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Endogenous morphine and codeine in the brain of non human primate

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Morphine is the most used compound among narcotic analgesics. Apart from its presence in the poppy plant, morphine has been shown to be endogenously present in different tissues of mammals and lower animals.

Materials/Methods: The presence of endogenous morphine and codeine was investigated by Gas Chromatography/ Mass Spectrometry (GC/MS) in the brain of non human primate. The release of endogenous morphine from monkey brain slices was studied *in vitro* in the presence of high potassium concentrations with and without calcium in the medium.

Results: Endogenous morphine, and its direct precursor codeine, was for the first time detected by GC/MS in the brain of non human primate. High potassium concentrations depolarized neurons releasing endogenous morphine twofold above basal line levels in a calcium dependent mechanism.

Conclusions: This finding confirms the presence of the endogenous alkaloid throughout the phylogenesis of the nerve system of mammals and lower animals and indicates that endogenous morphine might function as a neuromodulator/neurotransmitter agent in the central nervous system (CNS) of non human primates.

key words: **endogenous morphine • monkey • brain • gc-ms**

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BACKGROUND

Morphine alkaloids in animal tissues were first demonstrated by immunological recognition [1]. Subsequently, endogenous morphine-like compounds have been identified in mouse and calf brain as well as in human [2–4]. The molecular structure of the High Performance Liquid Chromatography (HPLC) purified compound was confirmed as morphine by liquid and gas chromatographic retention times and mass spectrometry in various tissues such as rat and rabbit skin, bovine brain, hypothalamus and adrenal glands, mouse brain, invertebrate and human tissues, mammalian lung and human plasma [1,5–12]. Codeine, morphine and their conjugate have also been identified in human cerebrospinal fluid [7]. Reticuline, thebaine, and codeine are some of the main intermediates of morphine biosynthesis in the poppy plant [13] and these compounds also have been found in mammals [9, 14–17].

The pathways of morphine biosynthesis have been established in the opium poppy and animal tissue starting from L-tyrosine. Radiolabeled reticuline was transformed into salutaridine by rat liver microsomes *in vitro*, and a similar conversion has been observed *in vivo* [18]. The conversion of salutaridine, thebaine and codeine into morphine has been demonstrated in several rat tissues, including the brain, providing evidence for a biosynthetic pathway of endogenous morphine (eM) in mammals [19,28]. The purpose of the present study was to demonstrate the presence of eM and its direct precursor, codeine, in a non human primate as well as to extend these observations to other mammals.

MATERIAL AND METHODS

Animals

Male adult non human primate Cercopitecus Aethiops of a weight of 3.7 Kg was previously quarantined and housed in a cage at 20–24°C. A 12 h light/dark cycle was in effect (lights on from 7:00 AM to 7:00 PM). The animal was maintained on a diet of fresh fruit and laboratory diet monkey pellet. The health of the monkey was periodically monitored by consulting veterinarians. The animal was anesthetized with intramuscular ketamine and xilazine (10–20 mg/Kg; 2 mg/Kg) and sacrificed by i.v. injection of Tanax (embutramide, mebenzonium iodide, and tetracaine hydrochloride). The procedures used in this study were in strict accordance with the European Council Legislation on the use and care of laboratory animals.

GC/MS determination

Monkey brain tissue, weighing 37 g, was placed into a beaker with an equal amount of bidistilled water (w/w) and cut with a blade homogenizer. Before GC/MS analysis, the brain tissue was pretreated by coupling a classical deproteinization method with solid-phase extraction according to the method described by other authors [20,21]. Deproteinization was achieved by adding to the homogenate 1.2 volumes of sulphate ammonia and heating it, after acidification with 3 ml of HCl 3N, at 121°C for 30 min. The sample was cooled, filtered through Buchner funnel using Whatman n°1 filter paper. The obtained fraction was set to pH 9 and then submitted to morphine extraction by solid phase on Bond Elut Certify columns (Varian, Harbor city, CA, USA).

In order to avoid a possible overload of the extraction phase, due to the volume of the filtrate, the sample was divided into three fractions which were singly extracted; the eluates were then collected into a unique sample. The extraction steps were: 1) activation of the extraction phase with 4 ml of methanol and 4 ml of distilled water; 2) passing of the sample through the phase (flow rate about 2 ml/min); 3) cleaning up of the sorbent phase using 4ml of distilled water; 4) change of the phase pH by adding 1 ml of 100 mM acetate buffer at pH 4; 5) cleaning up of the phase by adding 2 ml of methanol; 6) drying and eluting with 4 ml of methylene chloride/isopropyl alcohol/ NH_4OH (80/20/2) (v/v). New glassware was used for each experimental run and blank samples were also extracted in order to check for contamination of both reagents and solvents. Extraction solvents were collected into the same 120×80 glass tubes. Simultaneously, 1 ml of standard solution, containing both morphine and codeine (Carlo Erba, Milano, Italy) in concentrations ranging from 10 ng/ml to 0.5 ng/ml, was put into 120×80 glass tubes. 100 μl of 250 ng/ml nalorphine (S.A.L.A.R.S, Como, Italy) in methanol was added, as an external standard, to the samples and to the standard solutions.

Immediately before analysis, sample extracts and standard solutions were derivatized by adding 50 μl of a solution of N-Methyl-N-trimethyl-silyltrifluoroacetamide (Pierce, Rockford, IL, USA) 20% in toluene); 1 ml of each derivatized sample was submitted to GC/MS analysis which was performed using a Hewlett Packard 5890 Series II gaschromatograph coupled with a Hewlett Packard 5791 MS detector. The temperatures were respectively 250°C for the injector and 280°C for the detector. The oven temperature was programmed at 50°C for 2 min, up to 280°C at a heating rate of 35°C/min, the final temperature being held for 5 min. Helium was used as the carrier gas, the head pressure was 2 psi and the flow rate of the carrier was 0.6 ml/min. The injection mode was splitless, the purge valve was open after 1 min. Derivatized codeine, morphine and nalorphine RTs were respectively 11.22 min, 11.50 min and 12.35 min. The analysis was performed in selected ion monitoring (SIM) mode; derivatized morphine was identified by fragments 429 (quantifier ion), 414 and 236 (qualifier ions), derivatized codeine by fragments 371 (quantifier ion), 356 and 313 (qualifier ions), and derivatized nalorphine by fragments 455 (quantifier ion), 440 and 414 (qualifier ions). Previous GC/MS analysis of standard solutions of derivatized morphine, codeine and nalorphine did validate both the retention times and the characteristic ions for these substances [22,23]. The extraction recovery for morphine was established as previously described [11] and was estimated as 82–86%; in the same manner, the recovery for codeine was established, which is 86–90%. Morphine and codeine quantification was obtained by using multipoint calibration curves constructed using fragments 371, 429 and 455 for

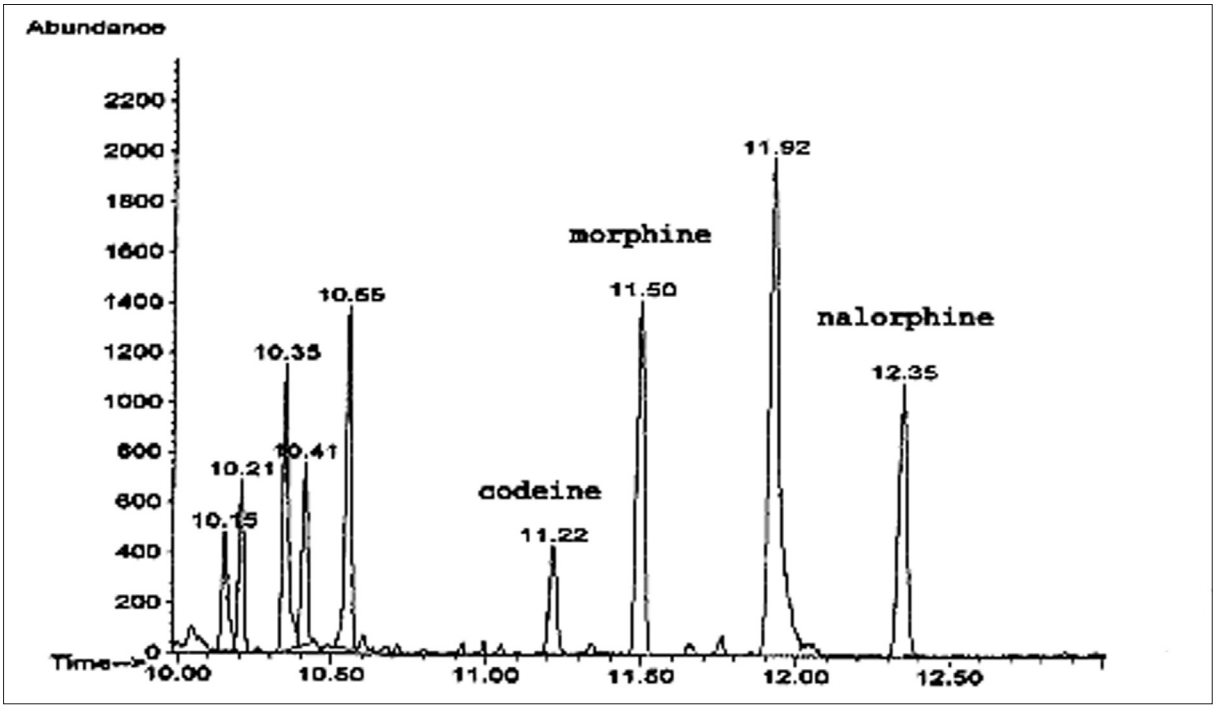

Figure 1. Chromatographic plot of monkey brain morphine and codeine content – Chromatographic plot of a monkey brain sample after derivatization. The retention times of derivatized morphine, codeine and nalorphine were respectively 11.50, 11.22 and 12.35 min.

codeine, morphine and nalorphine, respectively. The minimum detectable amounts of derivatized morphine and codeine were 10 pg injected for morphine and 15 pg injected for codeine (ratio signal-to-noise>5).

Release of endogenous morphine from monkey brain

The release of naturally occurring morphine from monkey brain was performed as previously described for rodent brain [11]. Briefly, monkey brain tissue from a hemisphere (35 g) was dissected into small blocks. Half of each tissue block was pooled and incubated for 30 min in Krebs bicarbonate medium (KS), and bubbled continuously in 5% CO^2/ 95% O^2 mixture in a superfusion chamber at 37°C. KS has the following composition: 118.3 mM NaCl, 4.7 mM HCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM$NaHCO_3$, 11.1 mM glucose, 0.0026 mM Ca-EDTA. The superfusion chamber was in a thermostatted water bath. The tissue blocks were initially superfused with KS for a 30 min period, during which the perfusate was discarded. Preincubation was followed by 10 min medium collection to establish the baseline of eM. The membrane depolarization was then produced by replacing normal KS with medium containing 50 mM K^+ for 5min and collecting during a single perfusate fraction. In a different experiment, the rest of brain tissue blocks were incubated according to the above protocol in Ca^+ deficient medium.

Results

Brain content of eM and codeine quantified by GC/MS were, respectively, 0.11 ng/g and 0.04 ng/g (Figure 1 and 2). The blank samples showed no morphine contamination as noted by the absence of morphine positive material. Morphine contamination was not found in food and water. The basal value of *in vitro* 10 min release of brain block morphine was 82.5 pg/g/min (Figure 3A). During the 5-min exposure of monkey brain blocks to the depolarization medium, the morphine concentration rose to 170 pg/g/min, which is 212.5% higher than basal values (Figure 3A). Basal morphine concentrations were restored by replacing the high potassium medium with normal KS. In the experiment in which Ca^+ was omitted from the superfusion medium, the depolarizing concentration of KCl failed to increase the rate of endogenous morphine release (Figure 3B). Samples of all chemicals and materials used in the incubation medium as well as the incubation medium exhibited no detectable morphine.

Discussion

Based on the belief that endogenous opiate alkaloids were not present in animals, previous studies have focused on the pharmacological effects of exogenous opiates, a family of important analgesic drugs. Apart from its presence in plants, recent studies indicated that morphine is endogenously present at a nanogram concentration per gram of tissue in different animal species, especially in nervous tissues [1,5,7,8,10–12,19]. Supporting this finding, we also detected eM, at a concentration of 0.11 ng/g, in the brain of a non human primate by using gas chromatography coupled to mass spectrometry analysis. The current finding of eM in monkey brain demonstrates the presence of morphine throughout the phylogenesis of the nerve system of mammals and lower ani-

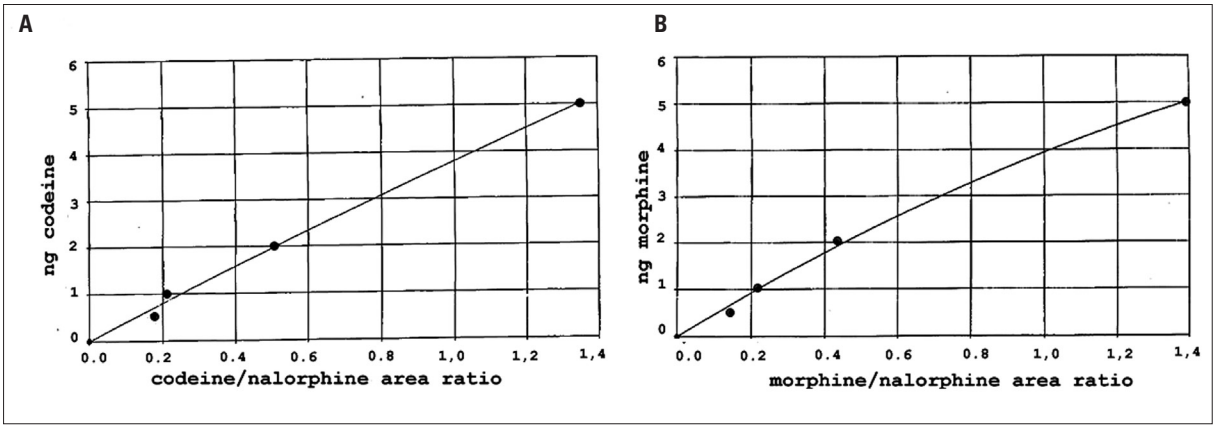

Figure 2. Calibration curves for morphine and codeine – Codeine (**A**) and morphine (**B**) calibration curve were constructed by plotting the codeine/nalorphine and morphine/nalorphine peak area ratio (X axis) versus the quantity of codeine and morphine (Y axis). The experimental points were fitted to the following equations: $y=-0.24\,x^2 + 4.04\,x$ for codeine; $y=-0.86\,x^2 + 4.79\,x$ for morphine.

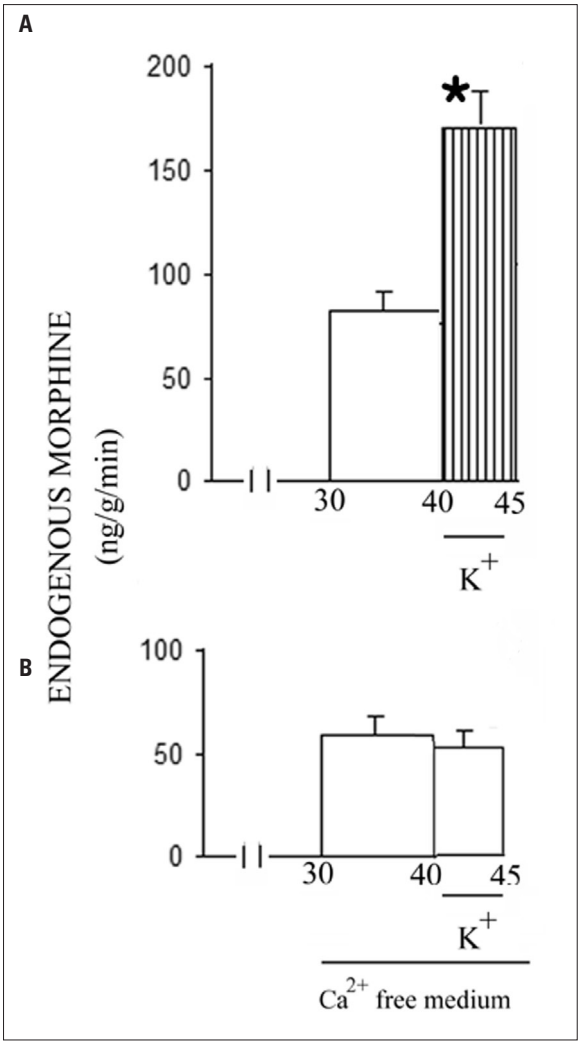

Figure 3. Release of eM from monkey brain in response to exposure to high K^+ medium with (**A**) and without Ca^{++} (**B**). Histograms are the mean+S.E.M. (bars) values of two independent experiments. * – significance at $\alpha=0.01$ according to Student t statistical test.

mals [24]. Furthermore, the detection of morphine precursors such as reticuline, salutaridine, thebaine and codeine strongly supports the hypothesis that animal tissue is able to synthesize morphine [9, 14–17]. Conversion of codeine to morphine has been demonstrated in several tissues of the rodent, including that of the brain [18,19,28]. In monkey brain, we detected codeine, which is the direct precursor of morphine in the biosynthetic pathway [13,14] at a concentration of 0.04 ng/g, establishing the presence of this necessary component for morphine synthesis also in non human primate.

The function of eM is a matter of speculation. However, a unified general immune, vascular and nervous role has been articulated based on earlier studies demonstrating physiological down regulation and restoring homeostasis following trauma [24–27]. The changes in morphine content during arthritis or food deprivation could reflect such adaptive processes that elevate the eM level by increasing the rate of alkaloid biosynthesis [6,29,30]. Endogenous morphine has been immunohistochemically detected in mammalian brain regions which are involved in modulation of pain transmission where opioids act by modulating the descending inhibitory pathway of nociceptive transmission [2,31]. Furthermore, it has also been shown that endogenous morphine alkaloids play a role in immunoregulation [24]. These immune studies have demonstrated the presence of a novel mu3 opiate receptor subtype that is opiate alkaloid selective and opioid peptide insensitive, supporting the presence of endogenous morphinergic signalling on immune, vascular and neural tissues [32].

These findings, including the present results, raise the question of the possible role of eM as a neurotransmitter and/or neurohormone. Indeed, the presence of morphine-like immunoreactivity was demomonstrated in perikarya, fibers, and terminals of neurons in discrete

areas of rodent brain [33], as well as in the ganglia of molluscs [27]. Morphine-like immunoreactivity has been localized by electron microscopy in nerve terminals, which appear to form synaptic contacts [34]. K^+ depolarization can cause the release of eM in a chemically detectable form from rat brain slices, an effect which is Ca^{++} dependent [11], further supporting a neurotransmitter role. In the present study eM was released from monkey brain to a value of 212.5% higher than the basal level of release in a Ca^{++} dependent process, demonstrating the specificity of the mechanism. These findings indicate that eM might function as a neuromodulator/neurotransmitter agent in the CNS of non human primates.

CONCLUSIONS

Given the presence of morphine, morphine precursors and opiate receptors with select responsivity to opiate alkaloids it must be concluded that this substance exists in invertebrates and mammals. Furthermore, its presence in many mammals, including primates, demonstrates and highlights its importance as a signalling molecule that has been conserved during evolution.

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