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Involvement of melatonin metabolites in the long-term inhibitory effect of the hormone on rat spinal nociceptive transmission

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Abstract

There is evidence that melatonin and its metabolites could bind to nuclear sites in neurones, suggesting that this hormone is able to exert long-term functional effects in the central nervous system via genomic mechanisms. This study was designed to investigate (i) whether systemically administered melatonin can exert long-term effects on spinal cord windup activity, and (ii) whether blockade of melatonin degradation with eserine could prevent this effect. Rats receiving melatonin (10 mg/kg ip), the same dose of melatonin plus eserine (0.5 mg/kg ip), or saline were studied. Seven days after administration of the drugs or saline, spinal windup of rats was assessed in a C-fiber reflex response paradigm. Results show that rats receiving melatonin exhibited a reduction in spinal windup activity. This was not observed in the animals receiving melatonin plus eserine or saline, suggesting a role for melatonin metabolites in long-term changes of nociceptive transmission in the rat spinal cord.

Keywords: Melatonin; Eserine; Windup; Spinal cord; Pain; Rat

1. Introduction

Melatonin, the main hormone produced by the pineal gland, has been shown to modulate pain sensation. In fact, physicians noticed that patients experienced less pain and therefore required fewer analgesics at night (Ebadi et al., 1998). Besides, rats submitted to alternating periods of 12 h of light and darkness exhibited diurnal variations in sensitivity to both heat- and pressure-elicited pain; the highest sensitivity occurred a few hours after the onset of photophase (John et al., 1994). In addition, it has been reported that exogenous melatonin induces antinociceptive effects in mice and rats (Golombek et al., 1991; Yu et al., 2000; Raghavendra et al., 2000). Since melatonin penetrates easily the blood-brain barrier (Vitte et al., 1988) and its receptors are widely distributed in the central nervous system (Stankov et al., 1991; Morgan et al., 1994), it is likely that melatonin-induced antinociception could be a consequence of its central effects on neurons relevant to pain transmission and/or control. This concept is further supported by the observation that intracerebroventricular administration of melatonin induces antinociception (Yu et al., 2000). In this regard, there is some evidence that relates melatonin-induced antinociception with the activity of opioid peptides since the antinociceptive effect of melatonin can be countered by the opiate antagonist naloxone (Golombek et al., 1991; Yu et al., 2000).

On the other hand, it has been reported that melatonin inhibits *N*-methyl-D-aspartate (NMDA) receptor-dependent synaptic mechanisms involved in both hippocampal and hypothalamic long-term potentiation (LTP) (Collins and Davies, 1997; Hogan et al., 2001; Fukunaga et al., 2002), as well as in spinal cord windup activity (Laurido et al., 2002). Windup, a synaptic potentiation phenomenon quite similar to hippocampal LTP (Dickenson et al., 1997), consists of a remarkable increase in the response of nociceptive dorsal horn neurons evoked by the repetition of constant intensity C fiber stimulus. Windup is especially important in the development and maintenance of chronic pain (Eide, 2000). The inhibitory effect of melatonin on spinal windup of rats, which can be evidenced 10 min after

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the systemical administration of the hormone, could be due to a direct effect of the hormone on melatonergic receptors that are abundant at this level (Pang et al., 1997) or to the inhibition of the activity and/or expression of key enzymes involved in neuronal NMDA receptor-dependent transduction mechanisms, such as nitric oxide synthase (Leon et al., 2000; Chang et al., 2000) and Ca^{2+}/calmodulin-dependent protein kinase II (Benitez-King et al., 1996; Fukunaga et al., 2002). However, they could also be the result of indirect effects produced by melatonin degradation products: in neural tissue, melatonin might be metabolized to 5-methoxytryptamine (5-MeOT) and thereby to pinoline (Hardeland et al., 1993), and both metabolites seem capable of interacting with neuronal function via genomic and nongenomic mechanisms (Pähkla and Rägo, 1999). Published data suggest that 5-MeOT is a good ligand for NMDA receptors in vitro (Worthen et al., 2001) and that this product could block the NMDA response of spinal neurons in vivo (Chesnoy-Marchais and Barthe, 1996). In addition, it has been shown that pinoline can be detected for longer than 2 days in the rodent retina after intravenous administration (Leino et al., 1983) and that up to 50% of the total label is located inside cell nuclei (Pähkla et al., 1996). On the basis of these antecedents, pinoline is expected to produce effects on neuronal function lasting longer than those induced by melatonin, which remains in the brain only for about 1 h after intravenous administration (Ferreira et al., 1996).

The present work was designed to test (i) whether systemic melatonin can exert long-term effects on windup activity in the spinal cord, and (ii) whether blockade of melatonin metabolization to 5-MeOT with eserine, a well-known inhibitor of aryl acylamidase and melatonin deacetylase, can modify the long-term effects of melatonin administration on spinal windup.

2. Materials and methods

The present study was performed in accordance with protocols approved by the Committee for the Ethical Use of Experimental Animals of the Institute of Nutrition and Food Technology (INTA) and was also in accordance with the ethical standards for investigations of experimental pain in animals (The Committee for Research and Ethical Issues of the International Association for the Study of Pain, 1980).

The experiments were carried out on adult, male, Sprague–Dawley rats weighing 200–300 g, maintained under controlled 12:12-h light–dark lighting conditions per day (light turned on at 07:00 h and off at 19:00 h). Male rats were used to avoid the fluctuations in melatonergic receptor sensitivity caused by changes in estrogen level (Witt-Enderby et al., 2003). Rats were separated into three groups: Mel rats ($n=6$) were injected with 10 mg/kg ip of *N*-acetyl-5-methoxytryptamine (melatonin) dissolved in ethanolic saline (0.5% ethanol); Mel/Ese rats ($n=6$) were injected with 0.5 mg/kg ip physostigmine sulfate (eserine) 15 min prior to the administration of 10 mg/kg ip melatonin; and Sal rats ($n=6$) were injected intraperitoneally with ethanolic saline solution in a volume equivalent to that used in the Mel and Mel/Ese groups. All injections were made 1 week prior to the experiments at 11:00 h. Melatonin and eserine were purchased from Sigma (St. Louis, MO). A dose of 10 mg/kg ip of melatonin was used in the present study on the basis that it can abolish spinal windup in rats (Laurido et al., 2002). Interestingly, it has been reported that a single injection of 5 mg/kg iv of melatonin is able of significantly increasing the availability of the hormone in the central nervous system (Crespi et al., 1994). A dose of 0.5 mg/kg ip of eserine was utilized in this study since it has been reported that comparable doses are effective in inhibiting by 50% the activity of butyrylcholinesterase (Somani and Khalique, 1986), an enzyme known to be less sensitive to anticholinesterase drugs than aryl acylamidase (Weitnauer et al., 1998). Seven days after the administration of the drugs or saline, at 11:00 h, rats were anesthetized with 1.1 g/kg ip urethane and spinal windup was evaluated in a C-fiber-evoked flexor reflex paradigm elicited in the left hind limb. The C nociceptive reflex is a useful tool to study spinal mechanisms involved in nociception (Strimbu-Gozariu et al., 1993). Briefly, rectangular electric pulses of sufficient strength for supramaximal activation of C-fibers (15 mA, 1 ms, 0.2 Hz) were initially applied to the sural nerve receptive field by means of two stainless steel needles inserted into the skin of toes 2 and 5 (Grass S11 stimulator equipped with a Grass SIU 5 stimulus isolation unit and a Grass CCU 1A constant current unit). The C-fiber-evoked reflex activity was recorded from the ipsilateral biceps femoris muscle via another pair of stainless steel needles. After amplification (Grass P511 amplifier), the electromyographic responses were full-wave rectified and integrated into a time window from 100 to 500 ms after the stimulus (Acer PC with 10 kHz sampling rate A/D converter card). Once stable C reflex responses were obtained, the stimulus strength was lowered and the current required for threshold activation of the C reflex determined (usually 6–7 mA for normal rats). Afterwards, a train of 12 stimuli at 0.6 Hz was delivered to the toes in order to develop windup activity. In the C reflex paradigm, windup consists of a remarkable stimulus frequency-dependent increase of the electromyographic-integrated response (Laurido et al., 2001). All responses were stored in a hard disk for later analysis. Least square regression lines were fitted among experimental points showing only incremental trend (prior to windup saturation at the 6th or 7th stimulus), discarding the remainder points (Microcal Origin 3.5 software). The slopes of the regression lines represent windup scores.

3. Results

Seven days after the administration of saline or drugs, application of 12 successive constant electric pulses at 0.6

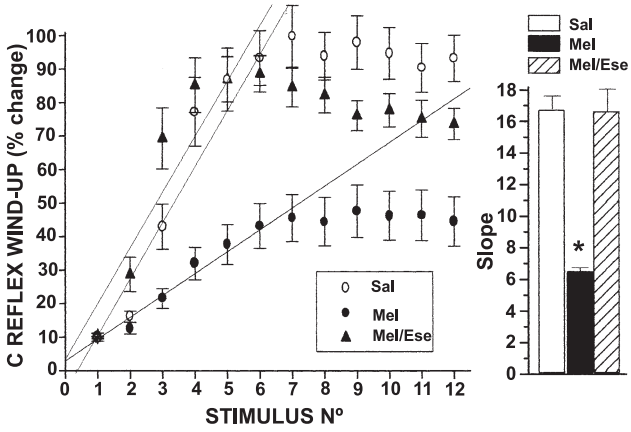

Fig. 1. Eserine-sensitive long-term effect of melatonin on spinal windup of rats. Left panel: abscissa, the stimulus number; ordinates, windup in terms of percentage change of C reflex integrated activity. The panel shows experimental points and regression lines. In all groups, the number of animals studied is six. Open circles, saline; closed circles, melatonin; closed triangles, melatonin plus eserine. Right panel: values are means of slopes ± S.E.M. Saline: 16.79 ± 0.83; melatonin: 6.54 ± 0.21; melatonin plus eserine: 16.64 ± 1.43. * $P < .001$ when Mel series was compared with either Sal or Mel/Ese series (Student–Newman–Keuls test).

Hz induced spinal windup in all rat groups, as revealed by the gradual but remarkable increase of the C reflex response induced by the repetitive stimuli. However, melatonin-injected rats showed decreased spinal windup, as revealed by the decreased slope of the regression line (54.3% decrease, $P < .001$) of the Mel group compared to Sal (Fig. 1). This reduction of windup was not evident in the group receiving eserine prior to melatonin (Fig. 1). In fact, the slope of the regression line in the Mel/Ese group was not significantly different to that obtained in the Sal animals, but they were significantly higher than the slope of the Mel group ($P < .001$). Fig. 2 shows original samples of windup development in the C reflex response paradigm before and 1 week after the different drug treatments. It can be observed that as windup develops, the C reflex response enhances in terms of both amplitude and duration (Fig. 2A). After full development of windup, the C discharge had amplitudes ranging from 150 to 200 μV and lasted for about 350–450 ms. Melatonin administration decreased windup activity, mainly in terms of a reduced duration of the C discharge. In these conditions, the C reflex response lasted less than 150 ms (Fig. 2B, second row of recordings). In contrast to the Mel group, Sal or Mel/Ese groups developed normal windup (Fig. 2B, first and third rows of recordings).

4. Discussion

The temporal pattern of the spinal windup has already been described by using both C-fiber reflex responses (Laurido et al., 2001) and single-unit recordings from convergent dorsal horn cells (Dickenson, 1990). In the present experiments, the windup recorded is completely consistent with those earlier descriptions. Previous studies with 1.25, 2.5, 5, and 10 mg/kg of intraperitoneally administered melatonin showed that it produced a dose-dependent

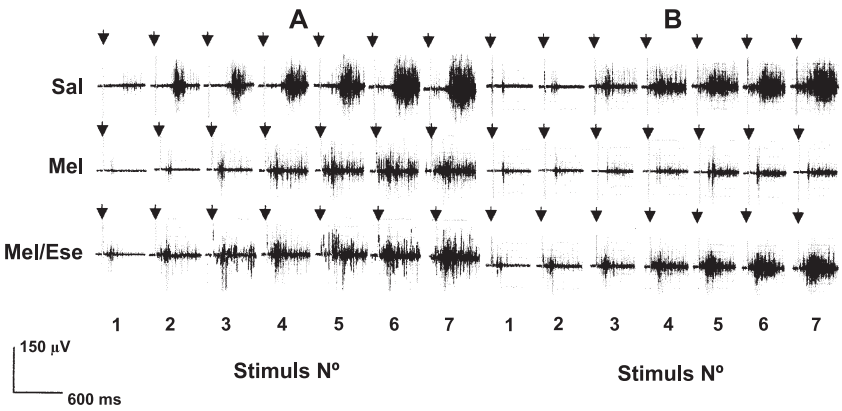

Fig. 2. Representative examples of windup development in the C fiber reflex paradigm, before (A) and 1 week after (B) the different treatments: Sal, saline; Mel, melatonin; Mel/Ese, melatonin plus eserine. Each recording is the electromyographic response to electrical stimulation of toes 2–5. Arrows indicate application of electrical stimulation. Calibration bars (lower left corner) represent time (horizontal) and electrical potential variations (vertical). Windup is depressed in the Mel group (B), while it is not affected in the Mel/Ese group (B).

inhibition of spinal windup in rats, as evidenced by a decreased C-fiber reflex gain 10 min after injecting melatonin. In these experiments, the maximum windup decrease was recorded with a melatonin dose of 10 mg/kg ip, which depressed completely windup activity (Laurido et al., 2002). Moreover, data from the literature show that administration of 10 mg/kg of melatonin completely blocks in mice the thermal hyperalgesia produced by lipopolysaccharide-induced inflammation (Raghavendra et al., 2000). These observations are consistent with the notion that windup could have a role in the development of hyperalgesia and in the maintenance of chronic pain (Dickenson et al., 1997; Eide, 2000) and also suggest that melatonin could be effective in inducing antinociception in chronic pain. Although in our study windup activity was not completely abolished 7 days after melatonin administration, an inhibitory effect still persisted as evidenced by the 54% decrease of windup with respect to the Sal group. This long-term effect of melatonin could only be explained by genomic changes, possibly induced by melatonin and/or its metabolites. In fact, melatonin appears to exert direct effects at the genomic level since exogenously administered melatonin has been reported to regulate melatonergic receptor mRNA expression in rats (Guerrero et al., 2000). Furthermore, specific high-affinity melatonin binding sites have been detected in the nucleus of rat liver cells, supporting the participation of melatonin in genomic regulation (Macias et al., 2003). However, in these studies, melatonin was administered daily and therefore the hormone was always available in the animal tissues until the date of the experiments. Although in the present study a direct genomic effect of melatonin cannot be excluded, the results show that long-term effects on spinal windup occur only when melatonin is injected without the previous administration of eserine. This observation suggests that the long-term functional effects of the hormone most probably are exerted through one of its metabolites, such as 5-MeOT and/or pinoline, which may remain for a much longer period of time in rat tissues (Leino et al., 1983). In fact, the formation of 5-MeOT from melatonin has been demonstrated in vivo in rat liver (Beck and Johnson, 1981). This metabolic pathway is also present in the brain of lizards where the conversion is mediated by melatonin deacetylase and production of 5-MeOT can be inhibited by eserine (Grace and Besharse, 1994). Melatonin deacetylation is also observed in amphibian retinal neurons where aryl acylamidase catalyzes the initial step in the degradation pathway releasing 5-MeOT. In this case, inhibition of the enzyme with eserine caused accumulation of endogenous melatonin, suggesting that this is the normal pathway for retinal melatonin (Cahill and Besharse, 1989). The genomic activities of 5-MeOT are still the subject of investigation, and experiments in mice have shown that this compound could stimulate the expression of genes related to immunomodulating cytokines (Liu et al., 2001). On the other hand, autoradiography studies in mice to explore the subcellular distribution of in vivo administered [3H]-radiolabeled pinoline show highly specific retention of pinoline in the nuclei of cerebral cortical cells (Pähkla et al., 1996), pointing to possible direct genomic activities of this metabolite. Melatonin has been reported to have a dominant role in the generation and regulation of several behavioral rhythms in mammals; although long-term signaling mechanisms subserved by melatonin and/or its metabolic products could hardly be involved in circadian rhythms of central neurons of vertebrates, they could be underlying the photoperiodic regulation of seasonal cycles, such as feeding and reproductive behaviors, as well as thermoregulation and hibernation. Pain could also be related to neuroendocrine rhythms such as male (Agmo, 1997) and female (McEwen et al., 1998) reproductive behavior, although the participation of melatonin in these events has not yet been established.

In conclusion, our results show that a single dose of melatonin can produce eserine-sensitive, long-term effects in spinal cord nociceptive transmission, this being the first study that suggests genomically mediated effects of melatonin metabolites on neuronal function. Further investigations addressed at the study of the direct effects of 5-MeOT and/or pinoline on spinal windup activity are required to elucidate the pathways and mechanisms involved in the long-term antinociceptive effects of melatonin metabolites.

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