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Research Articles

Participation of $ACTH_{1-10}$ and $ACTH_{4-10}$ on the melatonin modulation of benzodiazepine receptors in rat cerebral cortex

M. D. Gomar, B. Fernández, C. M. Del Aguila, J. L. Castillo, G. Escames and D. Acuña-Castroviejo*

Departamento de Fisiología, Facultad de Medicina, Universidad de Granada, Avda. de Madrid 11, E-18012 Granada (Spain)

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Abstract. In this study, we examined the effect of intracerebroventricular (i.c.v) injection of melatonin and/or $ACTH_{1-10}$ and $ACTH_{4-10}$ on [^{3}H]flunitrazepam binding sites in the cerebral cortex of hypophysectomized rats. Hypophysectomy increased the B_{max} (maximum number of binding sites) of benzodiazepine (BNZ) receptors for at least 7 days after surgery, without changing K_D (dissociation constant). The i.c.v. injection of melatonin to hypophysectomized rats significantly increased B_{max}, whereas the same doses of melatonin were ineffective in sham-operated animals. In both cases, K_D values were unchanged. The i.c.v injection of $ACTH_{1-10}$ to hypophysectomized animals significantly increased B_{max}, an effect that was enhanced by simultaneous i.c.v. injection of $ACTH_{1-10}$ + melatonin, reaching higher values of B_{max} than the i.c.v. injection of these hormones individually. No significant changes in K_D values were found after $ACTH_{1-10}$ and/or melatonin administration. However, the i.c.v. injection of $ACTH_{4-10}$ to hypophysectomized rats did not change B_{max}, although it significantly increased K_D values, indicating a decrease in the BNZ binding affinity. Melatonin injection counteracted this effect of $ACTH_{4-10}$, returning K_D to the control value. Moreover, although the lower dose of i.c.v. melatonin used, 10 ng, was unable to modify B_{max} of BNZ binding in the $ACTH_{4-10}$-injected group, the higher dose, 20 ng, significantly increased B_{max}. The results suggest that these ACTH-derived peptides can modulate the effect of melatonin on brain benzodiazepine receptors.

Key words. Melatonin; hypophysectomy; ACTH-derived peptides; benzodiazepine receptor; brain.

Several data suggest that the behavioural effects[1-4] of the pineal hormone melatonin (N-acetyl-5-methoxytryptamine, aMT) depend on its influence on the central-type brain benzodiazepine (BNZ) receptors, which increase $GABA_A$ neurotransmission in the central nervous system[5]. Pinealectomy, which induces proconvulsant activity in several animal species[6-8], is followed by a disruption in the $GABA_A$ and BNZ receptor circadian rhythms in rat brain[9,10]. Melatonin (aMT) administration has been shown to restore $GABA_A$ and BNZ receptor levels and their circadian rhythms. The minimal s.c. dose of melatonin required to counteract these pinealectomy-induced changes in $GABA_A$ and BNZ receptors was 50 μg/kg, suggesting a physiological effect of the hormone[9,10].

The aMT influence on $GABA_A$ and BNZ receptors, could be mediated by a corticoid mechanism related to the pineal-adrenal axis previously described[11]. Adrenal and pituitary hormones can influence central nervous system (CNS) activity[12,13]. In fact, glucocorticoids have been reported to affect certain behaviours in rodents[14], whereas BNZ receptors in rat cerebral cortex can be influenced by adrenal gland secretions[15], with corticosterone as a primary effector[16]. In this respect, pinealectomy[11] increased plasma ACTH and corticosterone levels and significantly decreased brain BNZ binding[10], whereas adrenalectomy increased it[16]. Moreover, ACTH is known to modulate brain GABA receptor binding[17,18], and corticosterone treatment decreased BNZ binding[16] and ACTH levels[11] in rats. ACTH, being a processing product of POMC[19] may in its turn function as a precursor from which smaller neuropeptides with differential behavioural and neurotropic activities can be enzymatically released[20]. Two of these peptides, $ACTH_{1-10}$ and $ACTH_{4-10}$ have been reported to have behavioural properties, and facilitate various forms of CNS plasticity[21]. Although the precise mechanism by which ACTH-like peptides influence plasticity is unknown, $ACTH_{4-10}$ is devoid of corticotropic action and therefore acts directly on the CNS[21]. Moreover, although $ACTH_{1-10}$ has slight corticotropic activity, stimulating steroidogenesis through the cAMP-linked receptors in vitro, it is accepted that the short ACTH fragments do not have the ability to release glucocorticoids in vivo[22]. We recently reported that in hypophysectomized rats aMT administration caused an increase in brain BNZ binding[23], although to a lesser extent than aMT administration to pinealectomized rats[24]. This effect is consis-

* To whom correspondence should be addressed.

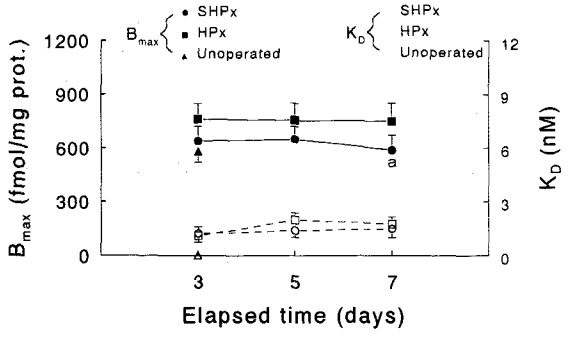

Figure 1. Changes in B_{max} and K_D of [^{3}H]FNZ binding in cerebral cortex membranes of rats subjects to SHPx or HPx 3, 5 and 7 days earlier. a: $p < 0.01$ vs SHPx.

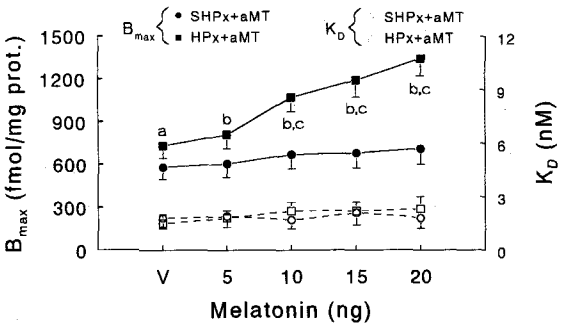

Figure 2. Effects of i.c.v. injection of vehicle or 5–20 ng of aMT on the B_{max} and K_D of [^{3}H]FNZ binding in cerebral cortex membranes of rats subjects to SHPx or HPx 7 days earlier. a: $p < 0.01$ vs SHPx; b: $p < 0.001$ vs SHPx; c: $p < 0.001$ vs a.

tent with the involvement of some compound(s) of pituitary origin in the aMT regulation of brain BNZ binding. We thus considered it worthwhile to examine the relationship between aMT and ACTH on brain BNZ binding in the rat cerebral cortex. To avoid the participation of corticotropin-induced adrenal corticosteroids the study was carried out in hypophysectomized rats, which were i.c.v. injected with the ACTH-derived peptides $ACTH_{1-10}$ and $ACTH_{4-10}$.

Materials and methods

Male Wistar rats (200–230 g) were housed 2–3 animals per clear plastic cage in a (22 ± 2 °C) controlled room temperature, with light from 07.00 to 19.00 h daily. Unless otherwise stated, experimental and control animals received tap water and a standard pellet diet ad libitum. Hypophysectomized (HPx) and sham operated (SHPx) animals were operated on under equithesin anaesthesia as previously reported[16]. Hypophysectomized animals were housed under normal conditions and had free access to food and a 5% sucrose-saline solution[17]. At the end of hypophysectomy, each rat was stereotaxically implanted with a single stainless steel 22-gauge thick wall guide cannula aimed at the lateral cerebral ventricle for subsequent i.c.v. injection of drugs[23]. At the end of the experiments, the rats were deeply anaesthetized and injected i.c.v. with 2 µl of black ink. The brains were removed and the success of the cannula placement could be verified by the presence of ink in the ventricular system.

Binding experiments were carried out on crude P_2 membrane fractions prepared from cerebral cortices of individual brains, as previously described[16]. The membranes (0.3–0.6 mg protein) were incubated in triplicate at 0 °C for 30 min in a total volume of 300 µl buffer with [^{3}H]flunitrazepam ([^{3}H]FNZ, 92.6 Ci mmol^{-1}, NEN) and in the presence (nonspecific binding) or absence (total binding) of 10 µM cold flunitrazepam (FNZ). The reaction was stopped by rapid filtration through Whatman GF/B glass fiber filters and the radioactivity was determined by liquid scintillation spectroscopy. In all experiments, FNZ-specific binding, i.e. the amounts of total binding displaced by excess unlabeled FNZ, was greater than 70% of total binding. The maximum number of binding sites (B_{max}) and dissociation constant (K_D) was calculated by Scatchard analysis using a Lig-and-PC program kindly provided by P. J. Munson (Laboratory of Theoretical and Physical Biology, NIH, Bethesda, MD, USA).

To study the effect of aMT on brain [^{3}H]FNZ binding, a dose-dependence experiment was performed in which SHPx and HPx rats were i.c.v. injected with vehicle (5 µl; n = 6) or aMT (5, 10, 15 and 20 ng; n = 7/dose). To study the participation of corticotropic hormone on aMT-dependent [^{3}H]FNZ binding, HPx rats were i.c.v. injection with $ACTH_{1-10}$ (10 and 20 ng; n = 6/dose); $ACTH_{4-10}$ (10 and 20 ng; n = 6/dose); aMT + $ACTH_{1-10}$ (10 and 20 ng of each compound; n = 7/dose), and aMT + $ACTH_{4-10}$ (10 and 20 ng of each compound; n = 7/dose). All drugs were dissolved in 5 µl fo 5:100 (v:v) ethanol:saline. Drugs were i.c.v. injection into 7-day HPx active rats at 09.00 h, and the animals were sacrificed 1 hour later. Brains were removed and their cerebral cortices were processed to assay brain BNZ receptors. Results are expressed as the mean ± SEM. Statistical analysis consisted of a two-way analysis of variance followed by Bonferroni's test.

Results and discussion

The changes in B_{max} and K_D of BNZ binding in cerebral cortex membranes of rats subject to HPx 3, 5 and 7 days earlier, and in their respective SHPx controls, are summarized in figure 1. Sham-operated animals exhibited a transient increase in B_{max} up to 5 days after surgery compared to unoperated animals, returning to normal values by the 7th day. Hypophysectomy increased B_{max} at 3, 5 and 7 days after surgery, the effect being significantly different from SHPx on the 7th day. Thus, to avoid interference with surgical effects, 7-day

Table. Effects of intracerebroventricular injection of vehicle or $ACTH_{1-10}$ and $ACTH_{4-10}$ alone or with aMT on B_{max} (fmol/mg prot.) and K_D (nM) of [^{3}H]FNZ binding to cerebral cortex of 7-day hypophysectomized rats

Group	Vehicle			10 ng		20 ng	
	n	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
Unoperated	6	1.1 ± 0.4	590 ± 39	-	-	-	-
SHPx	5	1.6 ± 0.6	619 ± 42	-	-	-	-
HPx	5	2.1 ± 0.6	710 ± 53^a	-	-	-	-
HPx + $ACTH_{1-10}$	6	-	-	3.2 ± 0.9	$1240 \pm 115^{a,b}$	3.8 ± 1.2^a	$1480 \pm 110^{a,b,c}$
HPx + $ACTH_{4-10}$	6	-	-	$5.2 \pm 1.1^{a,b}$	693 ± 94^a	$6.1 \pm 1.5^{a,b}$	685 ± 95^a
HPx + $ACTH_{1-10}$ + aMT	7	-	-	2.3 ± 0.9	$1780 \pm 130^{a,b}$	2.6 ± 0.9	$2470 \pm 135^{a,b,c}$
HPx + $ACTH_{4-10}$ + aMT	7	-	-	2.5 ± 0.8	690 ± 70^a	2.4 ± 0.7	$1100 \pm 120^{a,b,c}$

No significant differences were found between unoperated and 7-day hypophysectomized rats.
$^a p < 0.001$ vs SHPx + Vehicle; $^b p < 0.001$ vs HPx + Vehicle; $^c p < 0.001$ vs 10 ng.

HPx animals were chosen for subsequent experiments. No significant changes in K_D values were found in any group. Figure 2 shows the effects of i.c.v. administration of vehicle or 5, 10, 15 and 20 ng of aMT on the B_{max} and K_D of cerebral cortex [^{3}H]FNZ binding in 7-day SHPx and HPx rats. Whereas HPx increased B_{max} of BNZ binding, aMT administration significantly increased the BNZ binding sites in a dose-dependent manner, without changes in K_D. No significant changes in B_{max} or K_D were found in SHPx rats injected with the same doses of aMT. The effects of i.c.v. injection of $ACTH_{1-10}$ and $ACTH_{4-10}$ at doses of 10 or 20 ng each, alone or in combination with aMT in 7-day HPx rats, are shown in the table. The i.c.v. injection of $ACTH_{1-10}$ significantly increased B_{max} of [^{3}H]FNZ binding in a dose-dependent manner, with no changes in K_D. In contrast, i.c.v. injection of $ACTH_{4-10}$ was ineffective on B_{max}, but significantly increased K_D values at the doses used. Simultaneous i.c.v. injection of $ACTH_{1-10}$ + aMT (10 and 20 ng of each compound) significantly increased the B_{max} of cerebral cortex [^{3}H]FNZ binding, which reached higher values than following i.c.v. injection of aMT or $ACTH_{1-10}$ individually. No significant changes in K_D were found after $ACTH_{1-10}$ + aMT injection. When $ACTH_{4-10}$ + aMT were injected, only the higher dose used (20 ng) was effective in significantly increasing B_{max} of [^{3}H]FNZ binding, whereas 10 ng of $ACTH_{4-10}$ + aMT only slightly increased B_{max}. However, injection of aMT at both doses was able to counteract the increased K_D values found after $ACTH_{4-10}$ injection alone.

Our data show that i.c.v. injection of aMT significantly increases B_{max} of [^{3}H]FNZ binding to cerebral cortex of HPx rats, without significant changes in K_D. The stimulatory effect of aMT on brain BNZ binding in HPx rats was smaller than in pinealectomized animals[24], suggesting the participation of some compound(s) of pituitary origin in the regulation of BNZ receptors. Since corticosterone decreases brain BNZ binding[16], the depressed levels of glucocorticoids in HPx rats cannot be responsible for the lower effect of aMT on BNZ binding seen in HPx compared with pinealectomized rats. Because ACTH was reported to regulate the $GABA_A$-BNZ receptor complex in rat brain[17,18], it is likely that this hormone can interact with aMT to control brain BNZ binding. After i.c.v. injection of $ACTH_{1-10}$, B_{max} of [^{3}H]FNZ binding significantly increased, whereas i.c.v. injection of $ACTH_{4-10}$ had no effect. However, $ACTH_{4-10}$ significantly increase the K_D value of brain BNZ binding, suggesting a different and selective effect of each of these peptides on brain BNZ receptor regulation. The different effect of $ACTH_{4-10}$ may be due to a modification of the $GABA_A$ turnover or the activation or inhibition of other neurotransmitter systems that in turn modify BNZ binding sites[17]. The results showing that hypophysectomy increases B_{max} seem to indicate that ACTH normally down-regulates the BNZ receptor. Moreover, the effect of ACTH-derived peptides described here is similar to the effect reported in normal rats[26], and also to the effect reported for ACTH in $GABA_A$ regulation[17].

Although no information is available about the role played by ACTH or its peptides in the control of the central BNZ receptor, a modulation by CRH has been reported[27]. The different effect of the ACTH fragments on CRH secretion and/or endogenous ligand for central BNZ receptors may thus explain the different activity of several ACTH-derived peptides on B_{max} and K_D of brain BNZ binding, as described above. These differences in peptide activity are not likely to be due to differential effects of aMT on CRH secretion, because at physiological doses aMT had no effect on either basal or CRH-stimulated ACTH secreation[28]. At pharmacological doses, aMT significantly increases ACTH secretion stimulated by CRH, although the effect did not increase with an increase in the CRH dose, indicating the existence of certain intrinsic agonistic activity between aMT and CRH[28]. Regarding ACTH, previous studies have reported that ACTH is actually formed in the brain rather than transported there from the pituitary, because HPx does not influence the ACTH content of the rat brain[25]. This may account for the fact

that HPx animals also display an increase in brain BNZ binding, as do adrenalectomized rats. Hypophysectomy may change brain ACTH metabolism which in turn modifies the production of brain ACTH fragments inducing an increase rather than a decrease in BNZ binding, as expected by the decrease in ACTH circulating levels after HPx.

Taken together with prior evidence, our results suggest a complex interaction between ACTH fragments, aMT and central BNZ receptors. This also explains why in situations of stress, where the stress hormones ACTH and aMT increase, there is a rapid modification of $GABA_A$-BNZ receptor complex activity. ACTH fragments may thus act as neuroregulators or modulators of neurotransmission, aiding aMT in neuroregulation of neurotransmission. The ultimate action of ACTH-derived peptides may involve synthesis of neurosteroids, the brain steroids involved in the regulation of $GABA_A$-BNZ receptor complex[29], a possibility that requires further investigation.

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