Evidence that β-endorphin is synthesized in cells in the nucleus tractus solitarius: detection of POMC mRNA

David M. Bronstein, Martin K.-H. Schafer *, Stanley J. Watson and Huda Akil

Mental Health Research Institute, University of Michigan, Ann Arbor, MI 48109-0720 (USA)

Evidence from a number of sources indicates that the major site of pro-opiomelanocortin (POMC)-producing cells in the CNS is the arcuate nucleus of the hypothalamus. Using immunocytochemical techniques, a second, smaller group of POMC cells has been detected in the nucleus tractus solitarius (NTS) area of the caudal medulla. However, POMC mRNA has never been reported in the NTS even though it has been found in other extrahypothalamic brain regions. Thus, there is some uncertainty as to whether POMC peptides are actually synthesized de novo in the NTS. In the present study, we used biochemical and anatomical techniques to examine whether POMC mRNA is localized in the NTS. Using in situ hybridization, cells containing POMC mRNA were found in the caudal portion of the NTS. The nucleic acid distribution correlated well with the anatomical distribution of 16k POMC peptide immunoreactivity as determined by immunocytochemistry. Northern analysis revealed that the apparent size of POMC mRNA in the NTS was similar to that found in the arcuate nucleus or the pituitary gland. Results of RNase protection assays using a POMC riboprobe complementary to the 5' end of exon 3 suggested that POMC mRNA in the NTS and arcuate nucleus are identical in this region of the message at least. We also calculated POMC peptide product to mRNA ratios in different tissues and found that NTS cells appear to produce less peptide per mRNA molecule than those in the arcuate nucleus or pituitary gland. Taken together, these data provide the strongest evidence to date that POMC is synthesized de novo in the NTS and offer support for a role for POMC-derived opioid and non-opioid peptides in autonomic functions in the caudal medulla.

INTRODUCTION

The potent opioid peptide β-endorphin (βE) is derived from the precursor protein pro-opiomelanocortin (POMC). While there is abundant evidence that βE may be a physiologically important peptide transmitter involved in diverse behaviors such as stress, pain, feeding, and autonomic responses (for review, see refs. 2 and 13), it has been difficult to define specific POMC pathways and to associate them with particular functions. Part of this difficulty relates to confusion about the precise localization of POMC somata and the anatomical distribution of their axonal projections. The major POMC cell group is found in the arcuate region of the mediobasal hypothalamus [3,4,14,26,29,36-38]. Axonal fibers from this cell group project to a number of brain areas including the septum, amygdala, various hypothalamic and thalamic nuclei and the midbrain. The discovery that POMC mRNA was also found in the arcuate nucleus [10] suggested that these cells intrinsically synthesized POMC peptides (as opposed, for example, to taking them up from the extracellular space). This hypothesis was confirmed by two sets of data: first, Liotta et al. demonstrated by pulse labelling techniques that cells in the arcuate could synthesize and process POMC peptides de novo; and second, using immunocytochemical (ICC) and in situ hybridization (ISH) techniques, cells in the arcuate were shown to contain both POMC mRNA and peptide product [11].

At the present time, it is not clear whether other brain sites also contain cells which synthesize POMC peptide products de novo. Using ICC, several groups reported the existence of a second cluster of POMC cells in the nucleus tractus solitarius (NTS) area of the

caudal medulla [16,17,33]. It should be noted that, largely because of the small number of cells involved, this cell group was relatively difficult to find. It was not possible to detect POMC immunoreactivity in cell bodies in the NTS without prior treatment with large doses of colchicine (which inhibits peptide transport from the cell body down the axon). Recent findings [7,18], indicating that neuropeptide mRNA expression can be induced by colchicine, raised the possibility that POMC may not be expressed in the NTS under normal conditions. This possibility gains strength when one considers that POMC message has, to date, never been found in the NTS despite the fact that it has been detected in a number of extrahypothalamic brain regions including the amygdala, cerebral cortex, midbrain and cerebellum [10,30]. Paradoxically, these brain regions do not contain POMC-containing cell bodies, as determined by ICC. Thus, there is some uncertainty as to whether biosynthesis of POMC peptides actually occurs in the NTS. To address this question, we employed three different techniques (in situ hybridization histochemistry, Northern analysis, and RNase protection) to examine whether POMC mRNA could be detected in the brainstem. In addition, concentrations of βE-immunoreactive (βE-ir) peptides in the pituitary and different brain regions were measured in an attempt to compare the relative expression of POMC message and peptide.

MATERIALS AND METHODS

Nucleic acid analyses

Radiolabelled POMC cRNA probe. POMC cDNA corresponding to the last 35 bases of intron B and the first 395 bases of exon 3 of rat POMC (courtesy of J. Eberwine) was subcloned into PGEM4 plasmid. For Northerns and RNase protections, radiolabelled cRNA probe was transcribed using [^{32}P]CTP or [^{32}P]UTP and T7 RNA polymerase [31]. For ISH, transcription was identical except that [^{35}S]UTP was used as a substrate.

In situ hybridization (ISH). Whole rat brains were frozen in an isopentane-liquid nitrogen slurry, blocked and cut into 10 μm-thick sections on a Bright cryostat. Sections were then fixed in 4% phosphate-buffered formaldehyde for 1 h and washed in phosphate-buffered saline. Hybridization was carried out essentially as described previously [32]. Briefly, following standard pretreatment (proteinase K, acetic anhydride), 30 μl of hybridization mix (75% formamide) containing the POMC cRNA probe was applied per section. Following 12 h incubation at 60°C, sections were washed in 2×SSC (300 mM sodium chloride, 30 mM sodium citrate), treated with RNase A (200 μg/ml) for 30 min, then washed in decreasing concentrations of SSC buffer (2×, 1×, 0.5×, 0.1×). Tissues were rapidly dehydrated and air dried, then dipped in Kodak NTB 2 nuclear emulsion. Autoradiograms were developed (Kodak D19) after 5 weeks and examined with dark field microscopy.

Two negative and one positive controls were carried out to ascertain that the observed signal was specific for POMC mRNA. For the negative controls, sections were either pretreated with RNase to digest all single stranded RNA or were incubated with excess amounts of 'sense' strand POMC mRNA (i.e. identical to the POMC mRNA sequence) during the hybridization reaction. In both cases,

no specific signal was detectable. As a positive control, the POMC riboprobe was incubated with hypothalamic brain sections. Confirming previous results [11], many POMC-positive cells were observed in the arcuate nucleus (data not shown).

Northern analysis. Total RNA from different rat brain regions was extracted by the guanidium isothiocyanate (GITC)/LiCl method [6]. After tissues were homogenized in GITC buffer (5.0 M GITC, 8% mercaptoethanol, 10 mM EDTA, 50 mM Tris pH 7.5), 6 vols. of 4.0 M LiCl were added, and RNA was precipitated overnight at 4°C. Poly(A)$^+$ RNA was isolated by hybridization on oligo(dT)columns according to Sambrook et al. (1989). Different amounts of RNA (caudate 18 μg, NTS (total RNA) 28 μg, NTS (poly(A)$^+$) 2 μg, IP 1 μg, AP 10 μg, arcuate 33 μg) were run on a 1.5% agarose/formaldehyde gel, then passively transferred to Nytran filters. Filters were hybridized with POMC cRNA probe at 60°C for 20 h (hybridization buffer: 5% SDS, 400 mM NaPO$_4$, 1 mM EDTA, 1% BSA, 50% formamide), washed (0.1×SSC (15 mM sodium chloride, 1.5 mM sodium citrate), 0.1% SDS, 1 mM EDTA) 3 times at 70°C and exposed to X-ray film (Kodak X-Omat AR5) for different time periods with or without an intensifying screen (Dupont Cronex Lightning Plus).

RNase protection assay. Total RNA, poly(A)$^+$ RNA or poly(A)$^-$ RNA from different regions of rat brain (hypothalamus 35 μg, NTS 42 μg, poly(A)$^-$ NTS 6 μg, poly(A)$^+$ NTS 1 μg) were incubated in hybridization buffer (0.2 M PIPES, 2.0 M NaCl, 5.0 mM EDTA, 50% formamide) at 55°C for 16 h. Unprotected single stranded RNA was digested by RNase A (20 μg/ml in RNase buffer: 10 mM Tris-HCl, 5.0 mM EDTA, 0.2 M NaCl, 100 mM LiCl) for 1 h at room temperature. RNase activity was terminated by Proteinase K treatment (100 μg/ml in 1% SDS) at 37°C for 30 min. Protected mRNA:cRNA hybrids were precipitated in ethanol, then fractionated by electrophoresis on 4% non-denaturing polyacrylamide gels in TBE buffer (90 mM Tris-base, 90 mM boric acid, 2 mM EDTA). Gels were exposed to Kodak X-Omat AR5 X-ray film at −80°C with 1 intensifying screen.

Peptide analyses

Immunocytochemistry (ICC). Rats pretreated 48 h earlier with 400 μg of colchicine intraventricularly were perfused with 4% phosphate-buffered formaldehyde. Brains were removed, incubated in 15% sucrose solution overnight and then frozen in liquid nitrogen. 20 μm-thick sections were cut and processed for ICC. The primary antibody, specific for the POMC 16k fragment (courtesy of E. Eipper), was used at a final dilution of 1:10,000. Following 16 h incubation at 4°C, secondary biotinylated antibody (goat anti-rabbit) was applied for 30 min at 37°C. Detection followed using the avidin-anti-peroxidase complex (Vectastain) and DAB/H$_2$O$_2$ for substrates. Control sections, in which excess 16k peptide was added to the incubation, showed no reaction product, confirming the specificity of the immunoreactive signal detected.

βE radioimmunoassay (RIA). Samples resuspended in RIA buffer (150 mM phosphate buffer, 1% NaCl, 0.1% BSA) were incubated with primary antibody (Brenda) directed towards βE$_{17-27}$, used at a final concentration of 1:40,000. Following incubation at 4°C for 1–2 days with [^{125}I]N-acetyl-βE$_{1-27}$ as the radiolabelled tracer, second antibody (goat anti-rabbit) was added and incubated for 3 h at room temperature. Pellets were spun down, the supernatant was removed and the amount of primary antibody-bound trace was measured in a gamma counter. βE-ir concentrations were quantitated relative to a rat βE$_{1-31}$ standard curve. Under these conditions, Brenda reacted equally with β-LPH, βE$_{1-31}$, βE$_{1-27}$, βE$_{1-26}$ and their N-acetylated derivatives but had no affinity for any other peptides derived from any of the three opioid precursors [1].

RESULTS

ICC and ISH. ICC analysis revealed the presence of 16k POMC immunoreactivity in cell bodies in the NTS (Fig. 1A). As described previously [16,17,33], these cells

were localized in the caudal portion of the NTS in the vicinity of the commissural nucleus, extending as far back as the area postrema. ISH results indicated that POMC mRNA signal was also present in somata in the NTS (Fig. 1B). The distribution of POMC mRNA-containing cells closely paralleled that found by ICC, with cells restricted primarily to the caudal half of the NTS. While ICC and ISH analyses yielded very similar distribution of POMC-positive cells in the caudal medulla, our impression was that a fewer number of cells were detected with ISH compared with ICC. It is also noteworthy that 5 weeks exposure time was necessary to visualize the POMC mRNA signal in the NTS whereas POMC-positive cells in the arcuate nucleus were clearly detectable in 1 week by ISH (data not shown).

RNase protection assay. Hybridization of the 430 base POMC cRNA probe with RNA extracts from different POMC-containing tissues, followed by RNase digestion, produced a single protected band on polyacrylamide gels (Fig. 2). The size of this band was estimated to be roughly 400 bases, which corresponds to the length of the Exon 3 coding portion of the cRNA probe (i.e. the single stranded intronic portion of the probe was degraded by the RNase treatment). As expected, protected POMC signal was found in RNA extracted from the hypothalamus (Fig. 2) as well as the

Fig. 1. Presence of POMC peptide-immunoreactivity (A) and POMC mRNA (B) in the caudal NTS. A: perfused rat brainstem blocks were cut in 20 μm-thick sections and processed for ICC. A primary antibody specific for the 16k POMC fragment was incubated with sections for 16 h at 40°C. Biotinylated second antibody was applied and detected using avidin-anti-peroxidase complex and DAB/H_2O_2 as substrates ($\times$557). B: fresh frozen brainstem blocks were cut into 10 μm-thick sections, post-fixed in formaldehyde and processed for in situ hybridization histochemistry. Following proteinase K and acetic anhydride pretreatment, a [35]S-labelled POMC riboprobe was applied to sections in 30 μl of hybridization buffer. Sections were incubated for 12 h at 60°C, then digested with RNase A. Sections were washed, dehydrated and air-dried before being dipped in Kodak NTB 2 nuclear emulsion. Autoradiograms were developed after 5 weeks and are shown here in darkfield ($\times$244).
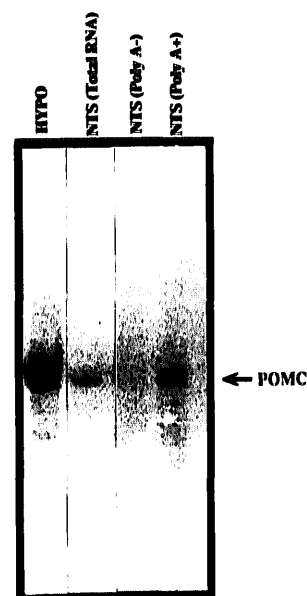

Fig. 2. Hypothalamus and NTS contain an identical nucleic acid sequence (approximately 395 bases) in the exon 3 region of POMC mRNA as determined by RNase protection. Total RNA, poly(A)[+] RNA or poly(A)[-] RNA from the hypothalmus or NTS was incubated with ^{32}P-labelled POMC cRNA for 16 h at 55°C. Single stranded RNA was digested by RNase A followed by proteinase K treatment. Protected mRNA:cRNA hybrids were precipitated in ethanol and fractionated by electrophoresis on a 4% non-denaturing polyacrylamide gel. The gel was exposed to X-ray film for 31 h at –80°C with 1 intensifying screen.
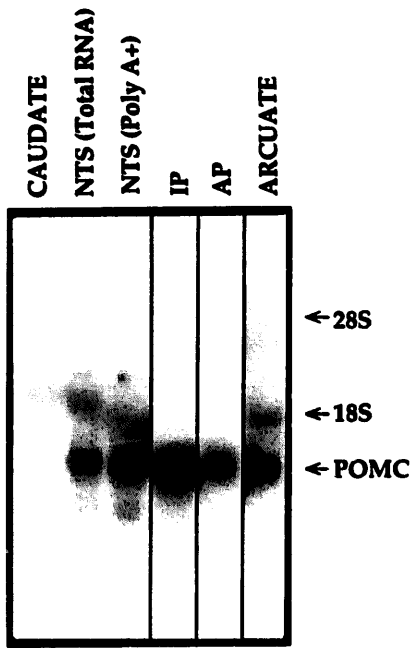
Fig. 3. Presence of POMC mRNA in the NTS, arcuate and pituitary (IP and AP) as determined by Northern analysis. Total RNA or poly(A)⁺ purified RNA from different tissues were loaded on a 1.5% agarose/formaldehyde gel, then passively transferred to Ny-tran filters. The amount of RNA loaded was: caudate 18 μg; NTS (total RNA) 28 μg; NTS (Poly A⁺) 2 μg; IP 1 μg; AP 10 μg; arcuate 33 μg. Filters were hybridized with a ³²P-labelled POMC cRNA for 20 h at 60°C, washed, and then exposed to X-ray films for different lengths of time. Exposure conditions were: caudate, NTS (total RNA) & NTS (poly(A)⁺) 94 h at −80°C with 1 intensifying screen: IP & AP 1.75 h at 25°C; arcuate 6 h at 25°C.

TABLE I

Comparison of relative amounts of POMC mRNA and β-endorphin in different tissues

Brain and pituitary regions were homogenized in GITC buffer. RNA was precipitated by addition of 4 M LiCl. Following centrifugation, RNA pellets were digested with proteinase K, extracted twice with phenol/chloroform and ethanol-precipitated. The protein-containing supernatant was processed on Sep-pak chromatography columns, dried down and resuspended in RIA buffer. POMC mRNA levels were determined by Northern and/or RNase protection assays and βE-ir was determined by RIA. Values, representing approximate amounts of mRNA or peptide per tissue, are expressed relative to anterior pituitary (= 1.0). Numbers in parentheses in β-Endorphin content column represent absolute concentrations (pmol/tissue) of βE-ir.

	POMC mRNA	β-Endorphin content	Ratio peptide mRNA
Anterior pituitary	1.0	1.0 (200 pm)	1.0
Intermediate pituitary	5.0	5.0 (1000 pm)	1.0
Arcuate nucleus	0.25	0.025 (5 pm)	0.1
Whole arcuate system (somata + nerve terminal regions)	–	0.25 (50 pm)	1.0
NTS	0.025	0.0025 (0.5 pm)	0.1
Whole NTS system (somata + nerve terminal regions)	–	0.01 (2 pm)	0.4

anterior (AP) and intermediate pituitary (IP) (data not shown). A similar size band was observed in total or poly(A)⁺ RNA from the NTS but not in the poly(A)⁻ RNA fraction (Fig. 2).

Northern analysis. Northern analysis confirmed the RNase protection data, demonstrating the presence of POMC mRNA in the NTS, in addition to its expected presence in both lobes of the pituitary gland and the arcuate nucleus; in contrast, no POMC signal was detected in total RNA from the caudate nucleus (Fig. 3). The length of time necessary for visualizing the POMC signal varied greatly between the different tissues and brain regions. Intense IP and AP signals were observed after less than 2 h exposure at room temperature; on the other hand, the NTS lanes were exposed for 94 h with an intensifying screen (at −80°C) before a quantifiable band was obtained. In all of the POMC-containing tissues, the size of the mRNA corresponded to roughly 1.2 kb. A larger molecular weight band (approx. 6 kb) can be faintly observed in the IP lane and most likely represents the full-length primary POMC transcript. Interestingly, a smaller POMC-positive band (approx. 800–900 bases in size) was observed in the NTS lanes (Fig. 3).

Peptide measures. Total amounts of βE-ir were determined in different tissues and compared to levels of POMC mRNA in these tissues. In general, there was a strong correlation between the relative amounts of POMC mRNA and βE-ir peptides found in a tissue, with intermediate pituitary containing the highest concentrations of both peptide and message, and the NTS containing the lowest. Peptide concentrations, expressed relative to AP levels, were calculated as a ratio of POMC mRNA concentrations (also expressed relative to AP levels) to provide a gross estimate of the amount of peptide produced per mRNA molecule in different tissues. Peptide/mRNA ratios in the anterior and intermediate pituitary lobes were more or less equal; in contrast, the relative amounts of peptide to mRNA in the arcuate nucleus or NTS were roughly 1/10 of that found in either lobe of the pituitary (Table I). However, since the bulk of peptides synthesized in a neuron are transported axonally away from the cell body (in contrast to pituitary cells), an estimate of the total amount of βE-ir in rostral and caudal nerve terminal projection regions was made for both the arcuate and NTS cell goups, respectively. Including βE-ir from all brain regions rostral to the midbrain/medulla increased peptide/mRNA ratios approximately 10-fold in the arcuate POMC system to levels found in the IP or AP. For the whole NTS system, even when the total βE-ir in the medulla and spinal cord was included, the peptide/mRNA ratio

remained less than half that observed in other POMC systems (Table I).

DISCUSSION

The results of this study provide strong evidence that there are POMC-producing cells in the NTS. The anatomical studies demonstrated that POMC mRNA and POMC peptide product (i.e. 16k POMC peptide) are both found in cells in the caudal medulla. Northern and solution hybridization analyses indicated that the POMC mRNA found in the NTS appears to be homologous to that found in the arcuate nucleus or pituitary gland. It has been shown previously that there are a number of cases, both in the brain and periphery, where the presence of POMC mRNA signal is not necessarily associated with POMC peptide biosynthesis. For example, POMC mRNA has been found in various brain regions (e.g. amygdala, midbrain, cortex) [10,30] where there is no evidence of POMC-containing cell bodies, as determined by ICC. Similarly, in the testes and other reproductive organs, only minute quantities of βE-ir are found [24] even though significant amounts of POMC mRNA are present [8,9,30]. In these cases, though, the apparent size of the POMC message is roughly 200 bases shorter than that observed in the pituitary or arcuate. It has been suggested that the shorter POMC mRNA may not be efficiently translated into protein [9]. Furthermore, since the 5' end (including exons 1 and 2 and intron 1) is missing from the truncated POMC message, even if a protein were translated, the absence of a signal-like sequence makes it unlikely that this protein would be processed and stored properly [20]. In contrast to the examples just mentioned, there are several reasons to believe that POMC mRNA in the NTS is translated into protein products. First, the vast majority of POMC mRNA in the NTS was approximately the same size as that found in the pituitary or arcuate. Second, the riboprobe containing the first 350 bases of exon 3 hybridized and was protected in NTS, arcuate and pituitary samples, suggesting that, at least in this coding region, these tissues have identical POMC mRNA sequences. Finally, POMC mRNA and 16k peptide immunoreactivity were observed in the same region of the NTS, presumably in the same cells. While co-localization studies of POMC mRNA and peptide would have provided conclusive evidence, it is difficult to imagine why (or how) cells containing POMC message without POMC peptides would reside adjacent to other cells which contain POMC peptides but no POMC mRNA. Taken together, the present data lead us to suggest that, in addition to the pituitary gland and

arcuate nucleus, there are POMC-synthesizing cells in the NTS. It should be noted that a lower molecular weight species (800–900 bases) of POMC mRNA was also observed in the NTS although it was much less abundant than the 1.2 kb size message. It would be interesting to determine the similarities between the truncated POMC mRNA in the NTS and the shortened species found in peripheral tissues, in terms of sequence homology, protein expression and regulation.

While ICC and in situ techniques demonstrated the presence of POMC peptide and mRNA in some NTS cells, there appeared to be some discrepancy between the results of these two methods. Although cell counts were not performed, our impression was that more cells were found to contain 16k POMC peptide immunoreactivity compared to the number expressing POMC mRNA signal. Even after 5 weeks exposure, relatively few cells showed POMC mRNA expression. One explanation for this could relate to the low level of POMC expression in these cells and the difficulty in detecting such low abundance messages. An alternative explanation for the discrepancy between the ICC and in situ data is that there was an induction in the number of cells expressing the 16k peptide because of the use of colchicine in the ICC studies. Colchicine is routinely used in ICC to block axonal transport of substances, thereby increasing the signal detectable in the cell body. However, it has been shown that colchicine induces neurons in some brain structures to express neuropeptide mRNA's which are not normally expressed [7,18]. It is possible that in the present studies, colchicine was responsible for the greater number of POMC cells which contain 16k peptide compared to those expressing POMC mRNA. If the in situ data are in fact representative of the normal basal status of the animal, they would suggest that the POMC cell group in the NTS is an extremely small one and could account for past difficulties in detecting POMC message in this brain region. In this regard, it should be noted that in each of the techniques employed in these studies, the detection of POMC mRNA signal in the NTS approached the limits of detection and at times produced variable results.

When the relative amounts of POMC mRNA and βE-ir peptides were compared in different tissues and brain regions, we found that the ratio of POMC peptide/mRNA in pituitary was approximately 10-fold greater than that found in brain tissue. This would seem to imply that pituitary cells produce much more peptide product per POMC mRNA molecule than neuronal cells. The reason for this tissue-specific difference is probably due, at least in part, to the fact that in endocrine cells, peptide biosynthesis and storage

274

occur in close proximity to one another whereas in neurons, peptide products are often transported far away from the site of biosynthesis for storage in nerve terminals. Thus, if peptide levels are measured only at the site of biosynthesis (i.e. in cell body regions), as they were in the brain, it is probable that peptide/mRNA ratios are greatly underestimated. If the total amount of βE-ir found in the rostral portion of the brain is estimated and assumes that it is all derived from the arcuate cell group, as results from lesion [19] or knife-cut studies [15,28] suggest, then the total amount of βE-ir produced by arcuate POMC cells increases approximately 10-fold and the POMC peptide/mRNA ratio increases concurrently to roughly the same levels seen in the pituitary. Analogous calculations are more difficult to justify in the caudal portion of the brain since βE-ir in the medulla, and possibly spinal cord, is derived from both the NTS and arcuate cell groups [15,28]. However, even if one assumes that all of the βE-ir found in the brainstem and spinal cord is produced solely by POMC cells in the NTS (with no contribution from arcuate neurons), the POMC peptide/mRNA ratio for the NTS cell group still remains less than half that seen in the arcuate or pituitary. While the precise reasons for this difference in peptide/mRNA ratios between arcuate and NTS neurons are not currently known, they may involve differences in translational efficiency, POMC processing, peptide storage or basal βE release.

The demonstration that POMC mRNA is found in the NTS, in the same areas where POMC peptides are localized, provides the strongest evidence to date that βE is synthesized in this brain region. The presence of βE-producing neurons in the NTS is consistent with a physiological role for βE-ir peptides, as well as other POMC products, in this brain region. For example, intracisternal injections of full length βE_{1-31} or the C-terminal shortened βE_{1-27} reduce mean arterial blood pressure [12] increase plasma concentrations of dopamine, norepinephrine and epinephrine [34,35] and depress respiratory function [25]. These data suggest a putative involvement of βE-related peptides in the regulation of autonomic function, including cardiovascular, respiratory, and sympathetic nervous system responses. Other data, showing that electrical stimulation in the NTS elicits opioid-mediated analgesia [21] indicate that βE might be part of an endogenous antinociceptive pathway in the brainstem. The present data, providing solid evidence of βE-producing cells in the NTS, places this opioid peptide at a brain site where autonomic and antinociceptive information converge. However, the precise role of βE and other POMC peptides in the NTS awaits further study.

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