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MELATONIN ENHANCEMENT OF [^{3}H]-γ-AMINOBUTYRIC ACID AND [^{3}H]MUSCIMOL BINDING IN RAT BRAIN

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Abstract—The pineal hormone, melatonin, enhanced the sodium-independent binding of [^{3}H]-γ-aminobutyric acid ([^{3}H]GABA) and [^{3}H]muscimol in the rat cerebral cortex *in vitro*. This effect was augmented by preincubation of synaptic membranes with melatonin but was abolished by preincubation with Triton X-100. Saturation binding studies using [^{3}H]GABA (2.5 to 1000 nM) indicated that the melatonin-induced enhancement of binding is due to an increase in low-affinity $GABA_A$ binding sites. These findings suggest that the central effects of melatonin involve modulation of GABAergic function.

Extensive pharmacological and biochemical studies of the GABA† receptor complex have led to the concept of a benzodiazepine–GABA receptor chloride ionophore complex [1, 2]. Electrophysiological studies have shown that benzodiazepines (BZs) and barbiturates are capable of enhancing GABAergic neurotransmission [3]. Coupled with these findings is recent biochemical evidence that BZs and barbiturates enhance GABA binding in brain membranes [4]. Thus, it has been suggested that the sedative and anticonvulsive effects of these drugs involve modulation of GABA receptor activity [4].

The pineal hormone, melatonin, has been shown to possess anticonvulsive, sedative and hypnotic activities in both humans and animals [5, 6]. However, the site(s) and mechanism of the actions of melatonin await clarification. Evidence that melatonin displaces [^{3}H]diazepam binding in rat brain membranes [7] suggests an interaction of this hormone with the GABA receptor complex. We have therefore examined the effect of melatonin on GABA binding *in vitro*.

MATERIALS AND METHODS

Radioligands and chemicals. [^{3}H]GABA (80 Ci/mmol) and [^{3}H]muscimol (15–30 Ci/mmol) were obtained from New England Nuclear. Melatonin and GABA were purchased from Sigma, and diazepam was donated by Hoffmann-LaRoche.

Preparation of synaptic membranes. Male Sprague–Dawley or Wistar rats (200–400 g) were decapitated, and brain tissues were rapidly dissected and prepared as previously described [8]. Synaptic membranes were used in the binding assay immediately after preparation or were frozen at −20° for at least 18–20 hr before use. In some experiments, tissues were treated with Triton X-100 as previously reported [8]. Membranes were resuspended in 20–50 vol. of either Tris–citrate or Tris–HCl (0.05 M, pH 7.1) buffer for binding assays.

Preincubation with melatonin or diazepam. Aliquots (0.9 or 0.45 ml) of synaptic membrane suspensions were preincubated in triplicate with melatonin or diazepam (as indicated) for 60 min at 0–4° as preliminary experiments indicated that the effects of melatonin on GABA binding were augmented under these conditions. Control samples were preincubated under identical conditions but no drugs were added to these tissues. During preincubation of both control and drug-treated samples, all assay components were present with the sole exception of the radioligand.

Binding assays. Following preincubation, samples were incubated with 50 nM [^{3}H]GABA for 10 min at 4°, or 2.5 nM [^{3}H]muscimol for 30 min at 0° in single-point binding experiments. A range of radioligand concentrations was used in saturation studies as indicated. Bound [^{3}H]muscimol was separated by rapid filtration as previously described [8]. Samples incubated with [^{3}H]GABA were centrifuged at 48,000 *g* for 10 min at 4° to separate bound ligand. The supernatant fraction was decanted, and the pellet was rinsed rapidly with 2 × 4 ml of ice-cold buffer. The pellets were then solubilized overnight in 0.25 ml Protosol (New England Nuclear), and radioactivity was determined as previously described [8]. Specific binding was defined as total bound radioactivity minus binding in the presence of excess (10^{-4} M) unlabeled GABA or bicuculline. Protein content was measured by the method of Lowry *et al.* [9].

Data analysis. Binding parameters were determined using a non-linear least squares curve-fitting program (BDATA, EMF software, Knoxville, TN). Statistical analysis was done by Student's *t*-test.

RESULTS

Effects of Triton X-100 or freezing. Melatonin at a concentration of 10^{-7} M increased the specific binding of [^{3}H]muscimol by about 24% in the rat frontal cortex. Pre-treatment of membranes with 0.05% Tri-

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† Abbreviations: GABA, γ-aminobutyric acid; and BZ, benzodiazepine.

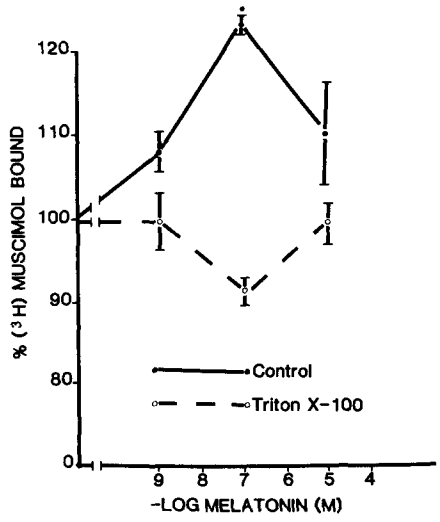

Fig. 1. [³H]Muscimol binding in fresh cortical synaptic membranes or Triton X-100-treated membranes as a function of melatonin concentration. Membranes were incubated in 0.05 M Tris–citrate buffer (pH 7.1) with 2.5 nM radioligand. Points represent means ± SEM of four experiments conducted in triplicate. Key: (*) $P < 0.01$ for Triton X-100-treated vs fresh membranes.

ton X-100 abolished the enhancing effect of melatonin as shown in Fig. 1. Freezing synaptic membrane preparations, like Triton X-100 treatment, resulted in an increase in specific GABA binding. However, in contrast to Triton X-100 pretreatment, a single freeze–thaw cycle augmented the effects of melatonin, whereas membranes subjected to two freeze–thaw cycles were less responsive to melatonin (data not shown).

Effects of preincubation with melatonin or diazepam. Maximal effects of melatonin on GABA binding were observed following preincubation of synaptic membranes with the hormone prior to assay. A 30-min preincubation with 10^{-4} M melatonin resulted in a 30% increase of [³H]GABA binding, while a 60-min preincubation yielded a 60% increase with the same melatonin concentration. The effects of diazepam were also enhanced by preincubation (Fig. 2).

Dose-dependent effects of melatonin and diazepam. Melatonin and diazepam (10^{-9} to 10^{-4} M) caused dose-dependent increases of 15–60% in [³H]GABA (50 nM) binding. The concentration (EC_{50}) of melatonin that caused half-maximal enhancement of binding was 1.5×10^{-5} M. Diazepam was about fourteen times more potent than melatonin and had an EC_{50} of 1.1×10^{-6} M.

Effects of melatonin on [³H]muscimol binding. Melatonin, at a concentration of 10^{-4} M, caused a significant increase of 96% in the density of high-affinity [³H]muscimol binding sites in rat cortical membranes. A concomitant decrease of more than 3-fold in binding affinity was also observed (Table 1).

Effects of melatonin on [³H]GABA binding. As indicated in Table 2, 10^{-4} M melatonin did not alter significantly high-affinity GABA binding. However, the density of low-affinity binding sites was increased significantly by about 120% in the presence of melatonin, which also caused a decrease in binding affinity.

Effects of diazepam on [³H]GABA binding. Diazepam increased the affinity of high-affinity sites but did not alter the density of these sites. As observed with melatonin,. diazepam at a concentration of 10^{-4} M, significantly enhanced low-affinity GABA binding. In the presence of diazepam,

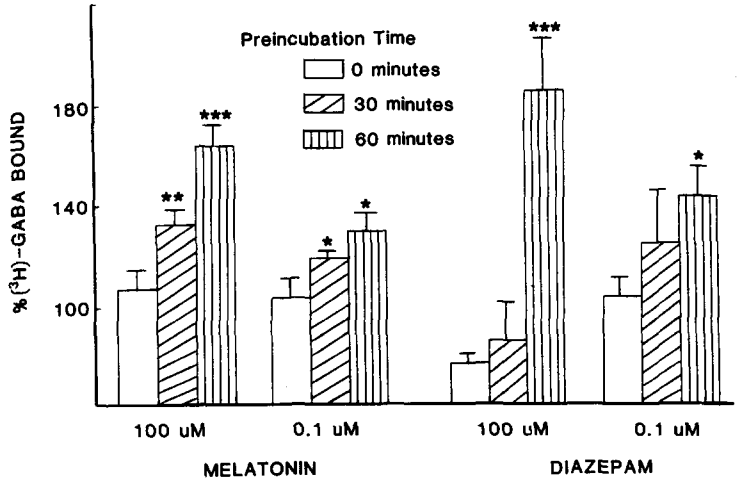

Fig. 2. Effects of preincubation with melatonin or diazepam on [³H]GABA binding in rat cerebral cortex. Previously frozen synaptic membranes were preincubated with melatonin or diazepam at 4° for the indicated periods and then incubated with 50 nM [³H]GABA as described in Materials and Methods. Means ± SEM of two to four experiments conducted in triplicate are presented. Values represent percent binding relative to control binding (in the absence of drugs) which was calculated as 100% for each condition. Key: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.005$ vs controls.

low-affinity sites exhibited an increase in binding affinity and about a 94% increase in density (Table 3).

DISCUSSION

The present findings indicate that the pineal hormone, melatonin, is capable of enhancing the *in vitro* binding of GABA in the rat brain. A similar *in vivo* enhancement of low-affinity GABA binding in the CNS has also been observed recently following chronic administration of melatonin to male rats [10]. Thus, the psychopharmacological effects of melatonin may be due to modulation of central GABA receptor function as previously reported for the benzodiazepines and barbiturates [4].

Since the range of [^{3}H]muscimol concentrations used in this study labeled primarily high-affinity sites, as indicated by the K_d values in Table 1, it appears that melatonin is capable of altering the characteristics of high-affinity GABA binding sites. However, saturation studies with [^{3}H]GABA indicate that the predominant effects of this hormone are on low-affinity GABA receptor sites. The results presented in Table 2 suggest that melatonin decreases the affinity of low-affinity sites while increasing the density of these sites. Nevertheless, an alternative explanation should also be considered. Recent reports have indicated the presence of a central GABA binding site with super-low affinity ($K_d = \sim 1\ \mu M$), which is not detected consistently in binding assays [11]. It is therefore possible that the effect of melatonin on GABA binding is due to the conversion of super-low affinity sites to low- (or intermediate) affinity sites, thus making them more readily detectable in binding assays. This is similar to the effect of barbiturates on GABA binding [11] and would account for the enhancement of low-affinity site density as indicated in Table 2.

The effects of melatonin were observed consistently in saturation binding studies, but we have had some difficulty in replicating these effects in single-point binding studies. This may be due to loss of binding during the separation procedure since melatonin appears to be primarily interacting with very-low-affinity sites as already discussed. It is conceivable that the random loss of low-affinity binding would have more significant effects on the con-

Table 1. Effects of melatonin on high-affinity binding of [^{3}H]muscimol in rat brain synaptic membranes with or without Triton X-100 treatment

	K_d (nM)		B_{max} (fmol/mg protein)	
Tissue treatment	Control	Melatonin	Control	Melatonin
None	6.9 ± 0.9	25 ± 1.8*	1372 ± 20	2689 ± 14*
Triton X-100	3.5 ± 0.2	3.9 ± 0.3	5316 ± 214	5306 ± 189

Frozen cortical membranes were thawed and treated with 0.05% Triton X-100 as previously reported [8]. Membranes were incubated with [^{3}H]muscimol (0.3 to 80 nM) at 0° for 30 min following preincubation with or without melatonin (10^{-4} M) at 0–4° for 60 min. Means ± SEM for four experiments conducted in duplicate are presented.

* $P < 0.05$ vs control.

Table 2. Effects of melatonin (10^{-4} M) on [^{3}H]GABA binding in rat brain

Treatment	K_{d_1} (nM)	B_{max_1} (fmol/mg protein)	K_{d_2} (nM)	B_{max_2} (fmol/mg protein)
Control	38 ± 4	637 ± 123	200 ± 19	3661 ± 348
Melatonin	28 ± 3	864 ± 132	394 ± 45*	8097 ± 817*

Frozen forebrain membranes were thawed, incubated at 37° for 30 min, and washed three times in the assay buffer before use in binding experiments. Membranes were preincubated with or without melatonin at 0–4° for 60 min. Saturation binding of [^{3}H]GABA was determined using a fixed concentration of radioligand and a range of nonradioactive GABA concentrations (2.5 to 1000 nM). Means ± SEM for four experiments conducted in triplicate are presented.

* $P < 0.01$ vs control.

Table 3. Effects of diazepam (10^{-4} M) on [^{3}H]GABA binding in rat brain

Treatment	K_{d_1} (nM)	B_{max_1} (fmol/mg protein)	K_{d_2} (nM)	B_{max_2} (fmol/mg protein)
Control	38 ± 6	810 ± 96	191 ± 23	1883 ± 183
Diazepam	17 ± 2*	731 ± 196	100 ± 7†	3645 ± 363†

Assays were carried out as described in Table 2. Means ± SEM for six experiments conducted in triplicate are presented.

* $P < 0.01$ vs control

† $P < 0.001$ vs control.

sistency of single-point studies with one concentration of radioligand, as compared with saturation studies utilizing several concentrations of radioligand.

The alteration in binding affinity induced by melatonin has also been reported for diazepam. Some investigators observed a decrease in the affinity of low-affinity GABA binding sites in the presence of diazepam [12], whereas others attributed the effects of diazepam to an increase in the affinity of low-affinity GABA receptor sites [13]. Our findings are in agreement with the latter report [13], as saturation experiments indicated that diazepam significantly enhanced the affinity of low-affinity sites for GABA (Table 3). Interestingly, although the effect of diazepam on the affinity of low-affinity sites for GABA appears to be opposite to that of melatonin, both of these compounds significantly increased the number of these sites. This similarity in the effects of diazepam and melatonin on GABA binding suggests that a common mechanism of action may be involved. Melatonin displaces [^{3}H]diazepam from BZ binding sites [7]; therefore, it may act on these sites to allosterically modulate GABA binding. In keeping with this view, we have found that the BZ antagonist, Ro15-1788, is capable of blocking the effect of melatonin on GABA binding (unpublished observations). These studies have been hampered by the ability of Ro15-1788 to alter baseline GABA binding, as previously reported by other investigators [14].

Melatonin competes for BZ binding sites in rat brain with an affinity of about 400 μM [7] which greatly exceeds circulating and brain levels of this hormone [15, 16]. Thus, only the pharmacological effects of melatonin involve BZ binding sites. However, the observation that nanomolar concentrations of melatonin are able to alter GABA binding indicates that high-affinity and physiologically relevant binding sites for melatonin [17] are also involved in modulating GABAergic function.

Given the similarities in their pharmacological effects and the fact that both diazepam and melatonin are highly lipophilic compounds, it is possible that like diazepam [18] melatonin increases membrane fluidity by stimulating phospholipid methylation. This may be the mechanism underlying melatonin's enhancement of GABA binding, since phospholipid methylation increases GABA binding in brain membranes [19]. The observation that preincubation augmented the effects of melatonin and diazepam on GABA binding suggests that maximal expression of the underlying mechanism requires more time than the typical 10-min incubation used in GABA binding experiments. This is in keeping with the suggestion of involvement of phospholipid methylation which is a time-dependent process. Further studies are required to determine the site(s) and mechanism of the modulatory effects of melatonin on central GABAergic function.

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