Hippocampal Melatonin Receptors Modulate Seizure Threshold

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Summary: Purpose: The pineal hormone melatonin has been shown to enhance hippocampal excitability. We therefore investigated whether inactivation of hippocampal melatonin receptors affects behavioral seizures.

Methods: Intrahippocampal infusions were performed in rats to study the effect of different melatonin receptor antagonists on behavioral activity, EEG, and seizure susceptibility. Experiments were conducted at 2 times of the day that coincided with the peak and trough of the daily melatonin rhythm.

Results: Local infusion of the Mel 1b receptor antagonist 4-phenyl-2-propionamidotetralin (4-P-PDOT) into the hippocampus, but not the overlying neocortex, significantly increased seizure latency and in some cases provided complete protection against seizure development. In addition, 4-P-PDOT suppressed open field activity and hippocampal EEG amplitude. The mixed Mel1a/Mel1b receptor antagonist luzindole also increased seizure latency but to a lesser degree than 4-P-PDOT. The behavioral effects of Mel1b receptor inhibition were comparable to those of the γ-aminobutyric acid (GABA)A receptor agonist muscimol and were observed during the dark phase (2400–0200 h) but not the light phase (1200–1400 h) of the daily photocycle. The anticonvulsant effect of intrahippocampal infusion of 4P-P-DOT was blocked by coadministration of the GABAA antagonist bicuculline.

Conclusions: Our results suggest that nocturnal activation of hippocampal Mel1b receptors depresses GABAA receptor function in the hippocampus and enhances seizure susceptibility.

Key Words: Mel1b receptor—Dorsal hippocampus—Seizure latency—GABA—Pilocarpine—Rat.
time-dependent manner. Preliminary results of this study have appeared in abstract form (11).

METHODS

Animals and surgery

At the start of the study, adult male Wistar rats (225–250 g; N = 67 total) were entrained to a 12 h/12 h light–dark cycle with lights on at 0800 h. Under pentobarbital (PTB) anesthesia, rats were implanted with four 23-gauge steel guide cannulae (two injection sites per hemisphere) dorsal to the hippocampus (from bregma in mm: P, –4.0; L, ±2.2; and P, –6.0; L, ±2.8) and stainless steel electrodes (125 µm, Teflon coated) bilaterally in the stratum radiatum of hippocampal CA1 (P, –4.0; L, ±2.2; V, 3.3 from skull surface). Rats were allowed ≥7 days of recovery after surgery. All experiments were conducted during either the mid-light phase (1200–1400 h) or mid-dark phase (2400–0200 hrs) of the daily photocycle.

Intrahippocampal drug injections and open field testing

Rats received bilateral microinfusion into the hippocampal fissure (V 3.8 for anterior site and V 4.0 for posterior site) of one of the following: (a) mixed Mel1a/Mel1b antagonist N-acetyl-2-benzyltryptamine or luzindole [LZ; 2 µg/µl/site in dimethyl sulfoxide (DMSO)], (b) Mel1b antagonist 4-phenyl-2-propionamidotetralin (4P-PDOT; 2 µg/µl/site in DMSO), (c) GABA A agonist muscimol (1 µg/µl/site in saline), or (d) an equivalent volume of saline or DMSO. Drugs were infused through an inner injection needle that projected 2 mm below the guide cannulae. All intrahippocampal injections were performed manually by using a Hamilton microsyringe at a rate of ∼0.5 µL/min. Twenty minutes after drug infusions, rats were removed from their home cages, and spontaneous locomotor activity (horizontal movement) in a 20 × 20 × 8-inch open field was quantified every minute for 15 consecutive minutes by using an infrared photobeam movement sensor (Columbus Instruments, Columbus, OH, U.S.A.).

Electroencephalographic (EEG) recordings

Hippocampal EEG was recorded in rats before drug infusions during awake immobility and 5 min before open field testing, irrespective of behavior (∼15 min after drug infusions). The EEG was amplified by a Grass Model 8–10 polygraph (filtered between 1 and 70 Hz) and sampled at 100 Hz by using a model 2800 digital oscilloscope (A-M Systems, Inc., Carlsborg, WA, U.S.A.). Power spectral analysis of the EEG was performed offline according to established methods (12).

Evaluation of seizure latency

No less than 2 days later, rats were administered scopolamine methyl nitrate (1 mg/ml, i.p.) followed 30 min later by pilocarpine (350 mg/ml, i.p.) to induce generalized convulsive seizures. With the same treatment groups, hippocampal drug infusions were performed 20 min before pilocarpine injection with two additional groups added: (a) LZ or 4-P-PDOT was injected dorsal to the hippocampus into the neocortex (V, 1.4 mm), and (b) coinfusion of 4-P-PDOT and the GABA A antagonist bicuculline methiodide (BIC; 400 ng/site in DMSO) into hippocampus. Seizure-onset latency was defined as the time elapsed from pilocarpine injection to the occurrence of an overt behavioral seizure of Racine stage 3 or higher (i.e., bilateral forelimb clonus with or without rearing and loss of posture) (13). Rats were observed for ≤60 min, after which they were assigned the ceiling measure of 60 min for seizure-onset latency. From our experience, rats that did not show behavioral seizures within this time frame seldom displayed any evidence of seizure activity later on.

Histology and statistical analyses

Electrode placement was verified histologically in 40-µm frozen sections stained with thionin. Drug-diffusion volume was estimated by perfusing rats 20 min after injecting dye through the inner cannulae (2% aqueous Congo Red). Measures of locomotor activity were compared by using a repeated measures analysis of variance (ANOVA). EEG power spectra [peak power in the theta (4–10 Hz) frequency band], and seizure-onset latencies were analyzed by using one-way ANOVA with post hoc t tests.

RESULTS

Histology

Histologic analysis confirmed that 24 electrodes from 12 rats were located in CA1 (Fig. 1). The depth of guide cannulae also was verified to be in shallow neocortex, dorsal to the hippocampus, with the injection cannulae lowered to 2 mm below the guide cannulae. Bilateral intrahippocampal dye infusions indicated that each drug injection may have affected a volume of ∼1-mm diameter 20 min after injection (see Methods). In a previous study, we suggested that this diffusion volume would affect ∼60% of the dorsal hippocampus (14).

Experiment 1: Effects of bilateral intrahippocampal infusion of melatonin receptor antagonists on EEG and open field behavior

During the light phase, baseline hippocampal EEG during behavioral immobility was characterized by irregular slow activity with low theta rhythm (4–10 Hz) amplitude. After injection of saline into the hippocampus, rats began to walk around their home cages, and their hippocampal EEG was characterized by high-amplitude theta activity. Thus after saline injection, the peak theta power was significantly higher than during baseline immobility, and this was found after LZ and 4-P-PDOT injections as well (Table 1). However, after muscimol (2 µg/site) injection, the theta rhythm was significantly suppressed compared with the other treatment groups (see Table 1). The EEG
at all frequencies was reduced after muscimol (Fig. 2A). After placement in the open field, control rats injected with saline engaged in exploratory behavior (thigmotaxis, rearing) and displayed a clear habituation to the test environment over time (Fig. 2B). Rats injected with LZ or 4-P-PDOT showed similar open field behavior as compared with saline controls. In contrast, muscimol-injected rats showed a marked decrease in locomotion, or a near-complete cessation of spontaneous movements, although the rats maintained an upright posture (righting reflex was intact) and were responsive to tactile stimulation and tail pinch. In a subgroup of rats (n = 3), DMSO vehicle did not affect hippocampal EEG or seizure latency compared with saline controls (data not shown).

When administered during the dark phase, both muscimol and 4-P-PDOT reduced locomotion in the open field as compared with that in LZ- and saline-treated animals (Fig. 2D). Similarly, muscimol and 4-P-PDOT injections resulted in a decrease in EEG power (Fig. 2C), including the theta rhythm (Table 1), as compared with LZ and saline. Thus the EEG and locomotor activities induced by 4-P-PDOT were different between the light and dark phases.

**Experiment 2: Effects of bilateral intrahippocampal infusion of melatonin-receptor antagonists on pilocarpine-induced motor seizures**

Pilocarpine-induced behavioral seizures were characterized by tonic–clonic contractions of the face, neck, and forelimbs with or without rearing and a loss of posture. Co-incident EEG activity was characterized by the emergence of high-amplitude and low-frequency (<2 Hz) paroxysmal activity. During the light phase, injection of LZ and 4-P-PDOT into the hippocampus did not affect seizure-onset latency as compared with saline controls, whereas muscimol completely blocked pilocarpine-induced seizures in five of seven rats tested and significantly increased seizure-onset latency in the other two rats (Fig. 3A).

### TABLE 1. Peak logarithm power of hippocampal EEG (mean ± S.E.M.) for the theta frequency band before and after intrahippocampal drug infusions during the light and dark phases

<table>
<thead>
<tr>
<th>Group</th>
<th>Light Phase</th>
<th>Dark Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Drug</td>
</tr>
<tr>
<td>Saline</td>
<td>4.59 ± 0.07</td>
<td>4.95 ± 0.09</td>
</tr>
<tr>
<td>Luzindole</td>
<td>4.57 ± 0.09</td>
<td>5.12 ± 0.08</td>
</tr>
<tr>
<td>4P-P-PDOT</td>
<td>4.55 ± 0.08</td>
<td>5.01 ± 0.12</td>
</tr>
<tr>
<td>Muscimol</td>
<td>4.60 ± 0.07</td>
<td>4.47 ± 0.02</td>
</tr>
</tbody>
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**A**P: level of significance for One-way ANOVA (down column)

*P<0.01 versus saline control for luzindole and 4-P-PDOT (Tukey’s post-hoc test)

+P<0.05 decrease of theta power after injection as compared to baseline immobility (Student’s t-test)

1 the increase in theta power after injection compared to baseline was attributed to increase in locomotion after injection EEG data for N ≥ 4 per treatment group.
**DISCUSSION**

Our results provide new evidence that hippocampal melatonin receptors modulate locomotor activity and seizure latency in a time-dependent manner. Intrahippocampal injections of the Mel$_{1b}$ antagonist 4-P-PDOT, but not the mixed Mel$_{1a}$/Mel$_{1b}$ receptor antagonist LZ, suppressed open field locomotion during the dark phase.

**During the dark phase,** the effect of LZ and 4-P-PDOT on pilocarpine seizures was different from that observed during the light phase. LZ and 4-P-PDOT injections into the hippocampus significantly increased seizure latency during the dark phase, the latter having the same effectiveness as muscimol treatment (Fig. 3B). Three of six 4-P-PDOT–treated rats did not display any evidence of seizure activity after pilocarpine administration during the dark phase. As a control procedure, LZ or 4-P-PDOT was infused 2 mm dorsal to the hippocampal fissure in the overlying neocortex, but these injections had no effect on seizure latency during either the light or the dark phase.

In light of the effects of 4-P-PDOT and GABA$_A$-receptor agonist muscimol, we examined whether the anticonvulsant action of 4-P-PDOT involved an enhancement of hippocampal GABA$_A$-receptor activity. Coadministration of the GABA$_A$-receptor antagonist BIC (400 ng/side) and 4-P-PDOT into the hippocampus abolished the effect of 4-P-PDOT on seizure latency during the dark phase (Fig. 3B). Injection of BIC alone into the hippocampus had no significant effect on seizure latency compared with controls and produced only minor behavioral alterations (i.e., wet-dog shakes and vocalization). A higher dose of BIC (1 µg/side) produced frequent myoclonic jerks and jumping and decreased seizure latency (data not shown).

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FIG. 3. Effects of bilateral infusion of luzindole, 4-phenyl-2-propionamidotetralin (4-P-PDOT), and muscimol into the hippocampus on pilocarpine seizure latency. Seizure activity was induced with a single injection of pilocarpine (350 mg/kg, i.p.). Twenty minutes before pilocarpine, each drug was infused bilaterally into the hippocampal fissure at a rostral and caudal site (two sites per side). A: Luzindole and 4-P-PDOT (4 µg/side) did not affect seizure latency during the light phase, whereas muscimol (2 µg/side) increased seizure latency compared with that in saline controls (only two of seven rats displayed evidence of seizures; the remaining rats were assigned the ceiling measure of 60 min). B: Luzindole, 4-P-PDOT and muscimol (half of rats displayed no seizures) all increased seizure latency during the dark phase compared with controls. Coadministration of bicusculline (BIC; 400 ng/side) with 4-P-PDOT blocked the anticonvulsant effect. Luzindole and 4-P-PDOT did not affect seizure latency during either period when injections were placed dorsally in cortex (Cx). Mean seizure onset latency ± SEM is shown. ∗Significant difference (p < 0.01) from the saline control group (analysis of variance and Tukey’s post hoc).

with a corresponding decrease in hippocampal theta rhythm amplitude. LZ or 4-P-PDOT also increased seizure latency during the dark phase but not light phase of the daily photocycle. The anticonvulsant effect of 4-P-PDOT was comparable to the GABA_A-receptor agonist muscimol and could be reversed by coadministration of the GABA_A-receptor antagonist bicusculline. Thus the anticonvulsant and locomotor activity effects of Mel1b inhibition may involve an enhancement of postsynaptic GABA_A-receptor function during the dark phase.

Blockade of hippocampal Mel1b receptor affects spontaneous locomotor activity

The hippocampus represents a major structure involved with the initiation of locomotor behavior through connections with the ventral striatum (15). During voluntary movement, the hippocampus exhibits rhythmical slow (theta) waves of 6–8 Hz (16). Increased locomotor activity was induced by stimulation of the hippocampus (15,17) and effects of various pharmacologic treatments on locomotion also are mediated in part by the hippocampus (18,19). In the present study, bilateral infusion of 4-P-PDOT, but not LZ, into the hippocampus decreased hippocampal EEG power and open field locomotion only during the dark phase. Intrahippocampal injection of muscimol produced similar effects on suppressing hippocampal EEG and locomotion during both light and dark phases.

The effect of intrahippocampal injection of muscimol on locomotion and other voluntary movements has been reported (14). The suppression of movements by muscimol or 4-P-PDOT was not related to muscle paralysis because righting and other sensorimotor behaviors were intact.

Blockade of hippocampal Mel1b receptor increases seizure latency

Our most important finding is that blockade of hippocampal Mel1b receptors delays the onset of pilocarpine seizures during the dark phase. This behavioral effect was comparable to that obtained after bilateral activation of GABA_A receptors in the hippocampus with muscimol. Drug infusions placed dorsally in the neocortex did not delay seizure onset at any time. In the pilocarpine model, deep temporal lobe structures such as the piriform cortex and amygdala, along with frontal and orbital cortices, were found to be focal points for seizure initiation (10), which subsequently spreads to the hippocampus. Consequently, the relative absence of electrographic seizures in CA1 after 4-P-PDOT or muscimol does not preclude the development of paroxysmal activity elsewhere. However, inactivation of the hippocampus with muscimol was sufficient to block convulsive seizures in a majority of rats in our study. Millan and coworkers (20) demonstrated that inrahippocampal injection of pilocarpine alone could evoke seizure activity, suggesting that the hippocampus
possesses the necessary connectivity to generate and sustain seizures.

LZ increased seizure latency during the dark phase despite having no effect on the locomotor activity or hippocampal theta activity during the same period. This may be attributed to the different neural circuits that may be responsible for locomotion and seizure development. In addition to projecting to the nucleus accumbens, the hippocampus can access the motor system through prefrontal cortex (15) and the dorsal striatum (21), both of which may contribute to the development of pilocarpine seizures (10). The selective Mel1b antagonist 4-P-PDOT suppressed locomotor and seizure activity during the dark phase, suggesting that the Mel1b receptors suppress GABA<sub>A</sub> function on pyramidal cells projecting in both pathways. However, because the Mel1a subtype in the dentate gyrus, CA3, and CA1 (6) is likely blocked by LZ (Fig. 3), inhibition of Mel1a receptors may evoke opposite effects on GABA<sub>A</sub>-receptor function in certain neurons, such as increased GABA<sub>A</sub>-mediated current amplitudes (8).

**Locomotor and anticonvulsant effects of Mel1b-receptor inhibition are time dependent**

Taken together, our findings are in agreement with the hypothesis that hippocampal melatonin receptors, specifically the Mel1b subtype, depress GABA<sub>A</sub> receptor-mediated inhibition. The behavioral effects of Mel1b inhibition also were restricted to the dark phase when melatonin levels are presumably highest. This may suggest different mechanisms of modulating hippocampal GABA<sub>A</sub> receptor during the dark versus light phase. At night, high melatonin levels could depress postsynaptic GABA<sub>A</sub> receptors and enhance hippocampal output, which is in turn translated into increased locomotor activity and seizure susceptibility. During the day when melatonin production is minimal, GABA<sub>A</sub> receptor may be controlled by other mechanisms, such as subcortical modulation of GABA<sub>A</sub> function (22).

Mel1b receptors on CA1 pyramidal cells suppress GABA<sub>A</sub>-receptor function through an unknown signaling pathway (16). Mel1b receptors are thought to stimulate phospholipid hydrolysis and possibly the mobilization of protein kinase C or the phosphatase calcineurin, whereas the Mel1a receptor is coupled to inhibition of adenyl cyclase and protein kinase A activity (3,4). General agreement exists that GABA<sub>A</sub> receptors are substrates for multiple protein kinases and phosphatases, which partially determine the efficacy of GABA<sub>A</sub> receptors and can exert diverse effects, depending on receptor subunit composition (23–25). The finding that the GABA<sub>A</sub> antagonist BIC was able to counteract the effect of 4-P-PDOT on seizure latency is consistent with Mel1b receptors mediating a disinhibitory effect. Further experiments are necessary to determine which intracellular signaling molecules are responsible for the interaction between the Mel1b and GABA<sub>A</sub> receptors.

**Role of melatonin in epilepsy**

To our knowledge, this is the first study to suggest a proconvulsant role for endogenous melatonin in experimental epilepsy. Our results appear to be at odds with a large body of literature describing antiepileptic properties for melatonin against different seizure types. For example, exogenous application of melatonin raised the afterdischarge threshold in amygdala-kindled rats (26), potentiated the efficacy of conventional antiepileptic drugs (27), and increased seizure latency in the pilocarpine model (28). Children administered daily melatonin either alone or as add-on therapy showed a decrease in the severity and/or frequency of epileptiform activity (29,30). Much of the available literature, however, details the effects of exogenous administration of melatonin, which would presumably raise its bioavailability several orders of magnitude above what is produced physiologically by the pineal gland. At these doses, the effects of melatonin may be confounded by interactions with other transmitter systems such as GABA and serotonin. Moreover, in a study of temporal lobe epilepsy patients in which melatonin levels were measured at different times after seizures, it was suggested that a postictal increase in melatonin serves to control further seizure activity (31). However, no evidence was provided to support this hypothesis, and it is likely that melatonin, like many other chemical messengers induced by excessive neural activity (32), may have little or no involvement in seizure control and represent an epiphenomenon at best. Despite these findings, the role of endogenous melatonin in epilepsy remains controversial, although convincing evidence seems to exist that melatonin is a potent neuroprotectant (33).

Another factor that can be taken into consideration is the circadian influence on seizure occurrence. It is possible that factors other than melatonin may modulate diurnal changes in seizure susceptibility. Although the present findings are consistent with our previous study, in which a daily rhythm of pilocarpine seizure susceptibility was characterized by lower seizure-onset times during the dark phase (34), once chronic seizures have been established in pilocarpine-treated animals, as well as in the kainate model, spontaneous seizures are more frequent during the light phase, often while the animal is asleep or inactive (35,36). In a similar study, Quigg and coworkers (37) showed that spontaneous seizures in self-sustained limbic status epilepticus rats followed a clear diurnal rhythm with a higher number of seizures observed during the light phase. They further demonstrated that seizure occurrence occurred as a true circadian rhythm by obtaining the same results after exposing epileptic rats to a period of constant darkness (37). Accordingly, these long-term studies do little to support to a proconvulsive role for endogenous
melatonin, because the peak of the daily seizure rhythm in three separate animal models of epilepsy occurred during the light phase when circulating melatonin is presumably low. They do suggest, however, that state (i.e., sleep–wake transitions, sleep stage, behavioral activity) plays an important role in the chronic epileptic brain, whereas myriad other factors may have an influence on the induction of seizure activity in the nonepileptic brain (38).

Technical considerations

It has been demonstrated that the hippocampus exhibits uptake of systemically administered $[^3]$H]melatonin (39) and expresses both Mel1a and Mel1b melatonin receptors (8). Although we did not determine the concentration of melatonin in the hippocampus during the times selected for our experiments, it is possible that the hippocampus may exhibit a daily melatonin rhythm similar to that described for other areas of the CNS, such as the hypothalamus, where both melatonin concentration and the number of melatonin receptor–binding sites increase at night (40,41). Our findings that the electrographic and behavioral effects of intrahippocampal infusions of melatonin receptor antagonists were exclusive to the dark phase support this view.

CONCLUSION

We have shown that hippocampal Mel1b receptors modulate locomotor activity and seizure latency in a time-dependent manner. Even without intrahippocampal melatonin injections, the behavioral effects of the Mel1a/b receptor antagonists are likely the result of blocking melatonin action, because currently no endogenous ligands are known for these receptor subtypes other than the pineal hormone. It is suggested that physiologic melatonin promotes epileptogenesis through inhibitory effects on hippocampal GABA<sub>A</sub>-receptor function. A similar mechanism may be at work in human temporal lobe epilepsy due to the presence of Mel1b receptors in the human hippocampus (42).

Acknowledgment: This research was supported by grants from the Natural Sciences and Engineering Council and Canadian Institutes of Health Research #MOP36421 (L.S.L.). L. Stewart was supported by the Savoy Foundation (Quebec, Canada). We thank Dr. B. Shen for assistance.

REFERENCES


Epilepsia, Vol. 46, No. 4, 2005


