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17. The protocol for this trial was approved by the Institutional Review Board of the National Cancer Institute. All subjects gave informed consent to participate in the study prior to entry. The ddI for this study was provided by the Developmental Therapeutics Program of the National Cancer Institute and by Bristol-Myers Co.
18. Of the ten AIDS patients, five had had *Pneumocystis carinii* pneumonia, one had had esophageal candidiasis, three had Kaposi's sarcoma, and one had wasting syndrome. Each of the ARC patients had oral candidiasis.
19. We specifically recruited patients who had received AZT for 4 months or less because of the concern that patients with end-stage AIDS who had been on AZT for a longer time might not have been able to manifest immunologic improvements upon being administered another anti-retroviral agent. As a result, a higher than usual percentage of AZT-intolerant patients had nausea, vomiting, or other constitutional symptoms as their principal AZT toxicity. Of the ten AZT-intolerant patients, two had AIDS and eight had ARC.
20. Prophylaxis against *P. carinii* pneumonia with trimethoprim-sulfamethoxazole was permitted throughout the protocol; 6 months into the study, prophylaxis with aerosolized pentamidine was also permitted. In dose groups A and B, one patient received trimethoprim-sulfamethoxazole throughout and one patient started receiving aerosolized pentamidine 4 months after entry; in dose groups C and D, two patients started receiving aerosolized pentamidine 2 months after entry and one patient started receiving trimethoprim-sulfamethoxazole 6 months after entry; in dose groups E and F, one patient received aerosolized pentamidine throughout; and in dose groups G and H, three patients received aerosolized pentamidine throughout.
21. The concentration of ddI in plasma samples was measured by high-performance liquid chromatography (N. R. Hartman, J. A. Kelly, D. G. Johns, unpublished data).
22. The three patients who did not complete the initial 10 weeks as planned were as follows. Patient 5 was diagnosed as having *Cryptococcal* meningitis after 1 week of therapy and was taken off ddI at that time; he was the patient not considered evaluable. Patient 18 could not undergo hospitalization because of an unexpected nonmedical problem and received only oral ddI. Patient 22 was temporarily taken off ddI at week 5, after a seizure; he subsequently resumed ddI therapy after initiating anti-seizure medication.
23. As specified in the original protocol, the statistical significance of the changes from baseline were assessed at week 6 with the two-sided Wilcoxon signed rank test for paired values. For serum HIV p24 antigen and CD4/CD8 T cell ratios, logarithmically transformed values were used.
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30. The ongoing trials of ddI are as follows: R. Dolin, F. Valentine and co-workers at the University of Rochester and New York University, co-sponsored by Bristol-Myers Co. and the NIAID; H. Liebman and co-workers at Boston University, sponsored by Bristol Myers Co.; J. Groopman and co-workers at The New England Deaconess Hospital, sponsored by Bristol-Myers Co.; P. Pizzo and co-workers in children at the National Cancer Institute, sponsored by the Cancer Therapy Evaluation Program. At the V International Conference on AIDS, investigators from the first two of these studies reported preliminary data showing a similar toxicity prolife of ddI at doses comparable to those reported here (J. Lambert *et al.*, *Abstracts of the V International Conference on AIDS, Montreal, June 4–9* (1989), p. 563; T. Cooley *et al.*, *ibid.*, p. 336.)
31. We thank C. McLaren, S. Steinberg, D. Venzon, W. W. Chamberlain, C. K. Grieshaber, K. P. Flora, J. Groopman, M. Bassilar, M. Leuther, J. Stewart, I. Naily, J. Tomaszewski; the Pharmacy staff of the NIH Clinical Center and the medical and nursing staffs of the Medicine Branch of the NCI for their help.

5 June 1989, accepted 28 June 1989

Brain Region and Gene Specificity of Neuropeptide Gene Expression in Cultured Astrocytes

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Astrocytes have many neuronal characteristics, such as neurotransmitter receptors, ion channels, and neurotransmitter uptake systems. Cultured astrocytes were shown to express certain neuropeptide genes, with specificity for both the gene expressed and the brain region from which the cells were prepared. Somatostatin messenger RNA and peptides were detected only in cerbellar astrocytes, whereas proenkephalin messenger RNA and enkephalin peptides were present in astrocytes of cortex, cerebellum, and striatum. Cholecystokinin was not expressed in any of the cells. These results support the hypothesis that peptides synthesized in astrocytes may play a role in the development of the central nervous system.

ASTROCYTES CARRY OUT VARIOUS functions originally thought to be neuronal in nature; these include maintenance of ionic balance, uptake and metabolism of certain transmitters such as excitatory amino acids, and the synthesis and secretion of a number of trophic agents. In addition, astrocytes contain functional neurotransmitter receptors and ion channels (*1*). We present evidence that neuropeptide genes not only are expressed in astrocytes but are expressed in gene- and brain region–specific ways. Furthermore, the proenkephalin gene is regulated by cyclic adenosine monophosphate (cAMP) in astrocytes just as it is in neurons. Thus astrocytes are capable of yet another set of neuronal functions.

Astrocytes were prepared from the cortex, cerebellum, and striatum of 3-day-old rat pups by a modification of the McCarthy-DeVellis technique (*2*). Cultures were 95 to 98% astrocytes (positive for glial fibrillary acidic protein by immunohistochemistry), with less than 1% contamination by microglia [antibody to Mac-I (*3*)] or oligodendrocytes [antibody to galactocerebroside (*4*)]. Analyses of mRNA and peptides were carried out 3 to 4 weeks after the cultures were prepared. RNA was isolated and analyzed by RNA blot or slot blot as previously described (*5*). Methionine enkephalin (met-enkephalin) and somatostatin were extracted and analyzed by specific radioimmunoassays (RIAs) (*6*).

RNA blot analysis of total or polyadenylated [poly(A)$^+$] RNA extracted from cortical, cerbellar, and striatal astrocytes demonstrated that somatostatin mRNA is present in cerebellar astrocytes but undetectable in the other two cultures (Fig. 1). Proenkephalin mRNA is present in approximately equal amounts in astrocytes from all three regions, in agreement with previous results (*7*). Both of these mRNAs are the size expected for the authentic brain mRNA; that is, 670 bases for somatostatin (*8*) and 1.4 kb for proenkephalin (*9*). In contrast, cholecystokinin mRNA could not be detected in either cortical or cerebellar astrocyte cultures, although it is readily detected in cortex of rat brain. Table 1 expresses the results of several RNA blots quantitatively.

These data demonstrated that astrocytes prepared from neonatal rat brain could express neuropeptide genes with a specificity for both the gene expressed and the brain region from which the astrocytes were derived. We then asked whether only the mRNA was synthesized or whether astrocytes could in fact translate the mRNA into precursor and process the precursor to the free bioactive peptides produced by neurons. Because proenkephalin gene transcription is stimulated by cAMP, via receptors

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that activate adenylate cyclase, in a variety of neural cell types (*10*), it was of interest to determine whether similar regulation occurs in astrocytes. Both cortical and striatal astrocytes contain met-enkephalin; the content is 1% of that in adult cortex and 10% of that in adult striatum, respectively (*9*) (Table 2). The specificity of the antibody used in the RIA is such that carboxyl-terminal–extended forms are recognized with less than 1% of the efficiency of free met-enkephalin, suggesting that the product being measured is authentic met-enkephalin. Furthermore, separation of a cortical cell extract (Bio-Gel P4 column) followed by trypsin–carboxypeptidase B digestion to release free met-enkephalin before RIA (*11*) revealed a fraction containing high molecular weight met-enkephalin but little additional met-enkephalin immunoactivity in the region where low molecular weight peptides are eluted. Thus, most of the free peptide present in the extracts is met-enkephalin. Both cortical and striatal astrocytes also secrete met-enkephalin into the culture medium (Table 2). Cerebellar astrocytes contain somatostatin (52 ± 4 pg per milligram of protein), approximately 5% of that seen in whole cerebellum (*12*), whereas no somatostatin was detected in cortical astrocytes.

Exposure of cortical astrocytes to 5 μ*M* forskolin, which elevates intracellular cAMP about 50-fold (*13*), resulted in a 90% increase in proenkephalin mRNA, accompanied by a 2.1-fold rise in cellular met-enkephalin after 3 hours (Table 2). Forskolin treatment of striatal astrocytes led to a 5.3-fold stimulation in proenkephalin mRNA within 3 hours; cellular met-enkephalin was still increased (30%) 24 hours later (Table 2). For both types of astrocytes, there was a corresponding increase in the content of met-enkephalin in the medium. Preliminary results show that stimulation of the β-adrenergic receptor present on cortical astrocytes (*14*), which activates adenylate cyclase, also increases proenkephalin mRNA and met-enkephalin; thus in cortex, norepinephrine may be the endogenous regulator of astrocyte enkephalin synthesis.

Table 1. Region-specific expression of neuropeptide genes in astrocytes. RNA was prepared from 3×10^6 to 4×10^6 astrocytes per region, separated on an agarose-formaldehyde gel, blotted to nitrocellulose, and analyzed sequentially for each of the mRNAs. In addition, each blot was hybridized with the probe 1B15 (cyclophilin), a nonchanging RNA (*22*), which serves as an internal standard to correct for mRNA recovery or degradation. The area of the peak for a specific peptide mRNA band, obtained by densitometric scan of the autoradiogram, was divided by the area of the peak for 1B15; the value obtained is expressed as units of the neuropeptide mRNA. The values are the mean ± SEM for (*n*) blots. ND, not done.

Cortex	Cerebellum	Striatum
	Somatostatin	
<0.01	1.01 ± 0.29 (*n* = 3)	<0.01
	Proenkephalin	
1.85 ± 0.24 (*n* = 3)	2.42 ± 0.24 (*n* = 3)	2.02 ± 0.37 (*n* = 2)
	Cholecystokinin	
<0.01	<0.01	ND

Table 2. Forskolin stimulation of enkephalin biosynthesis in cortical and striatal astrocytes. Astrocytes (~10^6 per dish) were incubated with or without 5 μ*M* forskolin for 3 hours (RNA and met-enkephalin peptides from cortex and RNA from striatum) or 24 hours (peptides from striatum) before harvesting for RNA (*5*) or peptides (*6*). Medium was passed through a Sep-Pak, and met-enkephalin was eluted with 60% acetonitrile and 0.1% trifluoroacetic acid. Samples were reconstituted in RIA buffer after lyophilization. RNA units are expressed relative to 1B15 mRNA ($n = 3$), and control values have been arbitrarily set equal to 1.0. Cellular met-enkephalin (ME) is expressed as picograms per milligram of protein ($n = 3$), and medium ME as picograms per hour per milligram of protein ($n = 3$). Values are mean ± SEM. The experiment was repeated twice. PE, proenkephalin.

Region	Treatment	PE mRNA	Cellular ME	Medium ME
Cortex	Control	1.00 ± 0.04	55.4 ± 13.9	2.7 ± 0.2
	Forskolin	1.88 ± 0.28*	117.1 ± 18.4*	5.1 ± 1.2
Striatum	Control	1.00 ± 0.22	452 ± 142	10.4 ± 1.0
	Forskolin	5.33 ± 0.96*	581 ± 264	14.9 ± 5.1

*$P < 0.05$ as compared to the control.

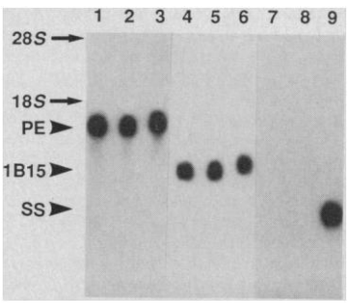

Fig. 1. RNA blot analysis of neuropeptide precursor mRNAs in cultured astrocytes. Total RNA was analyzed from cortical (8.8 μg; lanes 1, 4, and 7), striatal (11.2 μg; lanes 2, 5, and 8), and cerebellar (11.2 μg; lanes 3, 6, and 9) astrocytes. The blot was hybridized sequentially with probes for proenkephalin (PE) (lanes 1 to 3; 3.6×10^7 cpm per microgram of probe DNA; 1-day exposure), 1B15 (lanes 4 to 6; 8.8×10^7 cpm per microgram of probe DNA; 8-hour exposure), and somatostatin (SS) (lanes 7 to 9; 1.9×10^7 cpm per microgram of probe DNA; 7-day exposure). Cholecystokinin was hybridized to two other blots containing only cortical and cerebellar astrocyte RNA; no signal was observed after exposure for 10 days (6.9×10^7 cpm per microgram of probe DNA), whereas a strong signal was detected on a blot containing RNA from adult rat cortex and hippocampus (~10 μg of RNA; 6-day exposure), which was hybridized at the same time.

Our results show that (i) astrocytes in culture can express the genes for what had previously been considered to be neuronal peptide precursors, and (ii) the expression is specific for both the gene and the brain region. Somatostatin and its mRNA are detectable in cerebellar astrocytes only, whereas proenkephalin mRNA and met-enkephalin are present in astrocytes derived from cortex, cerebellum, and striatum. Cholecystokinin mRNA is not detectable. The gene and brain region specificities signify that the results are not an artifact of tissue culture but relate to specific functions in the brain. Furthermore, these patterns of expression show parallels to those seen in vivo and support the idea that astrocytes may synthesize peptides early in development for functions quite distinct from the neuronal neurotransmitter-neuromodulation function. In rat cerebellum, both somatostatin mRNA and peptide content decrease continuously from embryonic day 21 to adulthood (*12, 15*). These results suggest that somatostatin does not function as a neurotransmitter in adult cerebellum; the time course may reflect expression in astrocytes instead. A comparable expression of enkephalin has been noted early in the developing cerebellum, in glial-like cells (*16*). In contrast, in the cortex, somatostatin mRNA and peptides increase continuously from embryonic day 16 to adulthood (*15*), suggesting that expression may be limited to neurons. A similar rise is seen in total rat brain cholecystokinin mRNA and peptides from embryonic day 21 to adulthood (*17*), as well as in cortical enkephalin peptide levels (*18*). The developmental expression of proenkephalin mRNA in striatum shows a biphasic pattern, with an early peak of proenkephalin mRNA at postnatal day 2, a return to basal level by day 7, and a second rise to the adult content starting about postnatal day 14 (*19*). The results in this report further support the suggestion (*19*) that the early peak might represent proenkephalin expression in astrocytes, whereas the later rise, coinciding with a time of active synaptogenesis, would be the result of neuronal expression of proenkephalin.

Our data and those of others (*7, 16*) lead us to propose that astrocytes in specific brain regions can transcribe and translate specific neuropeptide precursor mRNAs and process the precursors to free peptides.

We further suggest that astrocyte expression of peptides occurs early and that the peptides produced may play a role in brain development. Exposure of animals or humans to opiates has been shown to affect development of the central nervous system (*20*); enkephalins might act endogenously in a similar capacity. Thus, the peptides may represent a new class of neurotrophic factors. Alternatively, some evidence exists that peptides can stimulate production of neurotrophic agents (*21*).

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13 March 1989; accepted 19 May 1989

Neurotoxicity of a Fragment of the Amyloid Precursor Associated with Alzheimer's Disease

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Amyloid deposition in senile plaques and the cerebral vasculature is a marker of Alzheimer's disease. Whether amyloid itself contributes to the neurodegenerative process or is simply a by-product of that process is unknown. Pheochromocytoma (PC12) and fibroblast (NIH 3T3) cell lines were transfected with portions of the gene for the human amyloid precursor protein. Stable PC12 cell transfectants expressing a specific amyloid-containing fragment of the precursor protein gradually degenerated when induced to differentiate into neuronal cells with nerve growth factor. Conditioned medium from these cells was toxic to neurons in primary hippocampal cultures, and the toxic agent could be removed by immunoabsorption with an antibody directed against the amyloid polypeptide. Thus, a peptide derived from the amyloid precursor may be neurotoxic.

AMYLOID IS A 4.2-KD POLYPEPTIDE (also called the β protein or the A4 peptide) that is deposited in neuritic plaques and along the walls of the cerebral vasculature in Alzheimer's disease, Down syndrome, and to a lesser extent in normal aging (*1*). Complementary DNA (cDNA) clones for the amyloid precursor protein (APP) encode a protein considerably larger than the amyloid polypeptide (*2*). One form of the precursor (APP-1), distinguished by the absence of a protease inhibitor domain (*3*), is selectively expressed in the nervous system (*4*). We designed three constructs containing different segments of APP-1 (Fig. 1) in the retroviral expression vector DO (*5*). The recombinant AS1 carries the entire APP-1 coding sequence, whereas the recombinants AB1 and AD1 both contain the internal amyloid sequence with different extents of the remaining COOH-terminal portion of APP-1. These constructs were transfected into PC12 and NIH 3T3 cells by a modified version of the calcium phosphate method (*6*). Stable transfectants were selected with the neomycin analog G418, and resistant colonies were subcloned and expanded.

Cells expressing the transfected constructs synthesize, under the control of the 5′ long terminal repeat of Moloney murine leukemia virus, a fusion mRNA that is transcribed from the cDNA insert and the neomycin resistance gene (*neo*). The fusion mRNA is then translated to give the peptide encoded by the cDNA insert. The *neo* gene product is predominantly synthesized from a second transcript originating from an internal simian virus 40 promoter (*5*). The RNA hybridization patterns for transfected PC12 cell clones expressing constructs AB1 and AS1 (designated PC12-AB1 and PC12-AS1, respectively) and transfected 3T3 cell clones expressing constructs AB1 and AD1 (designated 3T3-AB1 and 3T3-AD1, respectively) were examined (Fig. 2). The RNA blots were probed with ^{32}P-labeled APP cDNA. The probe hybridized to the 3.4- to 3.6-kb endogenous APP transcript present in normal PC12 and 3T3 cells and to the bands of higher molecular mass corresponding to the fusion RNA species transcribed from the transfected constructs (Fig. 2A). It was confirmed that the larger transcripts originated from the transfected vector by hybridization of identical RNA blots with ^{32}P-labeled cDNA for the *neo* gene (Fig. 2B). In PC12-AS1 cells, the predicted 7.1-kb RNA was identified (Fig. 2A). The blots were also hybridized with a ^{32}P-labeled Eco RI–Xho I fragment of the gene for APP, which represents the NH_2-terminal portion of APP and does not overlap with the AB1 or AD1 sequences. This NH_2-terminal probe hybridized with the transfected RNA only in PC12-AS1 cells and to the endogenous APP 3.4- to 3.6-kb band in all the clones (*7*). The PC12-DO clone was transfected with the DO vector alone and expressed the *neo* gene (Fig. 2B).

Immunoblot analysis of the transfected cells was performed with an affinity-purified

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