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Quantitation of proenkephalin A messenger RNA in bovine brain, pituitary and adrenal medulla: correlation between mRNA and peptide levels

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Concentrations of mRNA coding for the opioid peptide precursor proenkephalin A were measured in bovine brain areas, pituitary and adrenal medulla. In all tissues, a single hybridizable species of 1400 bases in size was found by Northern blot anlysis using as a probe a single-stranded (ss) cDNA complementary to bovine proenkephalin A mRNA. In solution hybridization experiments the distribution of the mRNA was quantified. Considerable differences were found for the abundance of proenkephalin A mRNA in the various tissues: from 0.023% in the adrenal medulla to 0.00002% in the adenohypophysis. Relative abundance in the various brain areas varied >20-fold, being highest in the caudate nucleus (0.0025%) and lowest in the thalamus and substantia nigra (0.0001%). Comparison with immunoreactive peptide concentrations in these tissues showed a close correlation between the levels of proenkephalin A mRNA and the immunoreactive peptides.

Key words: adrenal medulla/bovine brain-pituitary/Northern blotting/proenkephalin A/solution hybridization

Introduction

The structure of the three opioid peptide precursors (pro-opiomelanocortin, proenkephalin A, proenkephalin B/prodynorphin) were eluciated by recombinant DNA techniques [Nakanishi *et al.*, 1979; Noda *et al.*, 1983; Gubler *et al.*, 1982; Kakidani *et al.*, 1982; for a review, see Numa (1984)]. The primary structure of proenkephalin A (PENK A) has been obtained by cloning and sequencing the DNA complementary to its mRNA in bovine adrenal medulla and human pheochromocytoma (Noda *et al.*, 1982; Gubler *et al.*, 1982; Comb *et al.*, 1982). In addition, recent data indicate that a similar precursor protein also exists in bovine and rat brain (Jingami *et al.*, 1984; Tang *et al.*, 1983; Legon *et al.*, 1982; Kley *et al.* unpublished results). Peptides derived from this precursor protein by proteolytic cleavage are present in a large number of neurons throughout the brain, as indicated by radioimmunoassay and immunohistochemical studies (Pittius *et al.*, 1984; Watson *et al.*, 1984).

In contrast to the measurement of peptides cleaved from PENK A, determination of mRNA reflects the biosynthesis of the precursor molecule in cell bodies before its post-translational processing. This can be achieved either by *in situ* hybridization (e.g., Bloch *et al.*, 1984) or solution hybridization *in vitro*. In the present study the development of a solution hybridization assay for proenkephalin A enabled us to make a precise and quantitative analysis of the correlation between peptide and mRNA levels specific to the proenkephalin A system in bovine brain.

Results

Figure 1 shows the Northern blot analysis of poly(A)-rich RNA obtained from 1.5 – 1.8 g of bovine tissue. The bovine adrenal medulla and all brain regions examined revealed a single hybridizable species of a molecular size of ~1400 bases, suggesting a similar structure of the brain mRNA species to that in the adrenal medulla [coding, non-coding parts, poly(A) tail of 200 – 300 bases (Noda *et al.*, 1982)]. The amount present, as indicated by the intensity of blackening, was much higher in the adrenal medulla than in the brain. The highest levels in the brain were found in the neostriatum (caudate nucleus-putamen). Somewhat lower amounts were measured in the globus pallidus. Intermediate PENK A mRNA levels were seen in the hypothalamus and frontal cortex. Very low but still detectable amounts were found in all remaining areas, with the lowest levels in the hippocampus.

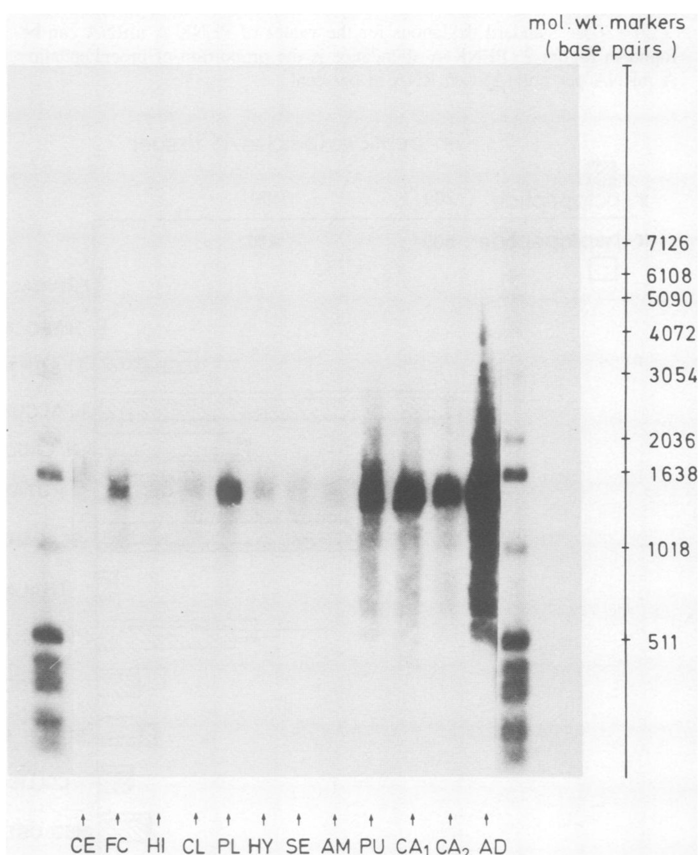

Fig. 1. Northern blot anlaysis of proenkephalin A mRNA in bovine tissue. Poly(A)-rich RNA obtained from 1.5 – 1.8 g tissue was separated by electrophoresis on a 1.2% agarose gel, blotted onto nitrocellulose and hybridized with ^{32}P-labeled M13$_{510}$PENK. CE: cerebellum [50 μg poly(A)-rich RNA], FC: frontal cortex (11 μg), CL: colliculi (6.3 μg), HI: hippocampus (3.1 μg), PL: pallidum (4.1 μg), HY: hypothalamus (6.7 μg), SE: septum (10 μg), AM: amygdala (4.1 μg), PU: putamen (5.6 μg), CA$_1$ and CA$_2$: two independent extractions of caudate (CA$_1$: 3.5 μg, CA$_2$: 2.5 μg), AD: adrenal medulla (1.4 μg, from 0.15 g tissue). Mol. wt. standards on both sides are ^{32}P end-labeled kilobase ladders. The blot shows a representative distribution of four separate extractions.

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Table I. Characteristics of PENK A mRNA in bovine brain, pituitary and adrenal medulla

Tissue	Total RNA (mg/g)	Poly(A)-rich mRNA (μg/g)	PENK A mRNA (pg/g)	Abundance (% x 10^3)
Frontal cortex	0.7	4.8	19	0.4
Hippocampus	0.5	2.4	5.0	0.2
Septum	0.5	6.0	25	0.4
Accumbens	0.4	6.0	67	1.1
Caudate	0.6	3.3	82	2.5
Putamen	0.5	2.4	30	1.3
Gl. pallidus	0.5	6.3	35	0.6
Thalamus	0.6	4.0	5.1	0.1
Hypothalamus	0.7	6.5	31	0.5
Amygdala	1.3	8.4	17	0.2
Substantia nigra	0.5	4.2	4.1	0.1
Colliculi	0.6	6.0	13	0.2
Med. obl. dors.	0.5	5.4	16	0.3
Cerebellum	1.5	30	31	0.1
Adrenal medulla	0.5	9.5	2200	23
Pituitary anter.	3.0	73	15	0.02
Pituit. neuroint.	2.4	76	68	0.09

Total RNA yield is given as mg/g tissue weight, poly(A)-rich RNA yield as μg/g; the values are means of 2–4 tissue extractions with a variation of ±20–30%. Standard deviations for the values of PENK A mRNA can be found in Figure 2. PENK A abundance is the proportion of proenkephalin A mRNA per poly(A)-rich RNA in per cent.

A quantitative analysis of the differential distribution is obtained if mRNA is measured by solution hybridization. In Table I the levels of PENK A mRNA are given in pg/g tissue, together with the yield of total RNA (in mg/g), the poly(A)-rich RNA in μg/g and the PENK A mRNA abundance in % of poly(A)-rich RNA. (The reliability of the values given was confirmed by a post-mortem degradation analysis, see Table II and Discussion.) Pronounced regional variations of the PENK A mRNA were found, ranging up to 20-fold between the caudate nucleus and the substantia nigra. Levels in the adrenal medulla were 20–400 times higher than in the brain.

These remarkable differences can only partly be attributed to a variation of cell body density in the various tissues. The total RNA yield, which might be regarded as a relative measure of cell body density, is relatively constant in the brain, ranging from 0.4 to 0.7 mg/g, the only exception with a higher density being the cerebellum and the amygdala (1.5 and 1.3 mg/g, respectively). In contrast, the pituitary possesses an ~5–10 times higher RNA content, reflecting the higher cell body density.

Somewhat larger differences were observed in the poly(A)-rich fraction of total RNA (3- to 4-fold variation within the brain). On the other hand, very large differences were found in the relative abundance of PENK A mRNA, that is the fraction of mRNA that is coding for PENK A, varying up to 20-fold within the brain. Interestingly, the concentrations of PENK A mRNA in the pituitary lobes were much lower than in the caudate, although their content in poly(A)-rich RNA was much higher,

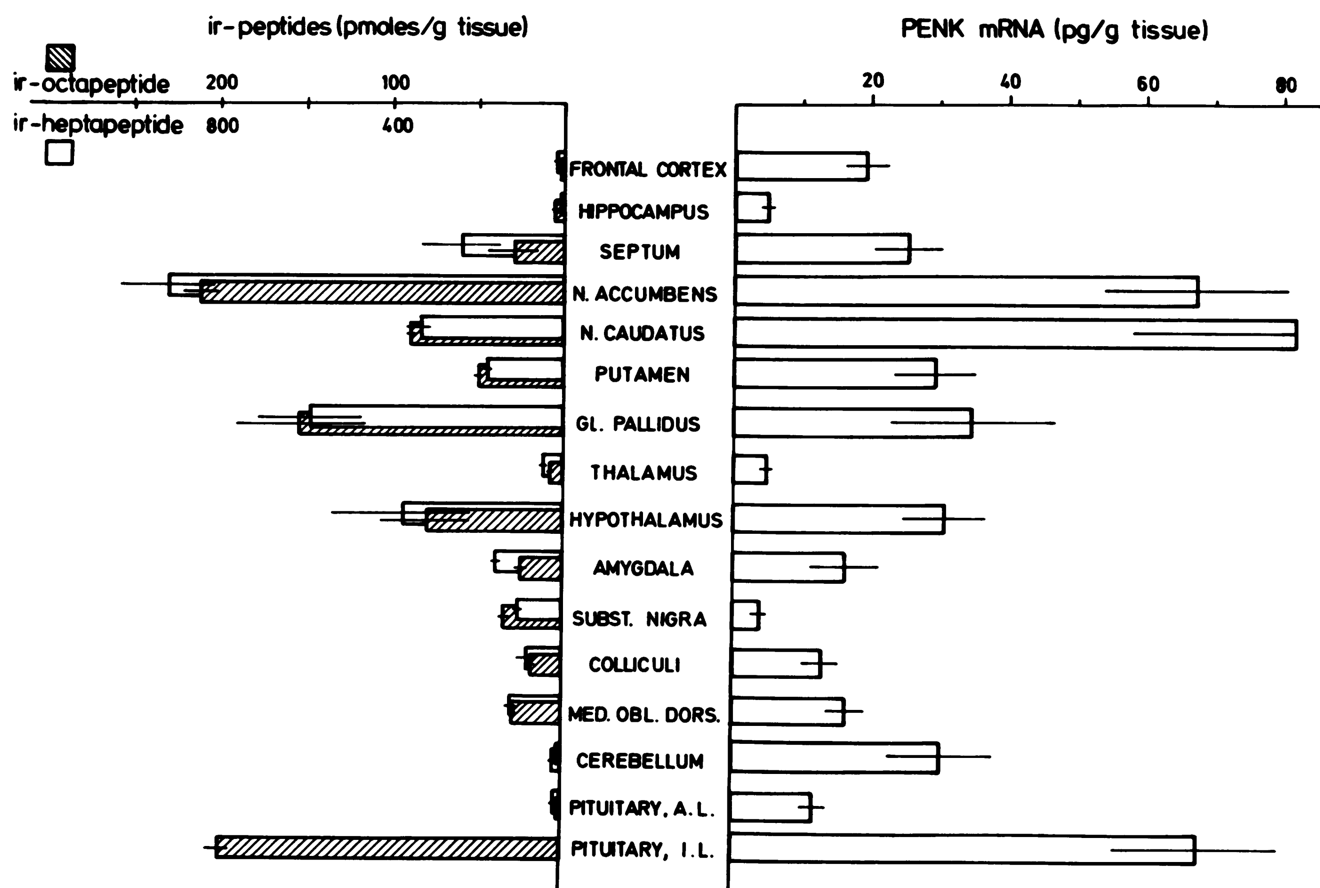

Fig. 2. Comparison of the concentrations of immunoreactive (ir-) [Met]enkephalin-Arg[6]-Phe[7] (heptapeptide) and [Met]enkephalin-Arg[6]-Gly[7]-Leu[8] (octapeptide) with that of proenkephalin A mRNA in bovine brain and pituitary. Ir-peptide levels (expressed as pmol peptide/g tissue weight) represent means ± S.E.M. of four determinations, mRNA concentrations determined by solution hybridization show means ± SD of two to four tissue extractions, expressed as pg PENK A mRNA/g tissue weight.

indicating that only a minor fraction of the mRNA pool in the pituitary represented PENK A mRNA.

In Figure 2 a comparison is made between the relative distribution of PENK A mRNA/g and two proenkephalin-A-derived peptides, the heptapeptide [Met]enkephalin-Arg^6-Phe^7 and the octapeptide [Met]enkephalin-Arg^6-Gly^7-Leu^8 (Gubler *et al.*, 1982). These two peptides are present in single copies in this precursor only and appear to be end products in the enzymatic processing of proenkephalin A in neurons [the heptapeptide levels were found to be four times higher than those of the octapeptide in all areas, indicating a possible further processing of the octapeptide to [Met]enkephalin (Pittius *et al.*, 1984)].

In general, a close correspondence seems to exist between the two, although regional differences were more pronounced for peptide levels (250-fold as compared with 20-fold). If the concentration of heptapeptide is plotted against the concentration of PENK A mRNA, a positive correlation with a correlation coefficient of 0.77 is found ($p < 0.01$, Pearson product-moment correlation analysis).

Discussion

We have determined the quantitative distribution of the mRNA coding for proenkephalin A and peptides derived from it in bovine brain, pituitary and adrenal medulla. Bovine tissue was used for the analysis, the advantage being that due to its large size it can readily be dissected into many defined areas and at the same time yields enough tissue for RNA and peptide extraction from each region. In addition, a cloned cDNA specific for bovine proenkephalin A was available (Noda *et al.*, 1982). A post-mortem degradation analysis has shown that no changes in the levels of hybridizable PENK A mRNA (Table II) and PENK A-derived peptides (Pittius *et al.*, 1984) have occurred by the time tissues were extracted for the quantitative distribution study, confirming the reliability of the absolute values determined.

Northern blot analysis as well as the more quantitative solution hybridization assay revealed a remarkable difference in the concentration of proenkephalin A mRNA in the various tissues. For instance, the proenkephalin A mRNA level in the adrenal medulla is several hundred times higher than in the substantia nigra, corresponding to an approximate abundance ranging from 0.023% to <0.0001% (Table I). The major site of synthesis in the brain appears to be in the neostriatum (accumbens-caudate-putamen). This area also contains the highest concentration of PENK A-derived peptides (Figure 2). This is in agreement with radioimmuno- and immunohistochemical studies in cats and rats, showing that the highest density of neuronal pericarya and processes staining for PENK A-derived peptides is in the neostriatum (Watson *et al.*, 1984; Bloch *et al.*, 1983; Boarder *et al.*, 1982). Lesion studies have established that most of the enkephalin-containing fibers from the neostriatum do project to the globus pallidus (Cuello and Paxinos, 1978). Moreover, no enkephalin-containing cell bodies were detected in the globus pallidus of rats after injection of colchicine, a drug which inhibits the transport of the peptides from cell bodies into the axons (Del Fiacco *et al.*, 1982). In contrast, our results clearly indicate the pallidum as a major site of synthesis of proenkephalin A in bovine brain, revealing large species differences.

Table II. Post-mortem degradation analysis of proenkephalin A mRNA

t = 0 (extrapol.)	100%
t = 20 min	98% ± 8%
t = 1 h	104% ± 23%
t = 2 h	79% ± 13%
t = 4 h	112% ± 8%
t = 8 h	107% ± 10%
t = 24 h	54% ± 12%

Relative PENK A mRNA concentrations in bovine striata, frozen at various times after death, are given relative to t = 0 (means ± SD of two tissues).

In addition, the fairly good correlation between the levels of immunoreactive peptides and PENK A mRNA (Figure 2) indicates that nerve fibers of PENK A-producing cells do not project over long distances to other brain areas as is the case with the pro-opiomelanocortin (POMC) system where the cell bodies synthesizing POMC are almost exclusively located in the hypothalamus but whose processes do project to other brain areas (Bloom *et al.*, 1978; Watson *et al.*, 1984; Civelli *et al.*, 1982).

An interesting observation is the demonstration of high levels of both PENK A mRNA and PENK A-derived peptides in the neurointermediate lobe of the pituitary. It has been suggested that enkephalin-like peptides in the posterior lobe originate from hypothalamic enkephalinergic neurons projecting to it (Rossier *et al.*, 1979). However, our results also provide evidence for the existence of proenkephalin A-producing cells in the intermediate/posterior lobe of the pituitary. The precise location of the synthesizing cell bodies in the intermediate or posterior lobe remains to be investigated.

The fact that the size of the PENK A mRNA appears to be the same in all tissues examined, indicates that there appears to be no alternative processing, generation of aberrant transcripts or alternate polyadenylation of PENK A mRNA in these tissues.

Materials and methods

Isolation of mRNA

Bovine brains and adrenal medullae were obtained from the slaughterhouse, dissected within 2 h and flash-frozen in liquid nitrogen. Tissues were homogenized in 10 volumes 0.5% SDS, 75 mM Tris, 25 mM EDTA pH 8.0 and phenol (1:1), followed by phenol/chloroform:isoamylalcohol (24:1) extraction according to Rosen and Monahan (1982). Following ethanol precipitation the pellet was washed with 3 M sodium acetate pH 5.5 to remove most of the remaining DNA. The yield of total RNA, as measured by u.v. absorption (1 A_{260}/ml = 40 μg), ranged from 0.4 to 3.0 mg/g, depending on the kind of tissue. Enrichment of poly(A) RNA by two passages over oligo(dT)-cellulose (Aviv and Leder, 1972) gave a final recovery of 0.5 – 3% poly(A)-rich RNA of total RNA.

Filter hybridization

Poly(A)-rich RNA was denatured with glyoxal (Thomas, 1980), run on a 1.2% agarose gel and transferred to nitrocellulose sheets. As a molecular size standard, a kilobase pair ladder, ^{32}P end-labeled with Klenow fragment of DNA polymerase I (Maniatis *et al.*, 1982), was used. RNA was fixed to the filters by baking them under reduced pressure at 80°C and pre-hybridized for 2 – 4 h according to Wahl *et al.* (1979). Following hybridization of a single-stranded cDNA probe (see below) to the RNA at 42°C, the sheets were washed four times in 2 x SSC (1 x SSC = 150 mM sodium chloride, 15 mM sodium citrate pH 7.0) containing 0.1% SDS at room temperature and three times in 0.1 x SSC containing 0.1% SDS at 50°C. Dried filters were autoradiographed at −70°C with intensifying screens.

Preparation of single-stranded (ss) probe

The proenkephalin A plasmid pENK5 which contains an 820-bp insert including the 324 base 3′-non-translated region of the mRNA (a generous gift of Dr S.Numa, Kyoto, Japan) was used as a source of cDNA (Noda *et al.*, 1982). Restriction with *Pst*I generates 510- and 310-bp fragments. The 510-bp fragment was subcloned into the *Pst*I site of M13(mp8). An M13 recombinant containing message-sense insert ('mDNA') was used as a template for cDNA synthesis by annealing a 15-base sequencing primer in 10 mM Tris, 10 mM $MgCl_2$, followed by elongation with Klenow fragment of DNA polymerase I in 10 mM Tris, 10 mM $MgCl_2$, 300 μM dTTP, dCTP, dGTP and 0.6 μM ^{32}P-labeled dATP (sp. act. ≥3000 Ci/mmol).

Recovery of the newly synthesized cDNA (sp. act. 1 – 2 x 10^9 c.p.m./μg) was obtained by denaturing Biogel A 50 chromatography (0.1 mM NaOH) as described by Durnam and Palmiter (1983). The resulting radiolabeled ss cDNA was analyzed by 1.5% agarose gel electrophoresis, indicating a size of ~450 bases in length.

Solution hybridization

Hybridization of ss cDNA to mRNA in excess was performed in 40% formamide, 0.6 M sodium chloride, 20 mM Tris, 4 mM EDTA according to Durnam and Palmiter (1983). The reactions were carried out under paraffin oil in sterile disposable polypropylene tubes at 42°C. The extent of hybridization was determined by treatment with S1 nuclease in 500 μl of 0.3 M sodium chloride, 30 mM sodium acetate pH 4.6, 3 mM zinc acetate, 100 μg/ml salmon sperm DNA and four units of S1 nuclease at 42°C for 2 h, followed by precipitation with 6 M trichloroacetic acid (TCA) on Whatman GF/C filters. Quantitation of PENK A mRNA in unknown samples was done by comparing the TCA-precipitable radioactivity with those in a standard curve constructed with 1 – 1000 pg of recombinant M13 ($M13_{510}$PENK) containing the message-sense insert ('mDNA'). Approximately 5 pg of 'mDNA' are equivalent to 1 pg of PENK A mRNA in sequence content under the chosen conditions. Concentrations of mRNA were expressed as pg/g 'mDNA' equivalents.

Radioimmunoassay

Tissue extraction and radioimmunoassay were performed as previously described (Pittius *et al.*, 1984). Antisera were kind gifts from Dr Weber, Stanford, USA.

Materials used

Kilobase pair ladder, enzymes, ultra-pure phenol, SDS were purchased from BRL (Karlsruhe, FRG), suprapure agarose and oligo(dT) from Pharmacia (Freiburg, FRG), nitrocellulose paper from Schleicher and Schuell (Dassel, FRG), [α-^{32}P]dATP from Amersham (Braunschweig, FRG). All other chemicals were from Sigma (Taufkirchen, FRG) or Merck (Darmstadt, FRG). All solutions and glass/ plasticware were autoclaved before use when working with RNA.

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References

Aviv,H. and Leder,P. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 1408-1412.

Bloch,B., Baird,A., Ling,N., Benoit,R. and Guillemin,R. (1983) *Brain Res.*, **263**, 251-257.

Bloch,B., Milner,R.J., Baird,A., Gubler,U., Reymond,C., Böhlen,P., Guellec,D. and Bloom,F.E. (1984) *Regul. Pept.*, **8**, 345-354.

Bloom,F.E., Battenberg,E., Rossier,J., Ling,N. and Guillemin,R. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1591-1595.

Boarder,M.R., Lockfeld,A.J. and Barchas,J.D. (1982) *J. Neurochem.*, **38**, 299-304.

Civelli,O., Birnberg,N. and Herbert,E. (1982) *J. Biol. Chem.*, **257**, 6783-6787.

Comb,M., Seeburg,P.H., Adelman,J., Eiden,L. and Herbert,E. (1982) *Nature*, **295**, 663-666.

Cuello,A.C. and Paxinos,G. (1978) *Nature*, **271**, 178-180.

Del Fiacco,M., Paxinos,G. and Cuello,A.C. (1982) *Brain Res.*, **231**, 1-17.

Durnam,D.M. and Palmiter,R.D. (1983) *Anal. Biochem.*, **131**, 385-393.

Gubler,U., Seeburg,P., Hoffman,B.J., Gage,L.P. and Udenfriend,S. (1982) *Nature*, **295**, 206-208.

Jingami,H., Nakanishi,S., Imura,H. and Numa,S. (1984) *Eur. J. Biochem.*, **142**, 441-447.

Kakidani,H., Furutani,Y., Takahashi,H., Noda,M., Morimoto,Y., Hirose,T., Asai,M., Inayama,S., Nakanishi,S. and Numa,S. (1982) *Nature*, **298**, 245-249.

Legon,S., Glover,D.M., Hughes,J., Lowry,P.J., Rigby,P.W.J. and Watson,C.J. (1982) *Nucleic Acids Res.*, **10**, 7905-7918.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.

Nakanishi,S., Inoue,A., Kita,T., Nakamura,M., Chang,A.C.Y., Cohen,S.N. and Numa,S. (1979) *Nature*, **278**, 423-427.

Noda,M., Furutani,Y., Takahashi,H., Toyosato,M., Hirose,T., Inayama,S., Nakanishi,S. and Numa,S. (1982) *Nature*, **295**, 202-206.

Numa,S. (1984) in Udenfriend,S. and Meienhofer,J. (eds.), *The Peptides: Analysis, Synthesis, Biology*, Academic Press, Orlando, USA, pp. 1-23.

Pittius,C.W., Seizinger,B.R., Pasi,A., Mehraein,P. and Herz,A. (1984) *Brain Res.*, **304**, 127-136.

Rosen,J.M. and Monahan,J. (1982) in Schrader,W.T. and O'Malley,B.W. (eds.), *Laboratory Methods Manual for Hormone Action and Molecular Endocrinology*, Texas Medical Center, Houston, TX, pp. 4-1 – 4-69.

Rossier,J., Battenberg,E., Pittman,Q., Bayon,A., Koda,L., Miller,R., Guillemin, R. and Bloom,F. (1979) *Nature*, **277**, 653-655.

Tang,F., Costa,E. and Schwartz,J.P. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3841-3844.

Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201-5205.

Wahl,G.M., Stern,M. and Stark,G.R. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3683-3687.

Watson,S.J., Akil,H., Khachaturian,H., Young,E. and Lewis,M.E. (1984) in Hughes,J., Collier,H.O.J., Rance,M.J. and Tyers,M.B. (eds.), *Opioids – Past, Present and Future*, Taylor and Francis, London and Philadelphia, pp. 145-178.

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