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Stimulatory effect of morphine on rat pineal melatonin synthesis via a cyclic AMP-dependent transcription pathway

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Abstract

The expression of mRNA of opioid receptors and the existence of opioid binding site in the rat pineal gland have been demonstrated previously. A major finding was that morphine enhanced the activity of the rate-limiting enzyme, *N*-acetyltransferase (NAT) and increased the level of melatonin in rat pineal gland. An attempt has been made in order to clarify the mechanism of this induction. In the present study, the stimulatory effect of morphine on the expression of NAT mRNA in the rat pineal gland has been demonstrated using semi-quantitative RT-PCR technique. The results showed that both acute and chronic morphine treatments significantly increased NAT mRNA expression in rat pineal gland. In addition, the effect of morphine on the phosphorylation of the transcription factors, cyclic AMP responsive element-binding protein (CREB) was investigated. Western blot analysis showed that morphine significantly increased phosphorylation of CREB. These results indicate that at least one downstream messenger pathway for the activation of opioidergic system on the induction of melatonin synthesis in the rat pineal gland acts via cyclic AMP-dependent cascade and transcription mechanism.

Keywords: Morphine; *N*-Acetyltransferase; Pineal gland; Opioid; Melatonin; P-CREB

Pineal melatonin synthesis is regulated by several neuronal pathways such as the well-known adrenergic pathway [15]. In addition, an association between the regulation of melatonin synthesis and opioid has been documented [9]. Furthermore, localization of opioid receptor sites and opioid receptor gene expression has been demonstrated in the central nervous system of rat [13,18].

In a previous study, the expressions of delta and mu opioid receptor mRNAs in rat pineal gland have been identified [4]. It has been demonstrated that morphine, an opioid receptor agonist, enhanced both *N*-acetyltransferase (NAT), a key regulatory enzyme in melatonin synthesis, activity and melatonin levels in the pineal gland [9].

In this study, we have attempted to clarify the possible mechanism of morphine on the induction of NAT activity in rat pineal gland. Sprague–Dawley male rats (200–250 g) were acute or chronic treatment by intraperitoneal injection of morphine sulfate, which was dissolved in sterile distilled water. Rats received a single dose (at 1:00 p.m.) of morphine sulfate at 40 mg/kg body weight in acute administration. In chronic administration, rats received morphine sulfate twice daily (at 6:00 a.m. and 6:00 p.m.) at 10, 20, 30, 40 and 50 mg/kg body weight for days 1–4, 5–6, 7–8, 9–10 and 11–14, respectively. Control animals received an equal volume of sterile distilled water. Rats were sacrificed 2 h after the morphine injection of acute or of the last dose of chronic treatments. Total cellular RNAs were isolated from rat pineal glands by a modification of the guanidine isothiocyanate–phenol–chloroform method using TRIzol reagent (GibcoBRL, Grand Island, NY, USA). The non-radioactive reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to yield the quantification of NAT mRNA from small tissue samples in relation to an internal standard (actin). The primers of NAT [2] and

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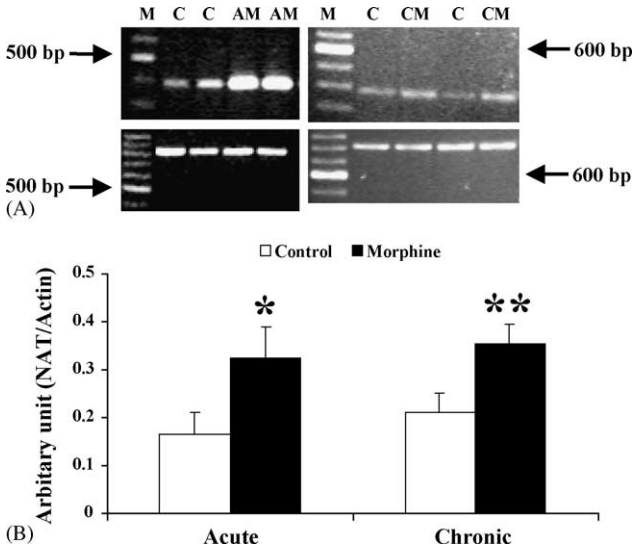

Fig. 1. (A) Gel electrophoresis of RT-PCR products from rat pineal gland mRNA. The products were analyzed on a 2% agarose gel stained with ethidium bromide. Upper panel is RT-PCR products of NAT (392 bp) in rat pineal gland. Lower panel is RT-PCR products of actin (830 bp) in rat pineal gland. M: 100 bp DNA ladders; C: control rat; AM: acute morphine-treated rats; and CM: chronic morphine-treated rats. (B) The DNA bands were quantified by densitometry and changes were represents in graph. RT-PCR products of NAT are expressed as a ratio of NAT/actin RT-PCR products. Each value represents mean ± S.E.M. of four independent experiments. Statistical evaluation of the data was performed by Student's *t*-test. The asterisk symbol (*, **) denotes significance between control and morphine treated rats at $p < 0.05$.

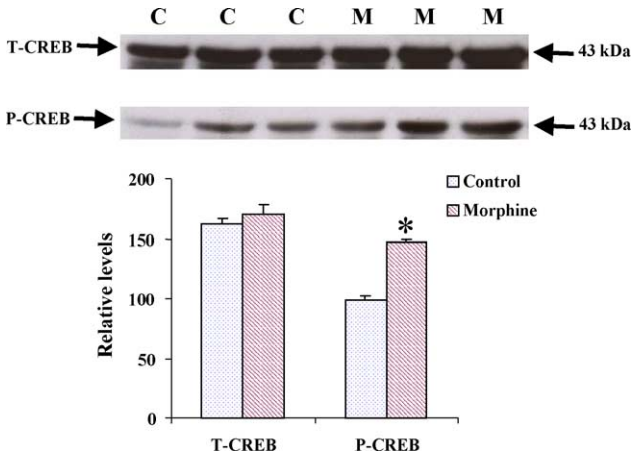

Fig. 2. Effect of morphine on phosphorylation of CREB (P-CREB) and total level of CREB (T-CREB) in rat pineal glands assessed by Western blot analysis. Each lane represents the protein extraction from two pineal glands. The P-CREB and T-CREB bands were quantified by densitometry and the changes were represented in graph. C and M are control and morphine-treated rats, respectively. Results are expressed as mean ± S.E.M. of four independent experiments. Statistical evaluation of the data was performed by Student's *t*-test. The asterisk symbol (*) denotes significance between control and morphine-treated rats at $p < 0.05$.

actin [4] were designed on the basis of published mRNA and primer sequences, respectively. The sequences of the primers used were: NAT-upper 5′ CGC CCT GGT GCC CTT CTA TG 3′, NAT-lower 5′ ACG TCC TGG TCT TGC CTT TG 3′, actin-upper 5′ CCC AGA GCA AGA GAG GCA TC 3′ and actin-lower 5′ CTC AGG AGG AGC AAT GAT CT 3′. Amplification of rat pineal gland with NAT and actin primers yielded the products of the expected size at 392 and 830 bp (Fig. 1A), respectively. Nucleotide sequencing done by using an automated fluorescent DNA sequencing confirmed that 392 bp cDNA fragment with the exclusion of amplimer bases, encode sequences which are identical (99%) to nucleotides 682–1040 of published rat *N*-acetyltransferase mRNA sequence (GenBank Accession Number: U40803) [2]. Acute and chronic morphine administrations significantly increased the expression of NAT mRNA (Fig. 1A, upper panel) without any change in actin mRNA expression (Fig. 1A, lower panel), when compared to those obtained from control rats (Fig. 1B). In order to clarify the possible mechanisms that morphine-enhanced expression of NAT mRNA, we have demonstrated that morphine exerts its effect by enhancing phosphorylation of cyclic AMP response element-binding protein (CREB), which plays a functional role as a transcription factor to induce expression of NAT mRNA in the present study. Rats were intraperitoneally injected with morphine sulfate (at 1:00 p.m.) at 40 mg/kg body weight for 2 h, then rats were sacrificed and pineal glands were collected. Total proteins were extracted from pineal glands and equal amounts of proteins were subjected to immunoblot analysis to determine the amount of phosphorylated CREB (P-CREB) and total level of CREB (T-CREB) using rabbit monoclonal antibodies against P-CREB at Ser133 (Cell Signaling, Beverly, MA, USA) and CREB (48H2) (Cell Signaling), respectively. The results showed that morphine significantly increased P-CREB but did not change T-CREB levels in rat pineal glands (Fig. 2).

An interaction between melatonin and opioids has been documented. Acute morphine administration has been reported to increase plasma melatonin concentration in the rat [7]. Claustrat et al. [6] has also shown that the chronic treatment of depressed and schizophrenic patients with des-tyrosine-γ-endorphine significantly increased the melatonin content in urine. Traditionally, chronic treatment of opioid leads to a "tolerance" phenomenon, but it does not happen in activating melatonin synthesis in pineal gland. Moreover, the administration of a met-enkephalin analogue, FK 33-828, enhanced melatonin level in human serum [12]. We have also found that the activation of opioid receptor by adding morphine into the cultured rat pineal explant increased of NAT enzyme activity and enhanced melatonin secretion [9].

These novel findings represent evidence that acute and chronic morphine administrations in rats increase NAT activity in the rat pineal gland, and it regulates at the mRNA levels. Several lines of evidence indicate that NAT enzyme activity closely relates to NAT protein levels, which is usually regulated by transcription and translation mechanisms [19]. In