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Effect of morphine treatment and withdrawal on endogenous methionine- and leucine-enkephalin levels in primate brain

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Methionine-enkephalin (ME) and leucine-enkephalin (LE) are two endogenous pentapeptides that bind to opioid receptors in brain and possess morphine-like pharmacological properties [1]. As yet there has been no account of the comparative concentration of ME and LE in monkey brain, although enkephalin-like immunoreactivity has been reported and mapped [2, 3]. This paper describes a specific method for measurement of ME and LE concentrations in brain tissue and the use of this technique to quantify the concentration of both peptides in monkey hippocampus and putamen. In addition, the concentrations of both enkephalins were measured in hippocampus of morphine-treated monkeys and monkeys in morphine withdrawal. Hippocampus was chosen for analysis because it receives a well-defined input from the noradrenergic nucleus, locus coeruleus [4], whose firing rate responds to morphine administration and morphine withdrawal [5], and because electrophysiological studies have shown that, despite the relatively small number of opioid receptors there, opioids have a powerful effect on the principal neurons of the hippocampus [6].

Materials and methods

Monkeys (five male, four female *Cercopithecus aethiops sabaeus*) were untreated for 10 days, except for participating in a daily sham subcutaneous pellet implant procedure. On day 10, 3 hr before being killed, three animals (one male, two female) received 20 mg/kg naltrexone via a nasogastric tube; the remaining monkeys of this group received saline by the same route. Other monkeys of the same species (three male, four female) received for 10 days a daily subcutaneous implant of a slow release preparation of morphine (75 mg/implant). Three of these morphine-treated monkeys (one male, two female) received naltrexone (20 mg/kg) 3 hr before being killed, as above; the others received saline. Prior to removal and dissection of brain, each animal was injected with ketamine (6–8 mg/kg, i.m.). Ten to fifteen minutes later sodium pentobarbital was infused until loss of corneal reflex. Further details of sacrifice and brain dissection have been published previously [7]. Brains were immediately chilled in an ice bath and then dissected on an ice-cold metal surface. Dissection was complete within 25 min of brain removal. Samples were frozen in liquid nitrogen until assay.

The whole of each brain sample from one side of the brain was weighed and then homogenized in 3 ml of ice-cold 1 M formic acid. The homogenizing tube was rinsed with a further 1 ml of 1 M formic acid. The homogenate plus rinsing was centrifuged at 12,500 *g* for 30 min at 4°. The supernatant fraction was passed through a small reverse-phase column ("C-18 Sep-Pak", Waters Associates, Milford, MA), which had been washed previously with 10 ml methanol followed by 20 ml water. The retained portion of the sample was washed with 10 ml of 1 M formic acid and then eluted with 2 ml methanol. After evaporating the eluate to dryness at 35° under a jet of nitrogen, the residue was reconstituted in 0.15 ml of a buffer comprising 0.04 M formic acid adjusted to pH 3.15 with triethylamine (TEAF) [8]. Fifty microliters of this extract was separated by high pressure liquid chromatography (HPLC), utilizing a 25 cm μ-Bondapak column (Waters Associates). Acetonitrile (20%) and TEAF (80%) buffer was pumped isocratically at 1 ml/min. The elution times of a number of peptides (Sigma Chemical Co., St. Louis, MO) detected by u.v. absorbance at 210 or 280 nm are shown in Table 1. Fractions corresponding to the retention times of ME and LE were collected and freeze dried. These residues were then assayed for ME and LE concentrations using radioimmunoassay (RIA) kits (Immuno Nuclear Corp., Stillwater, MN). Sensitivity of RIA detection in both assays was 8 pg/assay tube. Recovery was assessed by taking brain samples spiked with authentic unlabeled ME and LE through the procedure. This showed that 82% of ME and 84% of LE were recovered. Reported values are corrected for these losses.

Table 1. Separation of peptides by HPLC

Peptide	Retention time* (min)
Tyr-Tyr-Gly	2.5
Dynorphin (1-13)	3.5
des-Tyr-Met enkephalin (Gly-Gly-Phe-Met)	5.5
Met-enkephalin (Tyr-Gly-Gly-Phe-Met)	8.0
des-Tyr-Leu-enkephalin (Gly-Gly-Phe-Leu)	8.0
Alpha-endorphin (LPH 61-76)	8.75
Neurotensin	12.0
Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu)	12.75
Alpha MSH	18.0
Substance P	27.0
Gamma-endorphin-des-Tyr (LPH 62-77)	27.5
Beta-endorphin (LPH-61-91)	>30
ACTH	>30

Conditions: buffer, 20% acetonitrile and 80% TEAF; flow rate, 1 ml/min; and column, 25 cm μ-Bondapak C18. Abbreviations: LPH, beta-lipotropin; MSH, melanocyte stimulating hormone; and ACTH, adrenocorticotropic hormone.

* Solvent front, 2.5 min.

The cross-reactivity of the ME antibody used in this paper has been investigated by others ([9]; Dr. K. Jhamandas, personal communication; Immuno Nuclear Corp. data), and the ligands which have been characterized are listed in Table 2. Thus, none of the peptides listed in Table 1 or Table 2 interferes with the assay of ME by virtue of lack of cross-reactivity and/or because of different chromatographic retention on the HPLC column.

None of the peptides listed in Table 1 interferes with LE assay; neurotensin which eluted close to LE did not cross-react with the LE antibody when 500 pg was added to the RIA. In addition, it has been determined that somatostatin does not cross-react with this antibody (Immuno Nuclear Corp. data).

Results and discussion

ME and LE concentrations in hippocampus and putamen of sham-pelleted monkeys are shown in Table 3. Putamen contained concentrations of both peptides that were 40–50 times higher than in hippocampus. In both regions the content of ME was approximately 3-fold that of LE. There are very few reports of measurement of either enkephalin in primate brain. Simantov *et al.* [3] quantified enkephalin-like immunoreactivity in rhesus monkey brain as units of activity observed in a receptor displacement assay and reported that putamen contained 1.85 times more enkephalin than hippocampus. Emson *et al.* [10] and Taquet *et al.* [11] measured ME-like immunoreactivity in a group of control human post-mortem brains and reported values in hippocampus and putamen very similar to those found in the present study in the vervet monkey. Emson *et al.* [10], in addition, found LE concentration to be 4.48 times lower than ME concentration in putamen.

Naltrexone administration to sham-pelleted monkeys did not alter levels of either enkephalin compared with those sham-pelleted animals who received saline (Table 3); therefore, for all other statistical analyses these groups were merged to form a larger control group. Morphine treatment induced a statistically significant decrease (41%) in ME concentration (Table 3) in the hippocampus. In addition, the mean LE concentration of the morphine-treated monkeys was lower (52%) than in the sham-pelleted animals (Table 3), but this decrease did not reach statistical significance. Naltrexone treatment for 3 hr returned ME and LE levels to within the control range (Table 3).

Blind behavioral ratings immediately before sacrifice revealed that the morphine–naltrexone group of animals experienced the typical morphine-withdrawal syndrome of this species [12].

Although the usefulness of measuring static peptide concentration has not yet been established, the naltrexone-reversal of the morphine-induced decrease of ME concentration indicates that enkephalin levels are able to alter rapidly in response to an appropriate stimulus. It will be important to establish the time course of this effect in relation to the behavioral changes. Interestingly, a recent study in this laboratory [13] has shown that substance P levels were reduced significantly in the ventral tegmental area of the rat after as little as 20 min of exposure to mild footshock.

The present paper describes, for the first time, the effects of morphine treatment and morphine withdrawal on enkephalin levels in primate brain. Several studies have been performed previously in the rat, but these generally utilize less specific methods than that described in the present paper. Chronic morphine treatment of rats has been reported to reduce brain enkephalin concentration after 3 days [14] or to have no effect after 5 days [14–16], 10 days [17] or 21 days [14]. It may be important though that the study which treated rats for the longest period (30 days) [18] obtained the most striking effect, namely a marked decrease in ME and LE levels, similar to those found in the present study in the vervet monkey. In fact, Kosterlitz and Hughes [19] hypothesized that morphine dependence would lower the rate of enkephalin synthesis.

Table 2. Cross-reactivity of ligands with ME antisera and [^{125}I]ME

Ligand	% Cross-reactivity	Reference
ME	100	[9,*]
LE	0	[9]
des-Tyr-Leu-enkephalin	0	†
Met-(O)-enkephalin	19	[9]
Arg^6-Met-enkephalin	2	[9]
Arg^6-Phe^7-Met-enkephalin	<2	[9]
Tyr-D-Ala-Gly-Phe-Met-ol·CH_3COOH	0	[9]
Tyr-D-Ala-Gly-Phe-Met-(O)-CH_3COOH	0	[9]
Tyr-D-Met-Gly-Phe-Pro-NH_2CH_3COOH	0	[9]
Tyr-Phe-Arg-CH_3COOH	0	[9]
Tyr-Ala-Arg-CH_3COOH	0	[9]
Beta-Lipotropin (61-91)	0	[9]
Alpha-endorphin (61-77)	0	*
Alpha-neo-endorphin	0	*
Beta-endorphin (61-91)	0	[9,*]
Leu-beta-endorphin	0	[9]
Dynorphin	0	[9,*]
Cholecystokinin (1-8)	0	[9]
Vasoactive intestinal polypeptide	0	[9]
Substance P (1-11)	0	[9,*]

* Immuno Nuclear Corp. data.
† This paper.

Table 3. Enkephalin levels in vervet monkey brain

	Hippocampus		Putamen	
	ME	LE	ME	LE
Sham pellet and saline	27.7 ± 5.6 (6)	7.9 ± 3.2 (6)	1104 (2)	398 (2)
Sham pellet and naltrexone	27.4 ± 4.6 (3)	6.0 ± 2.1 (3)		
Morphine pellet and saline	16.4 ± 2.8* (3)	3.5 ± 0.5 (4)		
Morphine pellet and naltrexone	22.3 ± 2.4 (4)	7.5 ± 2.9 (4)		

Values are expressed as mean (±S.E.) ng/g wet weight, with the number of animals given in parentheses.
* Statistically different from sham-pellet animals ($P < 0.05$, Student's *t*-test).

In summary, a sensitive and relatively specific method employing reverse-phase high pressure liquid chromatography and radioimmunoassay has been used to quantify both methionine-enkephalin and leucine-enkephalin in monkey brain. Chronic morphine treatment resulted in a statistically significant decrease in methionine-enkephalin concentration, which was reversed by acute naltrexone treatment.

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Identification of two major reduction products of the hypoxic cell toxin 3-amino-1,2,4-benzotriazine-1,4-dioxide

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Recently, it has been shown that 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR-4233) exhibits a 50- to 200-fold greater toxicity for hypoxic relative to aerobic mammalian cells *in vitro* (E. M. Zeman and J. M. Brown, personal communication)*. Since the presence of hypoxic cells in solid tumors is believed to be a problem for the local control of tumors by radiation therapy, compounds that are selectively toxic for such radiation resistant cells have been proposed as adjuncts to radiation [1]. While two classes of compounds, the mitomycins [2] and the nitroimidazoles [3], are established as agents with selective toxicity toward hypoxic mammalian cells, SR-4233 represents a new class of these agents. Although the selectivity of SR-4233 implies that it is activated by reductive metabolism which is oxygen sensitive, little is known about the reduction chemistry of this molecule aside from applications to synthetic chemistry [4]. In an attempt to elucidate this chemistry, SR-4233 has been reduced in the absence of oxygen using radiation chemical, electrochemical and enzymatic systems. Two major reaction products of these systems have been isolated and characterized with the hope that this information may help in determining the nature of the cytotoxic agent or agents.

* Permission received from J. M. Brown, Stanford University, Palo Alto, CA, to cite this unpublished data.

Materials and methods

The compounds SR-4233, 3-amino-1,2,4-benzotriazine-1-oxide (SR-4317), and 3-amino-1,2,4-benzotriazine (SR-4330) were obtained from Dr. W. Lee of SRI International. Xanthine oxidase (grade III) and xanthine were purchased from the Sigma Chemical Co., St. Louis, MO, and DMSO-d_6 and trifluoroacetic acid-d_1 were obtained from MSD Isotopes, Montreal, P.Q.

Ultraviolet-visible spectra were recorded with a Varian 219 UV-visible spectrophotometer, and proton magnetic resonance (PMR) spectra were acquired with a Nicolet 360 MHz NMR spectrometer at the University of Toronto Biomedical NMR Centre. Analytical and preparative high pressure liquid chromatography (HPLC) were performed isocratically using Waters μBondapak C_{18} columns (3.9 mm × 30 cm; 7.8 mm × 30 cm) with a mobile phase of 20% methanol/water or 20% methanol/10% acetonitrile/water. A flow rate of 2 ml/min was used, and the eluant was passed through a u.v. detector set at 254 nm.

Reducing radicals were produced radiolytically using an AECL ^{60}Co gamma-cell (Atomic Energy of Canada Ltd., Chalk River, Ont.) (~3250 rads·min^{-1}). Solutions for irradiation were typically 0.5 mM in SR-4233, 100 mM in sodium formate, and 10 mM in sodium phosphate (pH 6.9) [5]. Small volumes (~1.5 ml) of the solutions were placed