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Running title; Effects of Melatonin and Diazepam in cats

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Abstract

The effects of an intravenous application of melatonin upon eye movements and muscular tonus were examined in acute decerebrate cats in order to elucidate whether melatonin can be beneficial as a therapeutic agent for treating sleep disturbance. The results were compared to those produced by an intravenous application of diazepam, one of the benzodiazepines, and from a microinjection of carbachol, a cholinergic agent resistant to cholinesterase, into the pontine reticular formation. The application of melatonin produced neither changes in muscle tone nor in eye movements in cats in which the decerebration was made at the level of precollicular-postmammillary level (mesencephalic cats). However, it did produce rapid eye movements (REM) with muscular atonia in the cats in which the decerebration was performed at the level of precollicular-precollicular-preoptic chiasma level (hypothalamic cats). In the latter preparation, the suprachiasmatic nucleus (SCN) was preserved. Although an application of diazepam abolished muscle tone, it did not change eye movements in either mesencephalic or hypothalamic cats. An intrapontine carbachol injection resulted in muscular atonia associated with REM in the mesencephalic cats.

These results suggest that the melatonin application activates SCN neurons, which, in turn, trigger REM sleep generating system in the brainstem. However, muscle tone suppression induced by the diazepam application may not be a result of the activation of the brainstem REM sleep generating system. We propose that melatonin would be a more useful and safe therapeutic agent for treating sleep disturbances than conventional drugs such as benzodiazepines.

Introduction

There has recently been considerable interest in melatonin as a drug for the treatment of sleep-wake rhythm disorders [1,2] and for its hypnotic effects [3]. Because

melatonin receptors are mainly located in the suprachiasmatic nucleus (SCN) [4,5], melatonin's effects are thought to be mediated by the activation of SCN neurons. However, evidence is lacking as to whether melatonin activates brainstem mechanisms generating REM sleep and whether the melatonin-induced effects are different from the effects induced by other hypnotic drugs such as benzodiazepines.

It has been shown that a microinjection of carbachol into the pontine reticular formation of cats induces REM sleep signs, i.e., cortical desynchronization, postural atonia, ponto-geniculo-occipital waves and rapid eye movements [6]. Even in acute decerebrate cats, intrapontine carbachol injection produced muscular atonia associating with REM [7,8]. Because it resembles the generalized motor inhibition during naturally occurring REM sleep, the decerebrate preparation is useful for understanding the brainstem-spinal cord mechanisms for generating REM sleep [7,9,10,11]. The present study was designed to elucidate whether an application of melatonin induces muscular tone and REM in two types of decerebrate preparations: in hypothalamic cats, in which the SCN was preserved; and in mesencephalic cats, which do not include the SCN. The effects of the melatonin application were further compared to those induced by an intravenous application of diazepam, one of benzodiazepines, and to those induced by the intrapontine injection of carbachol. Preliminary results have been reported [12].

Materials and Methods

All of the experimental procedures were approved by the Animal Studies Committee of Asahikawa Medical College in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Guide, revised 1996). Every attempt was made to minimize animal suffering and to reduce the number of animals used. The experiments were performed with 7 cats from the animal facility at Asahikawa Medical College which weighed 2.5 to 3.5kg. Under halothane-nitrous oxide gas anesthesia, the trachea was intubated and catheters were placed in the femoral artery to monitor blood pressure and in the cephalic vein for administrating either melatonin or diazepam. The electrodes for recording electrooculogram (EOG) were implanted on the skull, and bipolar stainless steel wires were also implanted into the bilateral soleus muscles to record electromyograms (EMG). The cats were decerebrated either at the precollicular-postmammillary level (mesencephalic cat, n=4, Fig.1A-a), or at the precollicular-preoptic chiasm level (hypothalamic cat, n=3, Fig.1A-b). The thalamic structure was further suctioned so that the thalamus was completely removed but the hypothalamus was preserved. Anesthesia was then discontinued. The head and the dorsal processes of T1-3 were fixed in a stereotaxic apparatus. The body was supported by a rubber hammock at the abdominal level. Either melatonin (1mg/kg), which was dissolved in distilled water, or diazepam (0.5mg/kg) was intravenously administrated in both the mesencephalic and hypothalamic cats. A glass micropipette (tip diameter 20 •m) filled with carbachol dissolved in saline was stereotaxically inserted into the brainstem through the cerebellum. Carbachol (4 •l/0.25•l) was injected into the rostral part of the pontine reticular formation over a period of more than 20 seconds by means of an oil-driven pressure pump attached to the injecting micropipettes. The location of injection sites corresponded

to the nucleus reticularis pontis oralis (NRPo) (Horsley-Clarke coordinates, P 2~2.5, LR 1.0~2.5, H -3.0~-5.5; Fig1C), where carbachol injection produced postural atonia. [8,13]. At the end of each experiment, injection sites were marked with injections of the same amount of Fast-Green solution. The cats were then sacrificed with an overdose of Nembutal anesthesia and the brainstem was removed and fixed in 10% formalin in saline. Frozen 50 •m parasagittal sections were then cut and stained with cresyl violet. The location of the SCN (Fig.1B) and carbachol injection site (Fig.1C) was identified with reference to the stereotaxic atlases of Berman [14] and Snider and Niemer [15].

Results

We first examined the effects of melatonin administration in the mesencephalic and hypothalamic cats (Fig.2). Tonic muscle contraction associated with sporadic eye movements was observed in the hypothalamic cat before melatonin application (left column in Fig.2A). However, an intravenous application of melatonin gradually decreased the level of bilateral soleus muscle activities and produced a burst of REM (right column in Fig.2A). It required 5-7 minutes to produce the state of a REM with atonia, and the state continued for more than 10minutes. Although it is not shown here, pinna stimulation, delivered by manually pinching the scapha, during the period of postural atonia did not restore muscle activity. Essentially the same findings were observed in 7 trials in 3 animals. In contrast, an administration of melatonin in the mesencephalic cats (4 trials in 3 animals) produced neither rapid eye movement nor reduced postural muscle tone (Fig.2B). The intravenous application was performed in 4 trials in 3 mesencephalic cats and 4 trials in 3 hypothalamic cats. The diazepam administration did not produce REM but bilaterally abolished

muscle tone (Fig.3A) in each type of animal preparation. Pinna stimulation easily elicited muscle contractions of soleus muscles (open triangles). By contrast, an intrapontine injection of carbachol in the mesencephalic cat resulted in suppression of postural muscle tone associating REM (Fig.3B). The muscular atonia persisted for more than 30 min, and pinna stimulation failed to elicit any muscular contractions during this period (not illustrated). The state of REM with atonia was produced by carbachol injection with a period between 2 and 7 minutes in 6 trials of 4 animals.

Throughout all experiments, acute respiratory side effects, such as dyspnea or apnea, were not observed after administration of melatonin. With histological analysis, we confirmed that the SCN was preserved in all the hypothalamic cats (Fig.1C), whereas it was not preserved in the mesencephalic cats.

Discussion

Melatonin is synthesized from serotonin by pinealocytes in the pineal grand. Its secretion depends on the circadian rhythm with, in human, the minimum during daytime and maximum secretion at night, being influenced by a photic input [16]. It is believed that melatonin plays an important role in reproductive function, regulation of circadian rhythms and generation of sleep cycles [17, 18, 19]. Melatonin receptors are found at several sites in the central nervous system, mainly in SCN [4, 5], but not in the brainstem and spinal cord [5]. Hypocretin neurons in the lateral hypothalamus send their axon to the pineal gland, and partially inhibit the beta-adrenergic-induced melatonin secretion [20]. Hypocretin neurons may also be regulated by the circadian and photo-neuroendocrine systems, and they may play a part in feedback regulation system of melatonin secretion rhythm.

Because REM with atonia was not induced by the melatonin administration in the mesencephalic cat but was in the hypothalamic cats, it may be that melatonin effects are produced via the SCN neurons. However, in *in vitro* slice preparation, the effect of melatonin on SCN neurons was mainly inhibitory [21, 22]. The massive efferent projection from the SCN terminates in the subparaventricular area and in the paraventricular nucleus [23], but there is little efferent projection to the ventrolateral preoptic area which has an important role in sleep onset and maintenance [24]. Novak et al. [25] reported that SCN neurons containing arginin vasopressin project to the paraventricular thalamic nucleus (PVT), and Peng et al. [26] demonstrated that expression of c-*fos* in the PVT increases at times of day when the animals are most active in both nocturnal rats and the diurnal rodent Arvicanthis niltoicus (Nile grass rat). These results suggest that SCN neurons mainly project to the regions involved in awakening rather than in sleep, and the sleep induction effect of melatonin could be produced by inhibiting SCN activities. The SCN also sends a few efferent projections to the midbrain [27]. However, neuronal connections between the SCN and the brainstem, which may be involved in the REM sleep generation, have not been confirmed. Nonetheless, this is the first report demonstrating the evidence of involvement of melatonin in triggering REM sleep mechanisms in the brainstem.

Intrapontine carbachol injection in the mesencephalic cats resulted in postural atonia that resembled that associated with REM sleep [7, 8]. Carbachol injection activated the reticulospinal system, which postsynaptically inhibited spinal alpha-motoneurons [13], indicating that motor inhibition during REM sleep is caused by the postsynaptic inhibition of alpha-motoneurons. On the other hand, diazepam-induced muscular atonia was not associated with REM. This indicates that diazepam does not activate the REM atonia system but decreases the activity of the descending excitatory pathways including the

coerulospinal tract [28]. The reduction in the activity of the excitatory descending system by diazepam could be produced by the enhancement of GABA receptors [29]. From these considerations, we postulate that melatonin activates REM sleep generating mechanisms in the brainstem, and the neuronal mechanism activated by the melatonin is essentially different from those activated by benzodiazepines.

In clinical applications, benzodiazepines have sedative and hypnotic effects in addition to their anticonvulsant and anxiety reducing effects. However, it has been reported that benzodiazepines increase REM-sleep latency, reduce slow-wave sleep (stages 3 and 4) and reduce REM-sleep period [30]. In contrast to this, and to barbiturates, melatonin dose not reduce REM-sleep [2, 3]. We clinically compared a combined therapy with flunitrazepam and melatonin and a monotherapy with flunitrazepam for sleep disturbances in a 14-year-old boy with spastic quadriplegia [31]. Polysommnographic studies showed that the combined therapy more preferentially decreased the number of awakenings, increased the total sleeping period, and increased the REM period which was associated with a decrease in the non-REM (stage I and II) period, than did the flunitrazepam monotherapy. Based on these clinical findings and in addition to the results obtained in our animal experiments, we speculate that the hypnotic effects induced by melatonin result from the activation of the sleep generating system, while benzodiazepines could non-specifically suppress the activity of both an alert system (descending excitatory system) and the sleep generating system (descending inhibitory system). Based on these considerations, we propose that melatonin is a more beneficial and safer therapeutic agent for treating sleep disturbances in comparison with the benzodiazepines or barbiturates. However, further investigations should be undertaken that focus on the clinical usage of melatonin for various types of sleep disturbances.

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Figure Legends

Figure 1

A: Decerebration level of the animal preparation in the present study. The suprachiasmatic nucleus (SCN) is removed in the mesencephalic cat (a), and is preserved in the hypothalamic cat (b). B: Location of the carbachol injection site. BC: brachium conjunctivum, IC: inferior colliculus, SC: superior colliculus, SCN: suprachiasmatic nucleus, NRPo: nucleus reticularis pontis oralis. P2 indicates Horsley-Clarke level.

Figure 2

Triceps surae muscle activities at intravenous administration of melatonin in mesencephalic cat (A) and hypothalamic cat (B). Neither rapid eye movement nor reduction of muscle activity was induced by melatonin in the mesencephalic cats. In the hypothalamic cats, however, rapid eye movement were induced with prompt reduction of muscle activity.

Figure 3

Triceps surae muscle activities and electrooculograms at intravenous administration of diazepam (A) and carbachol microinjection into the pontine reticular formation (B), in the mesencephalic cats. By diazepam administration, reduction of muscle activity was induced, and rapid eye movement was not induced. On the other hand, drastic reduction of muscle activity were rapidly induced by intrapontine carbachol injection with obvious rapid eye movement.