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Regulation of "Peripheral-Type" Binding Sites for Benzodiazepines in the Pineal Gland

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WEISSMAN, B. A., P. SKOLNICK AND D. C. KLEIN. *Regulation of "peripheral-type" binding sites for benzodiazepines in the pineal gland.* PHARMACOL BIOCHEM BEHAV 21(6) 821–824, 1984.—The density of "peripheral-type" binding sites for benzodiazepines (PBS) in the rat pineal gland was reduced by ~50% three weeks after superior cervical ganglionectomy or exposure to constant light; the apparent affinity of [^{3}H]Ro 5-4864 (the prototype ligand for these sites) remained unchanged. In contrast, neither the density of PBS nor the apparent affinity of [^{3}H]Ro 5-4864 for these sites was altered in crebral cortex by these procedures. The demonstration that PBS density is under neural control in the pineal gland suggests that these sites may have a physiologic role. The high density of PBS in pineal (~24 pmol/mg protein) suggests that this tissue may be a useful model to study both the physiologic and pharmacologic function of these sites.

Pineal Ro 5-4864 Superior cervical ganglionectomy Constant light

TWO physically and pharmacologically distinct classes of recognition sites for benzodiazepines have been described in the mammalian central nervous system (CNS). One class is found exclusively in the CNS and has been characterized as a pharmacologic receptor which mediates the principle therapeutic actions of the benzodiazepines [27]. A second class of sites was initially characterized in peripheral tissue [3] and subsequently shown to also be present in brain [5]. However, the apparent lack of stereospecificity of PBS [22,30] coupled with evidence of a non-neuronal localization of PBS in the CNS [5, 19, 23] has led some investigators to term these sites "acceptors" (that is, lacking physiologic or pharmacologic function) rather than receptors [12,24]. Recent autoradiographic studies have revealed a very high density of PBS in the pineal gland [18,29]. In addition, the pineal gland contains a nearly homogenous population of organotypic cells (80–90% pinealocytes) [28] and has a well defined neural input [6,8]. these observations prompted studies to determine whether PBS in the pineal gland are under neural control.

We now report that either superior cervical ganglionectomy (SCGX) or exposure to constant light, two experimental manipulations which reduce neural input to the pineal, results in a ~50% reduction in the density of PBS in this tissue with no concommitant change in the density of these sites in cerebral cortex. These observations suggest that PBS may have a physiological function, and that the pineal gland may be a valuable model study both for the role and regulation of these sites.

METHOD

Intact and SCGX male, Sprague-Dawley rats (200–250 g) obtained from Zivic-Miller (Allison Park, PA) were group housed with a 12 hour light-12 hour dark cycle for three weeks. SCGX was performed through an incision in the neck under ether anesthesia; each ganglion was exposed and removed carefully without damage to the adjacent tissue. Some of the intact animals were housed in constant light for three weeks. Light was provided by 34 W cool-white fluorescent tubes (Sylvania F40/CW/RS/SS); intensity was 200 μW/cm^2 at the level of the animals. Animals were killed by decapitation between 0900 and 1000 hours, and the pineal glands and brains placed in ice-cold potassium phosphate buffer (pH 7.4, 20mM). The binding of [^{3}H]Ro 5–4864 to pineal and brain was determined as previously described with minor modifications [30]. Membranes 20–24 pineal glands were used per experiment.

Pineal glands were disrupted in 5 ml assay buffer and centrifuged at 20,000 × g (4°C) for 20 min. The pellet was then resuspended in assay buffer in a volume of 1–1.2 ml/gland. Pineal membrane suspension (0.6 ml), assay buffer of drugs (0.1ml) and [^{3}H]Ro 5-4864 (0.1 ml) were incubated for 60 min (0–4°C) and the incubation terminated by filtration under partial vacuum as previously described [30]. [^{3}H]Ro 5-4864 binding in cerebral cortex was determined as previously reported [30] except that 20 mM potassium phosphate buffer (pH 7.4) was used rather than 50 mM Tris-HCl buffer (pH 7.4). In experiments to assess the pharmacologic profile

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TABLE 1

EFFECTS OF EXPOSURE TO CONSTANT LIGHT OR SUPERIOR CERVICAL GANGLIONECTOMY ON THE KINETIC PARAMETERS OF [^{3}H]Ro 5-4864 BINDING IN RAT CEREBRAL CORTEX AND PINEAL GLAND

	Pineal Gland		Cerebral Cortex	
	K_d	B_{max}	K_d	B_{max}
Control	2.46 ± 0.44	24.1 ± 2.6	1.50 ± 0.34	0.30 ± 0.03
Constant Light	1.74 ± 0.25	12.5 ± 0.18*	1.65 ± 0.24	0.26 ± 0.02
Superior Cervical Ganglionectomy	2.12 ± 0.25	12.7 ± 0.78*	1.34 ± 0.26	0.28 ± 0.03

Rats were exposed to constant light for three weeks or underwent a SCGX. Three weeks later, pineal glands and brains were removed and assayed for [^{3}H]Ro 5-4864 binding as described in the Method section. Each experiment consisted of a pool of pineal glands from 20–24 rats, or the cortex from one rat. Values represent the mean ± SEM from three experiments. B_{max} values are given as pmol/mg membrane protein, K_d values are in nM. *Significantly lower than control values, $p<0.01$.

of PBS in pineal, membranes were reconstituted at a concentration of 1 gland/1 ml, and a radioligand concentration of 2.5 nM was routinely used. Specific binding was defined as the difference in binding obtained in the presence and absence of 5 μM nonradioactive Ro 5-4864.

Pineal protein was determined using a microassay procedure (Biorad Protein Assay, Bio-Rad Laboratories, Richmond, CA). The values obtained using this method were normalized with bovine serum albumin as a standard to protein concentrations obtained using the Miller modification of the Lowry technique [11] in order to directly compare receptor density in pineal with values previously reported in other tissues using the same method for protein determination.

Benzodiazepines were provided by Hoffman-LaRoche, Nutley, NJ. PK 1195 was the gift of Dr. G. LeFur, Pharmuka Co., Genevilliers, France. [^{3}H]Ro 5-4864 (Sp. Act. 76.6 Ci/mmol) was purchased from New England Nuclear, Boston, MA.

TABLE 2

[^{3}H]Ro 5-4864 BINDING TO RAT PINEAL AND CEREBRAL CORTEX: EFFECTS OF PHARMACOLOGIC AGENTS

Tissue	Pineal Gland	Cerebral Cortex
Agent	Ki (M)	Ki (M)
Diazepam	5.3×10^{-8}	2.4×10^{-8}
PK 11195	4.8×10^{-9}	1.0×10^{-9}
Ro 5-4864	2.5×10^{-9}	1.6×10^{-9}
Clonazepam	$>10^{-5}$	2.5×10^{-6}
Ro 15-1788	$>10^{-5}$	$>10^{-5}$

Membranes were incubated for 60 min at 0°C with 10^{-10}–10^{-5} M drug as described in the Method section. K_i values were calculated from three or more experiments.

RESULTS

The density of PBS in rat pineal (~24 pmol/mg protein) (Table 1) is 5–10 fold higher than that reported in peripheral tissues such as heart and kidney [16, 22, 26] and 10–100 fold higher than in most CNS structures [22,29]. A low density of "brain-type" binding sites (B_{max} 35 fmol/mg protein) has been reported in bovine pineal glands using [^{3}H]3-carboethoxy-β-carboline as a ligand [9]. We observed an even lower site density using [^{3}H]Ro 15-1788 as a ligand (results not shown). Exposure to constant light and SCGX, procedures which block the neural stimulation of the pineal gland and increase the density of both α_1- and β_1-adrenoreceptor density [7,25], reduce the density of pineal PBS by about 50% ($p<0.01$, Table 1). In contrast, neither treatment altered the density of these sites in cerebral cortex. The apparent affinity of [^{3}H]Ro 5-4864 was unaltered by either manipulation in both tissues (Table 1).

The high density of PBS in pineal prompted a pharmacological comparison with PBS in other tissues. The pharmacological profile of PBS in the rat pineal gland appears qualitatively similar to that of other tissues since diazepam and PK 11195 have relatively high affinities for these sites, while clonazepam and Ro 15-1788, which have high affinities for "brain-type" benzodiazepine receptors, have relatively low affinities for pineal PBS (Table 2).

DISCUSSION

Sympathetic input to the pineal gland originates from the superior cervical ganglion [6]. Exposure to constant light and SCGX are well established procedures which severely reduce the production of melatonin by blocking the nocturnal increase in N-acetyl transferase activity and causing a gradual decrease in hydroxyindole-O-methyl transferase activity; these procedures also result in an increase in α_1- and β_1-adrenoceptors [7,8]. Furthermore, pharmacological manipulation of the pineal gland with adrenergic agents has been shown to mimick either denervation or stimulation of the superior cervical ganglion [8]. Thus, the finding that PBS in the rat pineal are under neural control, coupled with the well documented observation that norepinephrine is released from terminals in the pineal gland [6] suggests that PBS is associated with the sympathetic nervous system. No alteration in the kinetic parameters of [^{3}H]Ro 5-4864 binding to rat cortex was observed (Table 1) which strongly suggests that the reduction in pineal PBS caused by SCGX or exposure to constant light does not reflect a general effect, but rather a specific result of blocking of neural stimulation to the gland by sympathetic terminals. These findings do not permit a definitive localization of pineal PBS to either pre- or postsynaptic sites, since SCGX, which results in a complete degeneration of presynaptic elements, reduces the density of

pineal PBS by ~50%. However, it is likely that the PBS remaining after SCGX are located on postsynaptic structure.

The finding that pineal PBS are neurally regulated is of particular interest in view of the recent evidence that PBS may be under neural control in the olfactory bulb. Marked reductions in [^{3}H]Ro 5-4864 binding have been observed in the olfactory bulb after either surgical isolation of this tissue [2] or application of zinc sulfate to the nasal mucosa [1]. In addition, an apparent dramatic reduction in PBS has been observed when pinealocytes are cultured. Mathew *et al.* [10] recently reported that the density of PBS in monolayer cultures of pinealocytes is approximately 1.5 pmol/mg protein. The factors responsible for the more than 10-fold difference in density of PBS in freshly prepared pineal membranes and pinealocytes in culture are currently under investigation. The pharmacologic profile of PBS in the rat pineal gland did not appear to be qualitatively different from PBS in other tissues, including the CNS (Table 2 and [22]).

A clue to the functional significance of pineal PBS may be found in a series of recent reports demonstrating that Ro 5-4864 is a potent convulsant in rodents [4,31]. Pinealectomy has been shown to increase EEG activity in cortex and hippocampus [21], and elicit spontaneous convulsions in gerbils [15] and parathyroidectomized rats [13,17]. These observations suggest that pineal PBS may influence the ability of the gland to stabilize electrical activity in the CNS.

The high density of PBS in the rat pineal gland, coupled with the finding that these sites are under neural control makes the pineal gland a promising model to elucidate the physiological regulation and function of PBS.

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