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Altered MT1 and MT2 melatonin receptors expression in the hippocampus of pilocarpine-induced epileptic rats

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ABSTRACT

Clinical and experimental findings show that melatonin may be used as an adjuvant to the treatment of epilepsy-related complications by alleviates sleep disturbances, circadian alterations and attenuates seizures alone or in combination with AEDs. In addition, it has been observed that there is a circadian component on seizures, which cause changes in circadian system and in melatonin production. Nevertheless, the dynamic changes of the melatoninergic system, especially with regard to its membrane receptors (MT_1 and MT_2) in the natural course of TLE remain largely unknown. The aim of this study was to evaluate the 24-hour profile of MT_1 and MT_2 mRNA and protein expression in the hippocampus of rats submitted to the pilocarpine-induced epilepsy model analyzing the influence of the circadian rhythm in the expression pattern during the acute, silent, and chronic phases. Melatonin receptor MT_1 and MT_2 mRNA expression levels were increased in the hippocampus of rats few hours after SE, with MT_1 returning to normal levels and MT_2 reducing during the silent phase. During the chronic phase, mRNA expression levels of both receptors return to levels close to control, however, presenting a different daily profile, showing that there is a circadian change during the chronic phase. Also, during the acute and silent phase it was possible to verify MT_1 label only in CA2 hippocampal region with an increased expression only in the dark period of the acute phase. The MT_2 receptor was present in all hippocampal regions, however, it was reduced in the acute phase and it was found in astrocytes. In chronic animals, there is a reduction in the presence of both receptors especially in regions where there is a typical damage derived from epilepsy. Therefore, we conclude that SE induced by pilocarpine is able to change melatonin receptor MT_1 and MT_2 protein and mRNA expression levels in the hippocampus of rats few hours after SE as well as in silent and chronic phases.

1. Introduction

Temporal lobe epilepsy (TLE) is the most common type of epilepsy in adult humans [1] and could be initiated by a cerebral insult, *Status Epilepticus* (SE) or by genetic predispositions/syndromes [2,3]. The damaged areas in TLE include the limbic system where occurs neuronal degeneration, astrogliosis, aberrant sprouting and circuit reorganization in dentate gyrus CA3, CA1, and subiculum [4], increase of inflammatory cytokines and oxidative stress [5–20], and a reduction or loss of GABAergic inhibition [21–24]. TLE patients is highly refractory to pharmacological treatment since 25% of these patients have seizures that cannot be controlled by anti-epileptic drugs (AEDs) [25]. Therefore, many studies have aimed to understand TLE pathophysiological processes and to find strategies to improve its treatment. In this context, the melatonin may be a potential adjuvant for epilepsy treatment since it has anti-inflammatory, antioxidant, inhibitory and GABAergic activities [26–30] and low toxicity even at pharmacological doses.

Many clinical studies over recent years have demonstrated that, in epileptic children and adults, melatonin alleviates sleep disturbances, reduces circadian alterations [26,31–35], decreases the frequency of seizures, reduces the spicules in the electroencephalographic tracing, attenuates seizures alone or in combination with AEDs, increases activity of antioxidant enzymes (GPx and GRd) and improves the quality of life, regarding appetite, improvement in attention, memory, language and anxiety [31,36–39]. It has also been shown the neuroprotective effect of melatonin on seizures and pathological alterations induced by many experimental models of epilepsy such as induced by kainite, penicillin and pilocarpine [40–44].

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Melatonin is synthesized by the pineal gland in a circadian manner, with maximal production at nighttime darkness [45,46]. Many of the melatonin effects are mediated via G-protein-coupled receptors (MT_1 and MT_2) that are found in several structures of the Central Nervous System (CNS), including hippocampus [46–49]. Evidences show that MT_1 can inhibit calcium influx [50,51] and can have an acute inhibitory effect on neurons electrical activity. MT_2 is able to inhibit the formation of cAMP and cGMP [52,53], mediates the action of melatonin on phase shifts of the circadian rhythm [54] and increases the activity of PKC [55]. In rat hippocampus, melatonin inhibits long-term potentiation (LTP) and the excitability through the MT_2 receptor [56]. These data show that MT_1 and MT_2 receptors have a potential inhibitory effect on CNS. In this context, agomelatine, a MT_1 and MT_2 receptor agonist, has had anticonvulsant effects on seizures in experimental models of epilepsy [57,58].

In addition, it has been observed that there is a circadian component on seizures, which cause changes in circadian system and in melatonin production. Bazil et al. [59] observed that in adult patients with TLE, the interictal melatonin levels are significantly decreased compared to control patients. Moreover, temporal distribution of clinical seizures over 24-h has been shown both in human [60,61] and experimental models [62–67]. Furthermore, we demonstrated that epilepsy induced by pilocarpine is able to modify the circadian profile of the melatonin nuclear receptor ROR alpha, which has been considered a component of the circadian system [68].

Although clinical and experimental findings show that melatonin may be used as an adjuvant to the treatment of epilepsy-related complications, the dynamic changes of the melatoninergic system, especially with regard to its membrane receptors (MT_1 and MT_2) in the natural course of TLE remain largely unknown. Thus, to help understanding the molecular and functional mechanisms following the natural progress of the TLE that can lead to novel strategies for the treatment of this chronic disease with melatonin and/or melatonin agonists, the aim of this study was to evaluate the 24-hour profile of MT_1 and MT_2 mRNA and protein expression in the hippocampus of rats submitted to the pilocarpine-induced epilepsy model at different stages of the epileptic process.

2. Material and methods

2.1. Experimental design

All experimental procedures were approved by the Ethics Committee of Universidade Federal de São Paulo (CEP number 0403/11 protocol) and conducted in accordance with its guidelines. Male Wistar rats, 60 days-old, were housed under a light–dark schedule of 12 h of light and 12 h of dark, with food and water ad libitum and room temperature of 21–23 °C. Animals from all groups were euthanized in specific time points throughout the circadian cycle, according to the *zeitgeber time* (ZT). The ZT is a standardized nomenclature based on the period of a *zeitgeber*. In a light–dark cycle of 12–12 h the moment that lights goes on is usually defined as *zeitgeber time* zero (ZT0) and the moment lights goes off is defined as *zeitgeber time* twelve (ZT12). For PCR analysis, six animals (three control and three experimental ones for each time point) were euthanized every 3 h for 24 h totalizing 48 animals. Nine animals (five control and four experimental ones for each time point) were used for immunohistochemistry assays at ZT3 and ZT15. The euthanasia was carried out under a dim red light at the dark phase points which does not impair the production of endogenous melatonin (Fig. 1).

2.2. The pilocarpine model

Rats were injected, at ZT3, with 4% pilocarpine (350 mg/kg, i.p. Merck SA, USA) in order to induce temporal lobe epilepsy. To avoid the peripheral muscarinic effects, methyl-scopolamine (1 mg/kg, sc, Sigma Co., USA) was injected 30 min prior to each pilocarpine administration. Control animals received saline vehicle. The onset of the SE was defined as the beginning of category 4–5 of seizure severity. Seizure severity was rated by the Raccine scale [69]: category 1, immobility and facial twitch; category 2, head nodding; category 3, forelimb clonus; category 4, rearing; and category 5, rearing and falling. The presence of sustained *status epilepticus* (SE) was characterized by continuous seizure activity for 30 min (SE) what is considered as an effective response to pilocarpine. Animals were continuously monitored via video surveillance (Stellate system) to observe the latent period (no seizures and it may last from 4 to 44 days) and the occurrence of the first spontaneous seizure, inaugurating the chronic period (around 44 days after SE) [70,71].

2.3. Animal groups

2.3.1. Acute phase groups

As the experimental animals received pilocarpine at ZT3, in order to maintain the equivalence of the time after the SE and the time of the circadian cycle, we only included rats that presented SE no later than ZT4. For the PCR analysis animals were euthanized every 3 h starting from ZT6 (2 h after SE) to ZT3 of next day (23 h after SE), representing

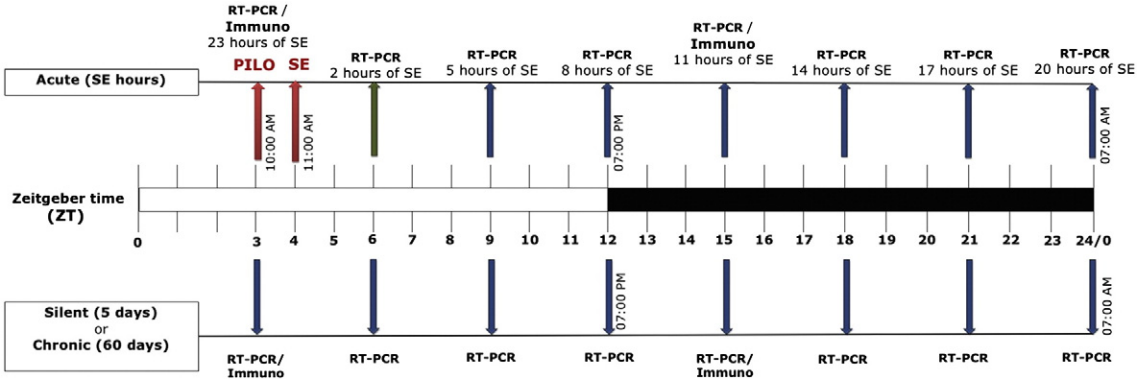

Fig. 1. Experimental design for acute, silent and chronic phase groups. Pilocarpine was injected at 10:00 am (ZT3). Rats which presented SE until 11:00 am (ZT4) were included in the experiments. Groups of animals were euthanized every 3 h around the 24-hour light–dark cycle starting at 1:00 pm (ZT6) for the RT-PCR assay, generating a total n of 48 (24 SE and 24 controls, n = 3 for each time point). For immunohistochemistry, the time points chosen were 10:00 pm(ZT15) and 10:00 am(ZT3) (total n of 18: control = 5, SE = 4 at each time point). Silent phase rats were euthanized 5 days after SE, and chronic phase animals were euthanized 60 days after SE, every 3 h along the 24-hour light–dark cycle for the RT-PCR assay, generating a total n of 48 for each phase (24 experimental and 24 controls, n = 3 for each time point). For immunohistochemistry, the time points chosen were 10:00 pm (ZT15) and 10:00 am(ZT3) (generating a total n of 18 for each phase (control = 5, experimental = 4 at each time point). IMMUNO = immunohistochemistry. PILO = pilocarpine; SE = *status epilepticus*;. The black box represents the dark period of the circadian cycle.