

BRES 15554

Effects of morphine treatment on pro-opiomelanocortin systems in rat brain

David M. Bronstein¹, Ryszard Przewlocki² and Huda Akil¹

¹Mental Health Research Institute, University of Michigan, Ann Arbor, MI 48109-0720 (U.S.A.) and ²Institute of Pharmacology, Polish Academy of Sciences, Krakow (Poland)

(Accepted 28 November 1989)

Key words: Morphine time course; Opiate treatment; Pro-opiomelanocortin mRNA; β -Endorphin; Brain pro-opiomelanocortin; Opioid peptide

In previous studies to determine whether chronic opiate administration might negatively feedback upon endogenous opioid systems in the CNS, investigators found no changes in steady-state concentrations of opioid peptides following morphine pelleting. However, since only steady-state levels were measured, it was still not clear whether morphine treatment altered the release and/or biosynthesis of opioid-containing neurons. The goal of the present study was to assess the effects of chronic morphine pelleting on the dynamics of β -endorphin (β E) biosynthesis in rats. Hence, at several times during a 7-day morphine treatment, concentrations of total β E-immunoreactivity (-ir), as well as chromatographically sieved forms of β E, were determined by RIA, and mRNA levels of pro-opiomelanocortin (POMC) were measured by a solution phase protection assay using a mouse or rat POMC ³²P-labelled riboprobe. Concentrations of total β E-ir or different forms of β E-ir peptides (i.e. β -lipotropin, β E₁₋₃₁, or β E₁₋₂₇/ β E₁₋₂₆) in the hypothalamus or midbrain following either 1 or 7 days of treatment were similar in morphine- and placebo-pelleted animals. However, a significant increase in total hypothalamic β E-ir was observed following 3 days of morphine pelleting; chromatographic analyses indicated that this was primarily due to a selective increase in the opiate inactive forms of β E, i.e. β E₁₋₂₇/ β E₁₋₂₆. After 7 days of pelleting, morphine-treated animals tended to have lower POMC mRNA levels than those of placebo controls (20 to 50% decrease in different studies). The accumulation of hypothalamic β E-ir at 3 days, and the apparent decline in POMC mRNA levels at 7 days, lend support to the hypothesis that morphine negatively feeds back upon POMC neurons in the brain by inhibiting β E release and biosynthesis.

INTRODUCTION

Over the past 15 years, investigators have pondered whether the tolerance and/or dependence induced by chronic opiate administration might be related to alterations in endogenous opioid systems in the brain. Shortly after the discovery of the enkephalins, Kosterlitz and Hughes²¹ hypothesized that chronic morphine treatment might result in agonist-induced feedback inhibition of endogenous opioid biosynthesis, a phenomenon well established in catecholamine systems^{30,31}. Early studies designed to test this hypothesis found no changes in the immunoreactive concentrations of β -endorphin (β E) or the enkephalins following a short-term (3–10 days) morphine treatment which produces morphine tolerance and dependence^{9,13,15,36}. However, these data were based solely upon steady-state peptide levels and provided no information about the 'turnover rate' of the endogenous opioids. Some measure of the relative rates of peptide synthesis, release, and degradation is essential to discriminate between systems which have increased, or

decreased, rates of both biosynthesis and release from those which have maintained established metabolic rates, since all 3 conditions could result in similar steady-state levels of peptide product. Thanks to the large increase in our understanding of peptide biosynthesis and to recent technological advances in molecular biology, we can now better estimate the dynamics of endogenous opioid biosynthesis. In the present studies, we re-examined the question of morphine's effects on brain opioid systems, focusing in particular on the pro-opiomelanocortin (POMC) opioid peptide family.

POMC is the precursor protein of the potent opioid peptide β E₁₋₃₁ as well as a number of other bioactive peptides, including beta-lipotropin (β -LPH), adrenocorticotropin (ACTH) and a family of melanocyte-stimulating hormones (MSH's) (see refs. 12, 29 for reviews). In the CNS, the major POMC cell group is situated in the arcuate nucleus of the medio-basal hypothalamus^{7,33,34}, with a second, smaller cluster of POMC cell bodies localized in the nucleus of the solitary tract (NTS) and nucleus commissuralis in the caudal medulla^{19,28}. POMC

Correspondence: D. Bronstein, Mental Health Research Institute, University of Michigan, 205 Washtenaw Place, Ann Arbor, MI 48109-0720, U.S.A.

projections from the arcuate nucleus innervate a diverse number of brain regions, including various hypothalamic nuclei, the periventricular nucleus of the thalamus, limbic structures such as the septum and amygdala, and the periaqueductal gray (PAG) through the diencephalon and midbrain^{17,18,33}. Although projection areas of POMC neurons in the NTS have not been well documented, it is possible that the NTS and arcuate groups may share some common terminal fields. While βE_{1-31} is a major cleavage product of POMC in all tissues, it can undergo further modification by carboxy-terminal cleavage or N-terminal acetylation^{2,11,12,37}. Thus, full length βE_{1-31} and the carboxy-terminal shortened forms, βE_{1-27} and βE_{1-26} , can exist in N-acetylated or non-acetylated forms. In all brain regions studied, βE_{1-31} is the major immunoreactive form of βE , with smaller amounts of βE_{1-27} , and no β -LPH, present^{14,35}. The great majority of βE -immunoreactive (βE -ir) species in the brain are not acetylated.

When the effects of chronic morphine treatment on brain levels of βE -ir were examined in earlier studies, investigators were unaware that at least 7 different peptides (β -LPH, and N-acetylated or non-acetylated forms of βE_{1-31} , βE_{1-27} and βE_{1-26}) might be contributing to the total βE -ir detected. It is possible that chronic morphine treatment affects POMC processing in a way which would result in alterations in the concentrations of differently processed βE -ir peptides without changing the total amount of βE -ir. Alterations in the relative amounts of βE_{1-31} and βE_{1-27} could be physiologically significant since post-translational modifications of βE_{1-31} radically alter the opioid characteristics of the peptide. Carboxy-terminal shortened forms of βE_{1-31} are 10 times less potent than βE_{1-31} in displacing specific opiate binding⁴ and fail to produce analgesia¹⁰. There is also evidence to suggest that βE_{1-27} is antagonistic to the opiate effects of βE_{1-31} ^{5,26}. Hence, one question that was addressed in these studies was whether chronic morphine treatment might cause increased processing of βE_{1-31} to βE_{1-27} , in effect reducing the intracellular concentration of endogenous opioid active material.

While measurements of different immunoreactive forms of βE are critical for understanding whether POMC processing might be altered following chronic morphine administration, they still represent steady-state concentrations and provide no information about the dynamics of the biosynthetic system (i.e. whether is has been activated or inhibited). The advent of molecular biological techniques which measure small amounts of POMC mRNA allowed us to determine a second parameter of biosynthetic activity. The concomitant measurements of peptide and mRNA levels make it possible to infer information about the dynamics of biosynthetic systems and to more accurately assess whether neural

activity has been altered²⁷. For example, Costa and his colleagues, based upon data showing that levels of POMC mRNA and POMC precursor protein both declined by 30–50% following 5–7 days of morphine pelleting, concluded that chronic morphine causes down-regulation of POMC biosynthesis and utilization in the hypothalamus^{23,24}. It should be noted, however, that not all laboratories have detected changes in POMC mRNA levels following morphine treatment¹⁶.

Another issue to keep in mind when studying the possible effects of morphine treatment on endogenous opioid systems is that it is likely that cells engage in a series of responses in adapting to changes in their neural activity. An important lesson learned from studies in the pituitary gland (see, for example, ref. 3) is that peptide systems appear to be regulated by several mechanisms which act at different levels of the biosynthetic pathway (e.g., transcription, translation, precursor processing, post-translational modifications) at different times. Thus, it is possible that some parameters of brain POMC biosynthesis may be altered during the early stages of morphine treatment, but these alterations may disappear at later points in the treatment as other regulatory mechanisms become more important. Hence, examining the POMC system at several time points following morphine treatment may be necessary to observe the full sequelae of possible effects of opiate administration on βE biosynthesis. In this paper, we attempted to provide a description of the effects of chronic morphine administration on the dynamics of brain βE biosynthesis by measuring different βE -ir peptide species and POMC mRNA levels in a cell body (arcuate nucleus) and nerve terminal (midbrain PAG) brain region at several time points following morphine pellet implantation. Preliminary results of these experiments have been reported⁸.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Charles River Co., Wilmington, MA), weighing 220–250 g at the start of the experiments, were maintained in groups of 5–6 per cage, with free access to food and water, in an environmentally controlled room (12:12 h light:dark cycle, lights on at 06.00 h).

Drug treatments

Animals lightly anesthetized with ether were implanted under the skin between the scapula with morphine (75 mg) or placebo (containing avicel PH-102, magnesium stearate, silicon dioxide, and water) pellets (provided by courtesy of the National Institute of Drug Addiction). In the initial study, animals received a single morphine or placebo pellet on Day 1 and 3 additional pellets on Day 4. On Day 7, morphine- or placebo-pelleted animals were injected i.p. with either naloxone (2.5 mg/kg) or saline vehicle and sacrificed 30 min later. The 4 experimental groups were designated as: Placebo pellets/saline injection (PS); Placebo pellets/naloxone in-

jection (PN); Morphine pellets/saline injection (MS); Morphine pellets/naloxone injection (MN). In the time course studies, all animals had one morphine or placebo pellet implanted on Day 1. One set of placebo and morphine animals was sacrificed 24 h later, a second set was killed 72 h after pellet implantation, while a third set of animals was implanted with 3 additional (morphine or placebo) pellets on Day 4 and sacrificed on Day 7. Two brain regions were dissected and frozen immediately on dry ice: (1) a hypothalamic region which contains the major POMC cell body group in the arcuate nucleus as well as a widespread distribution of β E-ir terminals; and (2) a midbrain PAG region rich in β E-ir terminals derived primarily from the arcuate nucleus^{17,22}. The hypothalamus was defined by the optic chiasm, the mammillary nuclei, the lateral aspects of the optic tract, and the top of the third ventricle — wet weight was roughly 30–40 mg. The midbrain dissection simply involved coronal cuts anterior to the superior colliculi and posterior to the inferior colliculi — wet weight was approximately 115 mg. Total β E-ir was determined in the crude extracts of individual animals. Gel chromatography followed by β E radioimmunoassay (RIA) was carried out on pooled samples from different experimental groups. POMC mRNA was quantitated by a solution phase-protection assay following extraction of nucleic acids from individual hypothalami.

Gel chromatography

Tissue was homogenized in acetone:0.2 N HCl (3:1) and the supernatant was dried down and resuspended in 1% formic acid containing 0.01% bovine serum albumin (BSA). After aliquots were removed for assay of total β E-ir, pooled extracts of 4–6 animals from each of the treatment groups were subjected to gel filtration chromatography to separate chemicals on the basis of molecular weight. Chromatography was carried out on a 1.5 × 90 cm Sephadex G-50-50 column developed with 1% formic acid containing 0.01% BSA. Fractions (1.3 ml) were collected and vacuum-dried for later assay for β E-ir. The column was precalibrated with blue dextran, cytochrome C, camel β E₁₋₃₁, camel β E₁₋₂₇, and cobalt chloride.

Peptide quantitation

β E-ir in tissue extracts and chromatographic fractions was measured by RIA using an antibody directed primarily against the midportion of β E₁₋₃₁ (i.e. β E₁₇₋₂₇). At the concentration (1:40,000) used in this assay, and using ¹²⁵I-N-acetyl- β E₁₋₂₇ as the radiolabelled tracer, the antibody was completely cross-reactive with β -LPH, β E₁₋₃₁, β E₁₋₂₇, β E₁₋₂₆ and their N-acetylated derivatives. The antibody showed no cross-reactivity with other POMC-derived peptides such as β E₁₋₁₆, β E₁₋₁₇, Des-tyrosine β E₁₋₁₇, β E₂₇₋₃₁, ACTH₁₋₃₉, α -MSH or γ -MSH, nor did it cross-react with peptides from other opioid precursors (e.g. Leu-enkephalin, Met-enkephalin, dynorphin A, dynorphin B, α -neo-endorphin). Sensitivity of the assay under equilibrium conditions was 2–3 fmol per tube, with an IC₅₀ of approximately 20 fmol. Samples were resuspended in RIA buffer (150 mM Na phosphate buffer with 1% NaCl and 0.1% BSA, pH 8.2) and were quantitated relative to a camel β E₁₋₃₁ standard curve.

POMC mRNA quantitation

POMC mRNA was quantitated in individual hypothalami of animals treated with placebo or morphine pellets for 3 or 7 days using a solution phase hybridization technique modified from Myers et al.²⁵. Total nucleic acids were extracted from hypothalami by homogenization in a LET buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% lithium dodecylsulfate). After aliquots were removed for β E-ir determinations, extracts were incubated with 0.1% proteinase K for 60 min at 37 °C and nucleic acids were recovered by phenol/chloroform extractions followed by ethanol precipitation. Radiolabelled antisense cRNA POMC probes were produced by transcribing linearized SP64 or pGEM plasmids containing a mouse or rat POMC cDNA insert, using α -³²P-cytosine triphosphate (CTP) as the radioactive label. The hybridization reaction was begun by incubating the radiolabelled cRNA and the extracted nucleic acids

in a hybridization buffer (5× hybridization buffer: 0.2 M PIPES, pH 6.4, 2.0 M NaCl, 5.0 mM EDTA) with deionized formamide for 5 min at 90 °C and then for a minimum of 10 h at 50 °C. Single stranded RNA was digested by ribonuclease A (R-5125, Sigma, St. Louis, MO; 10 μ g/ml in RNase buffer: 10 mM Tris-HCl, pH 7.5, 5.0 mM EDTA, 0.2 M NaCl, 0.1 M LiCl) at 22 °C for approximately 60 min. RNase activity was destroyed by Proteinase K (40 μ g/ml) treatment in 1% SDS at 37 °C for 30 min. Protected cRNA:mRNA hybrids were extracted with phenol/chloroform followed by ethanol precipitation and were fractionated by electrophoresis on 4% polyacrylamide non-denaturing gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). The gels were exposed via direct contact autoradiography on Kodak x-Omat XAR5 film and individual bands were quantitated by determining their total integrated optical density using a Loats Image Analysis System (Westminster, MD).

Two different POMC cDNA sequences were used. A mouse cDNA (courtesy of Dr. J. Roberts) contained the coding region for most of CLIP and all of β -LPH as well as approximately 100 bases of the 3' untranslated region which extended close to the polyadenylation tail site. A rat clone (courtesy of Dr. J. Eberwine) corresponded to the 3' portion of Intron 2 and approximately 365 bases of Exon 3 coding for amino acids 19-139 of the POMC precursor (i.e. ending just past the coding region of ACTH₁₋₃₉). While there is roughly 93% homology between the 440 bases of the mouse riboprobe and the rat mRNA sequence, there are several sites where base mismatches are clustered together (Fig. 1A). Assuming that the RNase treatment was most likely to nick double-stranded RNA hybrids in regions in which there are non-complementary bases (i.e. in effect making these regions single-stranded), we anticipated that there would be more than one protected band when the mouse cRNA was hybridized against rat POMC mRNA. Since the 3' tail of the mouse probe had several base deletions and additions compared to the rat message, the longest protected hybrid that was predicted was approximately 390 base pairs in length. In addition, we anticipated that protected hybrids corresponding to approximately 240, 210, 160 and 140 base pairs might be observed (Fig. 1A). There was a fairly good agreement between the predicted sizes of the protected bands (Fig. 1B) and what was actually observed when the protection assay and gel electrophoresis was carried out (Fig. 1C). The two most intense bands corresponded to hybrids of approximately 350–370 and 240 base pairs in length. In addition, there were fainter bands of roughly 300, 180 and 150 base pairs in size. When the rat riboprobe was hybridized against rat POMC mRNA, only a single hybrid band was observed in autoradiographs of the electrophoresis gels (Fig. 1D). This band, estimated to be approximately 370 base pairs long, was fully predicted from the hybridization of the portion of riboprobe containing Exon 3 to the complementary region of the mRNA sequence, followed by RNase digestion of the single stranded intronic portion of the probe.

Statistics

Differences between experimental and appropriate control groups were statistically evaluated by 2-tailed *t*-tests using a significance level of 0.05.

RESULTS

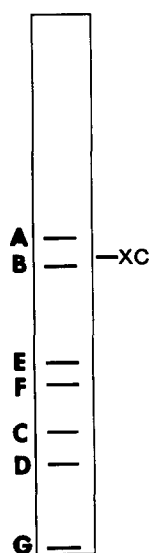
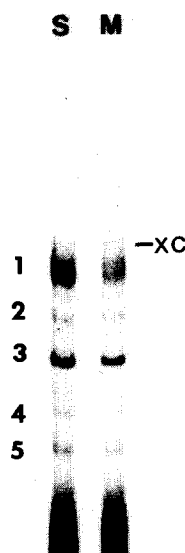
Seven-day morphine/precipitated withdrawal: effects on β E-ir peptides

In the first experiment, we examined the effect of chronic morphine pelleting on brain β E-ir, focusing in particular on whether the relative concentrations of the different β E-ir species might be altered by this paradigm. In addition, we were interested in possible changes in β E-ir peptides in morphine-tolerant animals who were

A. PREDICTED HYBRIDS OF MOUSE cRNA AND RAT mRNA



BAND	BASE PAIRS
A	440
B	390
C	160
D	134
E	240
F	210
G	20

B. MOUSE cRNA:RAT mRNA
(PREDICTED)C. MOUSE cRNA:RAT mRNA
(OBSERVED)

D. RAT cRNA:RAT mRNA

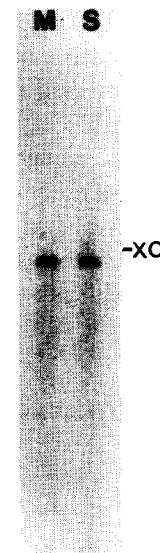
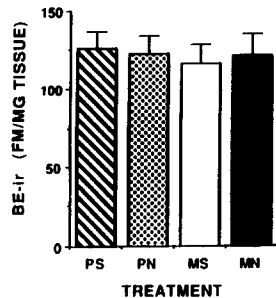


Fig. 1. Predicted and observed bands of mouse cRNA:rat mRNA and rat cRNA:rat mRNA hybridization following RNase digestion. A: schematic representations of the corresponding rat mRNA and mouse cRNA sequences. Regions where base mismatches between the two sequences are concentrated are indicated in black. The predicted double stranded mouse cRNA:rat mRNA hybrids which would be protected from RNase digestion are indicated in the lower portion, assuming that RNase attacks and cleaves the full length (440 bp) hybrid at regions where base mismatches are clustered (i.e. in effect making these regions single stranded RNA's). Note that it is highly unlikely that the 3' portion of the mouse:rat RNA hybrid is protected from RNase attack because of the presence of a number of single base additions or deletions in the 3' terminus of the mouse sequence (checkered region) compared to the rat sequence; hence, the longest protected hybrid which was expected was roughly 390 bp. B: schematic of a theoretical autoradiograph based upon the protected fragments predicted in part A of this figure. C: observed autoradiograph of protected mouse cRNA:rat mRNA bands. The largest protected fragment (band 1) was estimated to be 350–370 bp while the most intense band (band 3) was approximately 240 bp in length. Minor bands (bands 2, 4, 5) corresponded to roughly 300, 180 and 150 bp long, respectively. D: observed autoradiograph of protected rat POMC cRNA:rat mRNA hybrid. Note the presence of a single band corresponding to approximately 370 bp in length. M, morphine-pelleted animals; P, placebo-pelleted animals, XC, the position of the marker dye, xylene cyanol.

undergoing naloxone-precipitated withdrawal. Total β E-ir in crude extracts from the hypothalamus and midbrain was approximately the same in the 4 treatment groups (Fig. 2A,B). The absence of changes in β E-ir in any of the brain regions examined was reinforced by chroma-

tographic analyses. The chromatographic profiles of β E-ir from the PS, PN, MS and MN groups were extremely similar in both of the brain regions examined (Fig. 3). In each chromatograph, 3 main peaks of β E-ir were detected, which corresponded to the approximate

A. HYPOTHALAMUS



B. MIDBRAIN

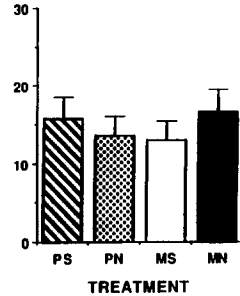


Fig. 2. Effect of 7 days of morphine treatment on concentrations of total β E-ir in A: hypothalamic and B: midbrain tissue extracts. Animals were implanted with 4 morphine (75 mg/pellet) or placebo pellets over a 7-day period and injected with either naloxone (2.5 mg/kg) or saline vehicle 30 min before sacrifice. β E-ir was determined by RIA and is expressed as pmol β E-ir/mg tissue (wet weight). The 4 experimental treatments were: PS, placebo/saline; PN, placebo/naloxone; MS, morphine/saline; MN, morphine/naloxone. Values represent the means \pm S.E.M. of 8 animals.

elution volumes of β -LPH, β E₁₋₃₁ and β E₁₋₂₇/ β E₁₋₂₆ (the chromatographic conditions used in these studies could not resolve β E₁₋₂₇ from β E₁₋₂₆). A small peak of immunoreactivity observed immediately after the void volume probably represents the precursor POMC. Total β E-ir associated with β E₁₋₃₁, and β E₁₋₂₇/ β E₁₋₂₆-sized peaks did not differ significantly between the 4 treatment groups in the two brain regions examined (Table I).

TABLE I

Effect of 7 days of morphine pelleting of POMC peptides in brain

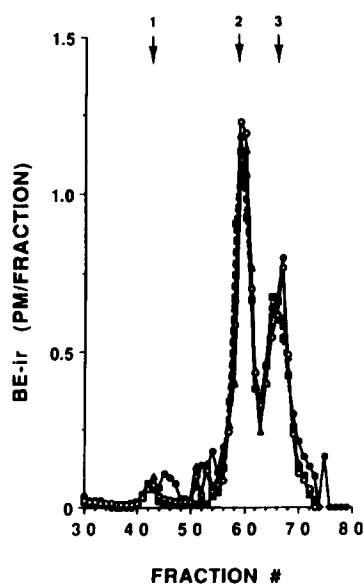
Total β E-ir in the two major peaks observed in the chromatographic profiles of Fig. 3. The two peaks corresponded to the elution positions of β E₁₋₃₁ and β E₁₋₂₇/ β E₁₋₂₆ (which could not be resolved from each other on this column). Values for β E-ir are expressed as pmol/peak. The ratios of β E₁₋₃₁ to the β E₁₋₂₇/ β E₁₋₂₆ peaks for the 4 treatment conditions (see Fig. 2 legend) are shown in the last column.

	β E ₁₋₃₁	β E ₁₋₂₇ / β E ₁₋₂₆	Ratio β E ₁₋₃₁ : β E ₁₋₂₇ / β E ₁₋₂₆
Hypothalamus			
PS	4.41	3.32	1.3:1
PN	4.65	3.82	1.2:1
MS	4.69	3.41	1.4:1
MN	4.70	3.41	1.4:1
Midbrain			
PS	3.39	1.35	2.5:1
PN	3.11	1.34	2.3:1
MS	2.75	1.14	2.4:1
MN	3.26	1.16	2.8:1

Three and seven days morphine treatment: effects on POMC mRNA

In this experiment, we measured the effects of 3 or 7 days of morphine pelleting on POMC mRNA levels in the hypothalamus. POMC mRNA was quantitated in 2 separate studies, using the mouse riboprobe in Study 1 and the rat probe in Study 2. In Study 1, each of the 5

A. HYPOTHALAMUS



B. MIDBRAIN

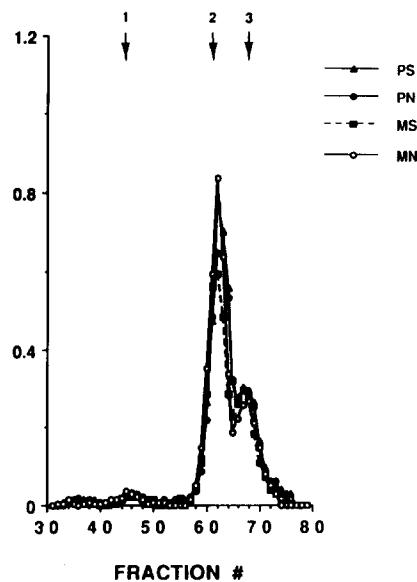


Fig. 3. Chromatographic profiles of β E-ir in A: hypothalamic and B: midbrain extracts. Pooled samples of 4–8 animals from the 4 treatment groups (PS, PN, MS, MN; see legend to Fig. 2) were applied to a Sephadex G-50-50 column and developed with 1% formic acid containing 0.01% BSA. Collected fractions (1.3 ml) were dried down and assayed for β E-ir. Values represent pmol β E-ir/fraction. Arrows at the top of the chromatographs refer to the predicted elution positions of β E-ir peptides: 1, β LPH; 2, β E₁₋₃₁; 3, β E₁₋₂₇/ β E₁₋₂₆.

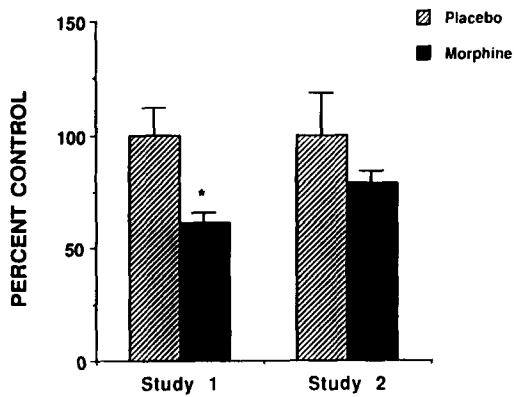


Fig. 4. Effect of 7-day morphine treatment on POMC mRNA levels in the hypothalamus. Total nucleic acids from hypothalami of morphine- or placebo-pelleted animals were extracted in LET buffer. Following hybridization at 50 °C with a radiolabelled riboprobe complementary to either mouse (Study 1) or rat (Study 2) POMC cDNA clones, 'unprotected' single-stranded RNA was digested by RNase A treatment. Protected cRNA:mRNA hybrids were extracted and fractionated on 4% polyacrylamide non-denaturing gels. Gels were exposed on Kodak x-OMAT XAR5 film and individual bands were quantitated densitometrically. The data for Study 1 shown in this figure were calculated for band 3 only (see Fig. 1C). Values represent the mean \pm S.E.M. of 5–6 animals and are expressed as a percent of the mean optical density of control animals. * $P < 0.01$ compared to placebo group.

bands observed in the autoradiographs was densitometrically analyzed (see Fig. 1C). In every case, the mean optical density of the bands of morphine-treated animals was lower than that of placebo-pelleted controls (although in the cases of Bands 1 and 2, this difference was not statistically significant). Quantitative analysis of the most intense hybrid band (Band 3) indicated that POMC mRNA levels in hypothalami from morphine-treated animals were approximately 50% lower than those seen in placebo-treated controls ($t = 4.5$, $P < 0.01$; Fig. 4).

In Study 2, using the rat POMC cRNA, a single band was protected following RNase digestion (see Fig. 1D). Densitometric quantitation of this band revealed a small (approximately 20%) decrease in POMC signal in animals morphine-treated for 7 days compared to placebo controls (Fig. 4), although this difference was not statistically significant. Similar amounts of POMC mRNA were detected in morphine and placebo animals following 3 days of treatment (data not shown).

Morphine time course: effects on β E-ir peptides

Although no changes in β E-ir peptide levels were found following 7 days of morphine treatment, the preceding data, showing that POMC mRNA tends to decline following chronic morphine, suggest the possibility that β E biosynthesis might be reduced in morphine-pelleted animals. Hence, we re-examined the effect of morphine pelleting on β E-ir peptide levels at several time points over the 7-day treatment, reasoning that if

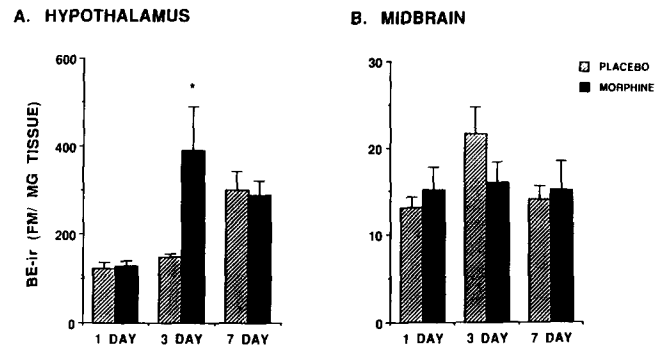


Fig. 5. Time course of effects of morphine pelleting on β E-ir concentrations in the A: hypothalamus or B: midbrain. Animals were implanted with one morphine or placebo pellet and sacrificed 24 (1 DAY) or 72 h (3 DAY) later; other groups of animals received one morphine or placebo pellet on Day 1, 3 more pellets on Day 4 and were sacrificed on Day 7 (7 DAY). β E-ir was determined by RIA and is expressed as pmol β E-ir/mg tissue (wet weight). Values represent the mean \pm S.E.M. of 5–14 animals. * $P < 0.05$ compared to 3 DAY placebo group.

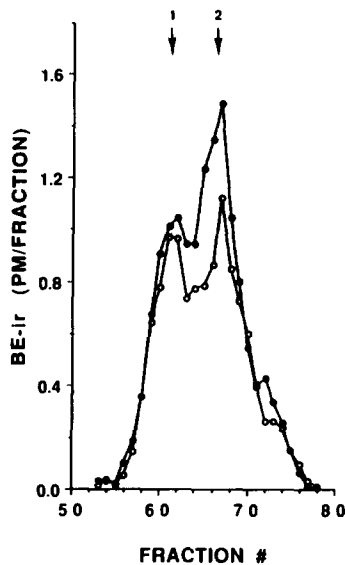
morphine decreased the release of β E-ir peptides, it might result in an intraneuronal accumulation of β E-ir at some point following morphine administration.

β E-ir concentrations in the hypothalami of morphine-treated animals were unchanged from control values following 1 day of pelleting but were significantly increased after 3 days of treatment (Fig. 5A). Interestingly, β E-ir concentrations after 7 days of morphine treatment were comparable to those found in animals who were morphine-pelleted for 3 days. However, the β E-ir content of placebo controls at 7 days post-pelleting increased relative to 1- or 3-day placebo control levels; hence, as observed previously, no significant treatment effect was detected at this time point. No significant differences between morphine- and placebo-treated animals were observed in midbrain concentrations of β E-ir at any of the time points examined (Fig. 5B).

When the relative amounts of different sized immunoreactive peptides in the hypothalamus were analyzed, we found that the increase in total hypothalamic β E-ir observed in 3-day morphine-treated animals appeared to be the result of a selective increase in β E₁₋₂₇/ β E₁₋₂₆ (Fig. 6A). The ratio of β E₁₋₃₁: β E₁₋₂₇/ β E₁₋₂₆ concentrations in placebo-treated animals (approximately 1:1) declined in morphine-treated animals (to roughly 1:2). In contrast to the hypothalamus, 3 days of morphine pelleting did not alter the relative amounts of β E₁₋₃₁ and β E₁₋₂₇/ β E₁₋₂₆ found in the midbrain (Fig. 6B). Following 1 or 7 days of treatment, morphine- and placebo-treated animals displayed similar chromatographic profiles of sieved hypothalamic or midbrain pools (data not shown).

The distribution of β E-ir in the brain following morphine treatment was assessed in a different way by calculating the ratio of the β E-ir content in the hypo-

A. HYPOTHALAMUS



B. MIDBRAIN

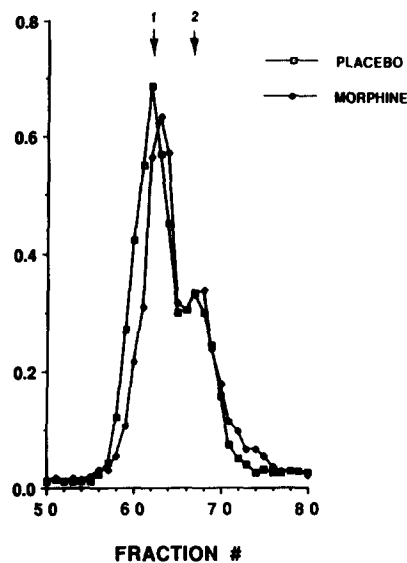


Fig. 6. Effect of 3 days of morphine pelleting on βE -ir chromatographic profiles in the A: hypothalamus and B: midbrain. Pooled samples of 5–6 morphine- or placebo-treated animals were developed on a Sephadex G-50-50 column with 1% formic acid containing 0.01% BSA. Collected fractions (1.3 ml) were dried down and assayed for βE -ir. Values represent pmol βE -ir/fraction. Arrows at the top refer to the elution positions of βE standards: 1, βE_{1-31} ; 2, βE_{1-27} .

thalamus relative to that found in the midbrain. Following 1 or 7 days of pelleting, there were no differences between the morphine and placebo groups in hypothalamic to midbrain ratios of total βE -ir. However, the hypothalamus:midbrain βE -ir ratio increased from 2:1 in 3-day placebo-treated animals to approximately 6:1 in morphine-pelleted animals. This indicates that morphine caused an apparent redistribution of βE -ir from nerve terminals in the midbrain to POMC cell bodies in the hypothalamus.

DISCUSSION

The main goal of these studies was to determine whether POMC biosynthesis was altered following chronic administration of the opiate agonist, morphine. By examining both peptide and mRNA levels, a more detailed description of the dynamics of POMC biosynthesis was provided compared to earlier reports, when steady-state tissue levels of total βE -ir were the sole measure taken. In agreement with the results of previous studies^{6,15,23,24}, total βE -ir in selected brain areas (i.e. the hypothalamus and midbrain) was not altered in animals treated for 7 days with morphine pellets or in morphine-treated animals undergoing naloxone-precipitated withdrawal. Furthermore, there does not appear to be any effect of chronic morphine treatment or precipitated withdrawal on the processing of the POMC precursor molecule as evidenced by the fact that the relative

amounts of β -LPH, βE_{1-31} and $\beta E_{1-27}/\beta E_{1-26}$ remained fairly constant across all treatment groups in the two brain regions examined. The sieving data support recent results showing that 4 days of morphine pelleting had no effect on the processing of βE -ir peptides in the hypothalamus or PAG⁶. If one relied solely upon peptide measurements at the 7-day time point, the evidence would suggest that chronic morphine administration had no effect on the biosynthesis of POMC-derived peptides.

When peptide levels were determined at different times over the course of the morphine treatment, an interesting result was observed in placebo-pelleted animals. Hypothalamic concentrations of βE -ir in animals placebo-pelleted for 7 days were approximately twice as high as those found in animals implanted with placebo pellets for 1 or 3 days (see Fig. 5). At the present time, it is not known whether the elevated βE -ir concentrations in the 7-day placebo group resulted from increased biosynthesis following increased neural activity or was due to an accumulation of βE -ir subsequent to reduced peptide release. The most obvious explanation for this 'placebo-pelleting' effect relates to differences in the handling of the placebo groups. Animals pelleted for 1 or 3 days were anesthetized and pelleted only once, while those treated for 7 days underwent the pelleting procedure a second time (i.e. on Day 4). The fact that hypothalamic βE -ir increased in the 7-day placebo-pelleted group relative to the 1- or 3-day groups suggests that some factor(s) associated with the second exposure

to the pelleting procedure affected β E-containing neurons. We are presently investigating whether a second exposure to ether anesthesia alters hypothalamic β E-ir levels relative to those found in animals anesthetized only once. Although the presence of the 'pelleting' effect precludes making meaningful comparisons about morphine's effects across different time points, valid comparisons between morphine and placebo groups can be evaluated at a given time point.

Keeping in mind the cautionary note just mentioned, we believe that the present studies did yield two pieces of data which lend support to the hypothesis that morphine inhibits β E biosynthesis in the brain. First, the amount of POMC mRNA detected in animals implanted with morphine pellets for 7 days was reduced (by roughly 50% in Study 1 and 20% in Study 2) compared to placebo-treated controls. That we observed a significant effect in one study (using the mouse riboprobe) but not in the other (using the rat POMC cRNA) reflects the confusion that already exists in the literature about whether chronic morphine treatment reduces^{23,24} or has no effect¹⁶ on hypothalamic POMC message. The variability in the magnitude of this effect across different studies may indicate that other factors (e.g. stress) have not remained constant in the different studies. In this regard, it is intriguing to note that there have also been conflicting reports on the effects of chronic morphine treatment on pro-enkephalin mRNA levels in the striatum, with some groups reporting that it reduces³² pro-enkephalin mRNA while others claim it has no effect²⁴. In future studies, it will be important to either eliminate or control for possible factors which might modulate morphine's effects on endogenous opioid systems. Although variable, the present results, together with earlier data^{23,24}, suggest that POMC mRNA levels tend to decline following chronic morphine pelleting, indicating that β E biosynthetic capacity in the hypothalamus might be reduced following 7 days of morphine treatment.

The second piece of evidence which indicates that morphine may inhibit brain POMC biosynthesis and neural activity is that although no changes in tissue content of β E-ir were observed following 7 days of morphine pelleting, significantly higher levels of β E-ir were detected in the hypothalami of 3-day morphine-pelleted animals compared to 3-day placebo-pelleted controls. While it is conceivable that the accumulation of β E-ir after 3 days of pelleting results from increased POMC biosynthesis, the fact that we observed no increases in POMC mRNA levels, or in any other parameter of β E biosynthesis or release, argues strongly against this possibility. Our interpretation of these data is that morphine reduced β E release from POMC neurons while peptide biosynthesis proceeded at a normal rate,

causing β E-ir to gradually accumulate in the hypothalamus. This explanation is supported by the fact that the hypothalamus:midbrain ratio of β E-ir concentrations was 3-fold greater in morphine- versus placebo-treated animals, suggesting that β E-ir was no longer being transported to nerve terminals in the midbrain but was accumulating in POMC cell bodies in the hypothalami of morphine-pelleted animals. One could speculate that when morphine treatment was continued beyond 3 days (i.e. to 7 days), homeostatic mechanisms caused POMC mRNA levels to decline to a point where the rate of β E biosynthesis once again matched the rate of β E release. Under this new equilibrium between synthesis and release, steady-state peptide levels in tissues of 7-day placebo- and morphine-treated animals would not differ. Also consistent with the notion that the accumulation of hypothalamic β E-ir after 3 days of morphine is due to inhibited peptide release is the finding that there was a relatively selective accumulation of smaller forms of β E (i.e. β E₁₋₂₆/ β E₁₋₂₇). It is generally believed that peptide processing is a time-dependent process; hence, post-translational modifications which occur later in the processing pathway (e.g. COOH-terminal cleavage of β E₁₋₃₁, C-terminal amidation, or N-terminal acetylation) may not proceed fully to completion. This appears to be the case for conversion of β E₁₋₃₁ to β E₁₋₂₇ and β E₁₋₂₆, as suggested by the fact that this processing event normally occurs less than 50% of the time in the brain and, under some circumstances, can become a rate-limiting biosynthetic step¹. Thus, it seems likely that the accumulation of β E₁₋₂₇/ β E₁₋₂₆-ir is indicative of increased storage time in granules (as a result of decreased release), leading to fuller processing. However, other interpretations of how hypothalamic β E₁₋₂₇/ β E₁₋₂₆ peptides increase following 3 days of morphine treatment are possible. For example, the enzyme responsible for converting β E₁₋₃₁ to β E₁₋₂₇ may be transiently activated by morphine treatment.

In addition to altering β E-ir peptide and POMC mRNA levels at different times during morphine administration, there is some evidence suggesting that morphine may inhibit β E release in the brain. First, concentrations of β E-ir in the hypophyseal portal plasma, which are thought to reflect release from hypothalamic POMC neurons, declined by over 60% following i.v. administration of morphine and this effect was blocked by naltrexone²⁰. Second, results from in vitro studies have shown that the basal and K⁺-stimulated release of β E-ir from hypothalamic slices into the incubation media is decreased in tissue of morphine-pelleted animals compared to placebo-pelleted controls (Bronstein and Akil, unpublished observations). These data, while preliminary, suggest that even though β E-ir levels appear to be comparable in morphine- and placebo-treated animals,

the peptide pools in morphine-treated animals may be less susceptible to release, or may be differently regulated, than those of control animals.

If it is substantiated that morphine does feedback to inhibit the biosynthesis of an endogenous opioid system, the precise function of the down-regulated system in opiate tolerance and withdrawal would have to be established. The finding that $\beta E_{1-27}/\beta E_{1-26}$ selectively accumulated in the hypothalamus after 3 days of morphine treatment could have a profound physiological effect, since βE_{1-26} and βE_{1-27} are much less potent peptides than βE_{1-31} ^{4,10}. The effects of changing the relative amounts of full length and processed βE -ir peptides may not be readily apparent as long as opiate receptors are occupied by morphine. However, once the exogenous opiate is removed, and activation of opiate receptors becomes dependent on endogenous stores of peptides, the decrease in the relative amount of βE_{1-31} could result in reduced opioid-mediated responses. It is possible that the decreased opioidergic tone in POMC (and perhaps other opioid) neurons which may follow cessation of long-term opiate treatment could play a role in the expression of various withdrawal symptoms.

REFERENCES

- Akil, H., Shiomi, H. and Matthews, J., Induction of the intermediate pituitary by stress: synthesis and release of a non-opioid form of β -endorphin, *Science*, 227 (1985) 424–426.
- Akil, H., Ueda, Y. and Lin, H.-L., A sensitive coupled HPLC/RIA technique for separation of endorphins: multiple forms of β -endorphin in rat pituitary intermediate vs anterior lobe, *Neuropeptides*, 1 (1981) 429–446.
- Akil, H., Young, E.A. and Watson, S.J., Pro-opiomelanocortin peptide secretion from anterior and intermediate lobe: effect of pre-translational and post-translational events. In N.A. Thorn, M. Treiman and O.H. Peterson (Eds.), *Molecular Mechanisms in Secretion*, Munksgaard, Copenhagen, 1988, pp. 507–520.
- Akil, H., Young, E., Watson, S.J. and Coy, D.H., Opiate binding properties of naturally occurring N- and C-terminus modified beta-endorphin, *Peptides*, 2 (1981) 289–292.
- Bals-Kubik, R., Herz, A. and Shippenberg, T.S., β -Endorphin-(1–27) is a naturally occurring antagonist of the reinforcing effects of opioids, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 338 (1988) 392–396.
- Berglund, L.A., Millington, W.R. and Simpkins, J.W., Gonadal steroid and chronic morphine treatment do not change the posttranslational processing of beta-endorphin in the rat brain, *Life Sci.*, 44 (1989) 591–601.
- Bloom, F., Battenberg, E., Rossier, J., Ling, N. and Guillemin, R., Neurons containing beta-endorphin in rat brain exist separately from those containing enkephalin: immunocytochemical studies, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 1591–1595.
- Bronstein, D.M. and Akil, H., Effects of chronic morphine treatment on beta-endorphin immunoreactive forms in rat brain, *Soc. Neurosci. Abstr.*, 13 (1987) 637.
- Childers, S.R., Simantov, R. and Snyder, S.H., Enkephalin: radioimmunoassay and radioreceptorassay in morphine-dependent rats, *Eur. J. Pharmacol.*, 46 (1977) 289–293.
- Deakin, J.F., Dostrovsky, J.O. and Smythe, D., Influence of N-terminal acetylation and C-terminal proteolysis on the analgesic activity of beta-endorphin, *Biochem. J.*, 189 (1980) 501–506.
- Dores, R.M., Jain, M. and Akil, H., Characterization of the forms of β -endorphin and α -MSH in the caudal medulla of the rat guinea pig, *Brain Research*, 377 (1986) 251–260.
- Eipper, B.A. and Mains, R.E., Structure and function of pro-adrenocorticotropin/endorphin and related peptides, *Endocrinol. Rev.*, 1 (1980) 247–262.
- Fratta, W., Yang, H.Y.T., Hong, J. and Costa, E., Stability of Met-enkephalin content in brain structures of morphine-dependent or footshock-stressed rats, *Nature (Lond.)*, 268 (1977) 452–453.
- Gramsch, C., Kleber, G., Volker, H., Pasi, A., Mehraein, P. and Herz, A., Pro-opiocortin fragments in human and rat brain: β -endorphin and α -MSH are the predominant peptides, *Brain Research*, 192 (1980) 109–119.
- Holtt, V., Przewlocki, R. and Herz, A., β -endorphin-like immunoreactivity in plasma, pituitaries and hypothalamus of rats following treatment with opiates, *Life Sci.*, 23 (1978) 1057–1066.
- Holtt, V., Morris, B., Haarmann, I., Reimer, S. and Becker, K.-F., Opioid peptide gene expression in rats treated with opiates. In J. Cros, J.-Cl. Meunier and M. Hamon (Eds.), *Progress in Opioid Research*, Pergamon, Oxford, 1989, pp. 711–714.
- Joseph, S.A., Immunoreactive adrenocorticotropin in rat brain: a neuroanatomical study using antiserum generated against ACTH_{1-39}}, *Am. J. Anat.*, 158 (1980) 533–548.
- Khachaturian, H., Lewis, M., Tsou, K. and Watson, S.J., β -Endorphin, α -MSH, ACTH, and related peptides. In A. Björklund and T. Hökfelt (Eds.), *Handbook of Chemical Neuroanatomy, Vol. 4: GABA and Neuropeptides in the CNS, Part I*, Elsevier, Amsterdam, 1985, pp. 216–272.
- Khachaturian, H., Alessi, N., Munfakh, N. and Watson, S.J., Ontogeny of opioid and related peptides in the rat CNS and pituitary: an immunocytochemical study, *Life Sci.*, 33 suppl. I (1983) 61–64.
- Koenig, J.I., Meltzer, H.Y. and Gudelsky, G.A., Morphine or capsaicin administration alters the secretion of beta-endorphin into the hypophyseal portal vasculature of the rat, *Neuroendo-*

The present results reveal the importance of measuring several parameters of biosynthetic activity, and sampling at multiple time points, when studying the effects of pharmacological (or other) manipulations on endogenous peptide biosynthesis. If peptide measurements of tissue and sieved extracts were determined at the 7-day time point only, one would most likely conclude that exogenous opiate administration had no effect on POMC biosynthesis. However, by measuring POMC mRNA levels and determining peptide levels at different times during the morphine treatment, we have presented evidence that morphine treatment may inhibit the activity of βE -ir-containing neurons. In future studies, it will be interesting to examine whether these findings will generalize to other morphine administration paradigms.

Acknowledgements. We would like to thank the National Institute for Drug Abuse (NIDA) for providing us with morphine and placebo pellets, Dr J. Roberts for the mouse POMC cDNA, and Dr J. Eberwine for the rat POMC cDNA. This research was supported in part by NIDA (DA02265) to H.A., the Medical Research Council of Canada, the University of Michigan Gastrointestinal Peptide Research Center, and an Endocrinology, and Metabolism training grant to D.M.B.

- crinology*, 43 (1986) 611–617.
- 21 Kosterlitz, H.W. and Hughes, J., Some thoughts on the significance of enkephalin, the endogenous ligand, *Life Sci.*, 17 (1975) 91–96.
 - 22 Millan, M.H., Millan, M.J. and Przewlocki, R., Lesions of the hypothalamic arcuate nucleus modify discrete brain pools of dynorphin in addition to β -endorphin in the rat, *Neurosci. Lett.*, 48 (1984) 149–154.
 - 23 Mochetti, I. and Costa, E., Down regulation of hypothalamic pro-opiomelanocortin system during morphine tolerance, *Clin. Neuropharmacol.*, 9 Suppl. 1 (1986) 125–127.
 - 24 Mochetti, I., Ritter, A. and Costa, E., Down-regulation of pro-opiomelanocortin synthesis and beta-endorphin utilization in hypothalamus of morphine-tolerant rats, *J. Mol. Neurosci.*, 1 (1989) 33–38.
 - 25 Myers, R.M., Lerman, L.S. and Maniatis, T., A general method for saturation mutagenesis of cloned DNA fragments, *Science*, 229 (1985) 242–247.
 - 26 Nicolas, P., Hammonds Jr., R.G. and Li, C.H., Beta-endorphin analgesia is inhibited by synthetic analogs of beta-endorphin, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 3074–3077.
 - 27 Schwartz, J.P. and Costa, E., Hybridization approaches to the study of neuropeptides, *Ann. Rev. Neurosci.*, 9 (1986) 277–304.
 - 28 Schwartzberg, D.G. and Nakane, P.K., ACTH-related peptide containing neurons within the medulla oblongata of the rat, *Brain Research*, 276 (1983) 351–356.
 - 29 Smith, A.I. and Funder, J.W., Pro-opiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues, *Endocrine Rev.*, 9 (1988) 159–179.
 - 30 Starke, K., Regulation of noradrenaline release by presynaptic receptor systems, *Rev. Physiol. Biochem. Pharmacol.*, 77 (1977) 1–124.
 - 31 Trendelenburg, U., Mechanisms of supersensitivity and subsensitivity to sympathomimetic amines, *Pharmacol. Rev.*, 18 (1966) 629–640.
 - 32 Uhl, G.R., Ryan, J.P. and Schwartz, J.P., Morphine alters preproenkephalin gene expression, *Brain Research*, 458 (1988) 391–397.
 - 33 Watson, S.J., Barchas, J.D. and Li, C.H., Beta-lipotropin: localization in cells and axons in rat brain by immunocytochemistry, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 733–738.
 - 34 Watson, S.J., Akil, H., Richard III, C.W. and Barchas, J.D., Evidence for two separate opiate peptide neuronal systems, *Nature (Lond.)*, 275 (1978) 226–228.
 - 35 Weber, E., Evans, C.J. and Barchas, J.D., Acetylated and non-acetylated forms of beta-endorphin in rat brain and pituitary, *Biochem. Biophys. Res. Commun.*, 103 (1981) 698–705.
 - 36 Wesche, D., Holtt, V. and Herz, A., Radioimmunoassay of enkephalins. Regional distribution in rat brain after morphine treatment and hypophysectomy, *Arch. Pharmacol.*, 301 (1977) 79–82.
 - 37 Zakarian, S. and Smythe, D., Distribution of active and inactive forms of endorphins in rat pituitary and brain, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 5972–5976.