact and homogenized preparations and mature and immature cultures. *J. Neurochem.*, **46,** 1186–1189.

WILKIN, G.P., MARRIOTT, D.R. & CHOLEWINSKI, A.J. (1990). Astrocyte heterogeneity. *Trends. Neurosci.*, **13,** 43–46.

(Received August 23, 1996
Revised October 14, 1996
Accepted October 23, 1996)