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Chronic Administration of Diazepam Downregulates Adenosine Receptors in the Rat Brain

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HAWKINS, M., M. PRAVICA AND M. RADULOVACKI. *Chronic administration of diazepam downregulates adenosine receptors in the rat brain.* PHARMACOL BIOCHEM BEHAV 30(2) 303–308, 1988.—Following chronic administration (10 or 20 days) of diazepam (5 mg/kg/day, subcutaneous pellets) or RO 15-1788 (5 mg/kg/day, intraperitoneally), adenosine and benzodiazepine receptors in different rat brain areas were assessed by radioligand binding studies using [^{3}H]R-PIA for A_1 receptors, [^{3}H]NECA and [^{3}H]R-PIA for A_2 receptors and [^{3}H]FNZ for benzodiazepine receptors. Chronic administration of diazepam for 10, but not for 20 days, decreased A_2 receptors in the striatum by 46% ($p<0.05$) and A_1 receptors in the hippocampus by 13% ($p<0.05$). Administration of diazepam for 10 days and 20 days failed to alter [^{3}H]FNZ binding in all brain areas studied. However, 20 days of diazepam administration decreased the magnitude of GABA enhancement of [^{3}H]FNZ binding in the cortex by 25% ($p<0.05$). In contrast, chronic administration of RO 15-1788 failed to alter [^{3}H]R-PIA, [^{3}H]NECA and [^{3}H]FNZ binding in all brain areas. These results suggest that adenosine receptors may play a role in the CNS actions of benzodiazepines.

Diazepam RO 15-1788 Adenosine A_1 and A_2 receptors Benzodiazepine receptors Striatum Hippocampus Rat

ADENOSINE and its analogs have sedative, hypnotic, anticonvulsant and analgesic activities [7]. They potently depress spontaneous and evoked firing of various neurons in the CNS [21]. These findings suggest an important modulatory role for adenosine in regulating neuronal activity. The brain contains two major subtypes of adenosine receptors: adenosine A_1 receptors which demonstrate high affinity (nM) for adenosine and mediate inhibition of adenylate cyclase, and adenosine A_2 receptors with low affinity (μM) for adenosine which mediate stimulation of adenylate cyclase [17, 31, 32]. High affinity adenosine uptake sites for rapid inactivation of extracellular adenosine have also been characterized in both neuronal and glial cells [2].

Benzodiazepines inhibit adenosine uptake [1, 13, 19] and thus potentiate many actions of adenosine, i.e., depression of spontaneous firing of neurons [18], elevation of cAMP [28,33] and inhibition of acetylcholine release [20]. On the other hand, blockade of benzodiazepine receptors by RO 15-1788, a specific benzodiazepine receptor antagonist, does not antagonize the effect of benzodiazepines on adenosine uptake [16]. In addition, RO 15-1788 is also an inhibitor of adenosine uptake [15,22]. Furthermore, several effects of benzodiazepines are antagonized by caffeine and theophylline which are also adenosine receptor antagonists [23].

Since some of the actions of benzodiazepines may involve adenosine uptake inhibition, chronic administration of diazepam may alter adenosine receptor binding. In the present study we examined the effects of chronic administration of diazepam and RO 15-1788 on brain adenosine A_1 and A_2 receptor binding. In addition, the effects of these treatments were also assessed on benzodiazepine receptor binding and GABA facilitation of flunitrazepam binding.

METHOD

Diazepam and RO 15-1788 were kindly supplied by Hoffmann-La Roche Inc. (Nutley, NJ). Adenosine-5′-N-ethylcarboxamide ([^{3}H]NECA, 20 Ci/mmol), N^6-R-phenylisopropyladenosine ([^{3}H]R-PIA, 49.9 Ci/mmol) and [^{3}H]flunitrazepam ([^{3}H]FNZ, 79.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA); all other chemicals were obtained from Sigma (St. Louis, MO).

Adult male Sprague-Dawley rats (300–350 g) were housed two in a cage (1 control and 1 experimental) on a 12 hr/12 hr light-dark cycle (lights on at 08.00 hr). Seven days were allowed for adaptation and handling. On the eighth day, diazepam pellets, which deliver 5 mg/kg/day diazepam, were implanted subcutaneously into one group of rats under ether anesthesia. Control animals for this treatment received a vehicle pellet (cholesterol: methyl cellulose: alpha-lactose).

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ABBREVIATIONS

R-PIA	N^6-[(R)-1-methyl-phenylethyl] adenosine
NECA	5'-N-ethylcarboxamide adenosine
FNZ	flunitrazepam
EDTA	ethylenediamine tetraacetic acid
GABA	gama-amino butyric acid
B_{max}	total number of receptor sites
K_d	dissociation constant

Another group of rats was injected intraperitoneally every day between 9 a.m.–10 a.m., during 10 or 20 days with a freshly prepared suspension of RO 15-1788 (5 mg/kg) in tween 40 (2 drops in 10 ml). Control animals for this treatment received 1 ml/kg tween 40 in water. Rats receiving diazepam were sacrificed 48 hr after removal of the pellet, whereas those receiving RO 15-1788 were killed 24 hr after the last injection. The brains were rapidly removed and cortex, hippocampus, cerebellum and striatum were dissected and stored at −70°C. Subsequently, cortex and cerebellum were separated into two halves and each half was used to either study adenosine or benzodiazepine binding.

Preparation of Membranes

Crude membrane preparations of each brain structure were prepared for (a) adenosine receptor binding [32] and (b) benzodiazepine receptor binding. Briefly, brain structures for adenosine receptor binding were homogenized in approximately 50 volumes of ice-cold 10 mM L-histidine containing 1 mM EDTA (pH 7.5). The homogenate was incubated with adenosine deaminase (5 U/ml) for 15 min at 37°C, diluted further to approximately 10–12 ml with the homogenizing buffer and centrifuged at 1200×g for 15 min. The resulting pellet was lysed in 100 volumes of 1 mM $NaHCO_3$ containing 1 mM EDTA (pH 7.5) for 30 min at 0°C. The lysate was then centrifuged (27,000×g for 15 min) and the pellet resuspended in the L-histidine/EDTA buffer to give a final protein concentration of 1–2 mg/ml. Brain structures for benzodiazepine receptor binding were homogenized in 0.1 M Tris-Citrate buffer, pH 7.1 at 0°C (1:20, wt./vol.), using a glass homogenizer-teflon pestle. The homogenate was centrifuged at 16,000×g for 10 min and the pellet washed four times. The final pellet was resuspended in 0.1 M Tris-Citrate buffer to give a final protein concentration of 1–2 mg/ml and stored at −20°C for at least 18 hr before use. The suspension was dispersed by sonication for 5 seconds using a Polytron homogenizer at setting 5 prior to adding to the incubation. Protein in all cases was determined by the method of Lowry *et al.* [12].

Adenosine A_1 Receptor Binding in Cortex, Cerebellum and Hippocampus

Radioligand binding reactions for [^{3}H]R-PIA saturation curves contained 10 mM L-histidine, 4 mM $MgSO_4$, 5 U/ml adenosine deaminase, 100–150 μg membrane protein and one of six concentrations of [^{3}H]R-PIA ranging from 0.5 to 25 nM. Nonspecific binding was defined in the presence of 10 μM unlabeled R-PIA. Each assay was performed in duplicate. Reactions were incubated at 37°C for 15 min to attain equilibrium. Bound and free ligand were separated by vacuum filtration with a modified cell harvester (Brandel, Gaithersburg, MD), using three 3 ml washes with ice-cold wash buffer (1 mM glycylglycine, pH 7.5, 1 mM $MgSO_4$). Whatman GF/A filters were used.

Adenosine A_1 Receptor and A_2 Receptor Binding in Striatum

The striatum contains both A_1 and A_2 adenosine receptors [32]. A single, saturating concentration (40 nM) of [^{3}H]R-PIA was used to assess A_1 receptor binding, while a single saturating concentration (200 nM) of [^{3}H]NECA was used to assess the sum total of A_1 and A_2 receptor binding. An estimate of A_2 receptor binding was determined by subtracting the amount of [^{3}H]R-PIA bound from the amount of [^{3}H]NECA bound (NECA minus PIA assay). Choca *et al.* [5] and Porter *et al.* [24] have determined that this technique provides an adequate estimate of A_2 receptors in tissues that contain both A_1 and A_2 receptors. These assays were performed in triplicate and 50 μM unlabeled R-PIA was used to define nonspecific binding. Bound and free were separated as described above.

Benzodiazepine Receptor Binding in Cortex and Cerebellum

Radioligand binding reactions for [^{3}H]FNZ saturation curves contained 0.1 M Tris-Citrate pH 7.1 at 0°–4°C, 100–150 μg membrane protein and one of six concentrations of [^{3}H]FNZ ranging from 0.5 to 30 nM. Nonspecific binding was defined in the presence of 3 μM clonazepam. Binding was linear at least up to 250 μg protein. Each assay was performed in duplicate. Reactions were incubated at 0–4°C for 60 min to attain equilibrium. Bound and free ligand were separated as described above using ice-cold 0.1 M Tris-Citrate pH 7.1 as wash buffer.

GABA Facilitation of [^{3}H]FNZ Binding in Cortex and Cerebellum

Assays were carried out in the presence of 0.1 M Tris-Citrate pH 7.1, 40–50 μg membrane protein, a single concentration (0.5 nM) of [^{3}H]FNZ and in the presence or absence of 100 μM GABA plus 150 mM NaCl. Nonspecific binding was defined in the presence of 3 μM clonazepam. Each assay was performed in triplicate. Reactions were incubated and terminated as described in benzodiazepine binding.

Scintillation fluid (Budget-Solve) was added to all the filters and then counted in a Beckman scintillation counter at approximately 43% efficiency.

Statistical Analysis

Binding parameters for adenosine A_1 and benzodiazepine receptor binding were determined by least squares analysis of Scatchard plots. In the striatum adenosine A_2 receptor binding was determined indirectly by the "NECA-minus-PIA" method.

All preparative and experimental procedures were carried out with adherence to a paired killing and assay schedule of control and experimental animals. Therefore, the significance of differences between control and treated groups were determined using a randomized complete blocks design with two treatment levels (paired comparisons). The Student's *t*-test was used for all other comparisons.

RESULTS

Effect of Diazepam Pretreatment on Adenosine A_1 Receptors in Cortex, Hippocampus and Cerebellum

Adenosine A_1 receptors were assayed in membranes isolated from different brain areas of control rats and rats pretreated with diazepam. Results of Scatchard analysis of [^{3}H]R-PIA binding are presented in Table 1 which shows [^{3}H]R-PIA bound to cortical, cerebellar and hippocampal

TABLE 1

[³H]R-PIA AND [³H]FNZ BINDING IN DIFFERENT BRAIN AREAS AFTER 10 AND 20 DAYS OF DIAZEPAM

Days	Brain Area		[³H]R-PIA Binding		[³H]FNZ Binding	
			Control	Diazepam	Control	Diazepam
10	CTX	B_{max}	552 ± 18	557 ± 9	1655 ± 49†	1642 ± 140†
		K_d	1.4 ± 0.09	1.5 ± 0.11	2.7 ± 0.05	2.5 ± 0.19
	CRB	B_{max}	649 ± 90	599 ± 54	724 ± 38‡	727 ± 27‡
		K_d	1.1 ± 0.07	1.2 ± 0.04	2.7 ± 0.06	2.9 ± 0.18
	HIP	B_{max}	512 ± 21	446 ± 23*		
		K_d	1.3 ± 0.04	1.2 ± 0.05		
20	CTX	B_{max}	364 ± 33	378 ± 27	1361 ± 69†	1472 ± 83†
		K_d	2.4 ± 0.36	2.2 ± 0.31	2.8 ± 0.19	2.6 ± 0.14
	CRB	B_{max}	630 ± 33	560 ± 25	636 ± 31‡	635 ± 34‡
		K_d	1.1 ± 0.04	1.1 ± 0.04	2.5 ± 0.20	2.5 ± 0.13
	HIP	B_{max}	550 ± 16	493 ± 25		
		K_d	1.3 ± 0.11	1.3 ± 0.04		

Cortical and cerebellar membranes for [³H]R-PIA and [³H]FNZ binding were prepared from the same brain structure. The B_{max} (fmol/mg protein) and K_d (nM) values were determined from Scatchard analysis of saturation binding experiments. The data represent the mean ± S.E.M.; n=5–7 rats per group. *$p<0.05$ vs. control (paired comparisons), †$p<0.01$ vs. [³H]R-PIA binding in CTX (Student's t-test), ‡$p<0.01$ vs. [³H]FNZ binding in CTX (Student's t-test). CTX=cortex, CRB=cerebellum, HIP=hippocampus.

membranes with similar high affinities. The affinity of [³H]R-PIA for A_1 receptors was not affected in animals pretreated with diazepam. However, the B_{max} of [³H]R-PIA binding in hippocampal membranes decreased by 13% ($p<0.05$) in animals pretreated with diazepam for 10 days (Table 1). No difference in B_{max} for [³H]R-PIA was observed in other brain regions. By 20 days, no difference was observed in B_{max} for [³H]R-PIA in all regions studied (Table 1).

Effect of Diazepam Pretreatment on Adenosine A_1 and A_2 Receptors in the Striatum

A_2 receptor number was estimated by the difference in binding between [³H]NECA and [³H]R-PIA as described in the Method section. The results show that B_{max} values of [³H]NECA were almost twice the values for [³H]R-PIA (Fig. 1). This difference between the two ligands was significantly reduced by 46% ($p<0.05$) in striatal membranes prepared from rats pretreated with diazepam for 10 days. Diazepam pretreatment for 10 days did not affect [³H]R-PIA binding to A_1 receptors. Therefore, reduced [³H]NECA binding was due to a decrease in adenosine A_2 receptors (Fig. 1). In contrast, diazepam pretreatment for 20 days did not affect radioligand binding to either A_1 or A_2 receptors (Fig. 1).

Effect of Diazepam Pretreatment on Benzodiazepine Receptors and GABA Facilitation of [³H]FNZ Binding

[³H]FNZ binding and GABA facilitation of [³H]FNZ binding were simultaneously assayed in membranes isolated from cortex and cerebellum of control rats and rats pretreated with diazepam. Table 1 shows that [³H]FNZ bound to cortical and cerebellar membranes with similar high affinities. The affinity of [³H]FNZ for benzodiazepine receptors was not affected in animals pretreated with diazepam. Unlike adenosine A_1 receptors which did not show regional variation in number, [³H]FNZ binding was higher in cortex than in cerebellum ($p<0.01$), both after 10 or 20 days of diazepam pretreatment (Table 1). Furthermore, [³H]FNZ binding in cortex was higher than [³H]R-PIA binding ($p<0.01$), indicating that the rat cortex contains more benzodiazepine binding sites than adenosine A_1 sites (Table 1). Diazepam pretreatment with both protocols did not affect either of these two characteristics of [³H]FNZ binding.

The magnitude of GABA enhancement of [³H]FNZ binding in cortical membranes decreased by 25% ($p<0.05$) in animals pretreated with diazepam for 20 days, no changes were observed after 10 days. In the absence of GABA and NaCl the average number of binding sites for [³H]FNZ in membranes prepared from control rats and rats pretreated with diazepam for 20 days was 230±66 fmol/mg protein. In the presence of GABA and NaCl the data were analyzed as % increase of [³H]FNZ binding. [³H]FNZ binding increased by 83±13% in cortical membranes from control animals, whereas in membranes from rats pretreated with diazepam [³H]FNZ binding increased only by 60±8%. This indicates that although there were no changes in benzodiazepine binding site density or affinity, the allosteric interaction between the GABA and the benzodiazepine components of the receptor complex was affected after 20 days of diazepam pretreatment.

Effect of RO 15-1788 Pretreatment of Adenosine A_1 and A_2 Receptors, Benzodiazepine Receptors and GABA Facilitation of [³H]FNZ Binding in Specific Brain Structures

Assays were carried out in membranes isolated from different brain areas of control rats and rats pretreated with RO 15-1788. K_d and B_{max} values were similar to those reported in Table 1, and were unaffected by this treatment. In contrast to treatment with diazepam (Fig. 1), pretreatment with RO 15-1788 did not affect [³H]NECA binding in striatal membranes, since B_{max} values for [³H]NECA were twice that for

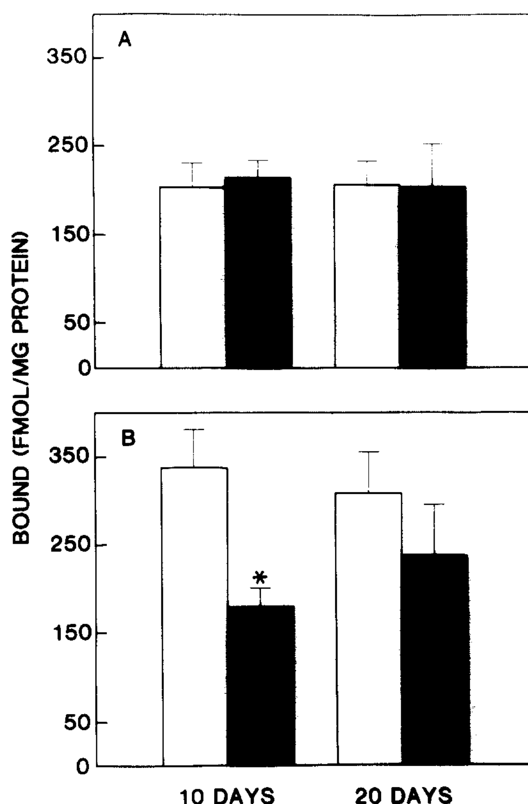

FIG. 1. Adenosine receptor binding in striatal membranes from control rats and rats pretreated with diazepam for 10 and 20 days. Membranes were simultaneously prepared from control and treated rats. Radioligand binding to adenosine A_1 (A) and A_2 (B) receptors was estimated by the [^{3}H]NECA minus [^{3}H]R-PIA assay as described in the Method section. The data are mean±S.E.M. of 4 rats per group and represent the amount of ligand specifically bound. Each treated group is compared to its own control. Binding to A_2 receptors was significantly reduced in rats pretreated with diazepam during 10 days (*$p<0.05$). The average number of binding sites for [^{3}H]NECA (A_1+A_2) and [^{3}H]R-PIA (A_1) in all control groups was 530±36, 205±18 fmol/mg protein, respectively.

[^{3}H]R-PIA, both in control and RO 15-1788-treated rats (data not shown). Scatchard analysis of [^{3}H]FNZ binding to cortical and cerebellar membranes from control, diazepam (Table 1) and RO 15-1788 (data not shown) pretreated rats had similar characteristics as measured by the K_d and B_{max} values of the radioligand. In addition, RO 15-1788 pretreatment for 10 or 20 days did not affect GABA facilitation of [^{3}H]FNZ binding (data not shown).

DISCUSSION

Chronic administration of diazepam for 10 days decreased [^{3}H]R-PIA binding to A_1 receptors in the hippocampus as well as [^{3}H]NECA binding to A_2 receptors in the striatum, at a dose that failed to alter benzodiazepine receptor binding. These results provide the first evidence of the ability of a benzodiazepine to modify adenosine A_1 and A_2 receptor binding in the rat brain after chronic administration.

Diazepam was administered via pellets, which are a reliable sustained release dosage form comparable to the constant-rate regimen produced by mini-osmotic pumps [11]. Using a similar technique, Gallager *et al.* [10] showed that 5 mg/kg day of diazepam produces fairly constant levels of diazepam in the rat brain capable of eliciting electrophysiological, biochemical and behavioral changes.

Diazepam potently inhibits adenosine uptake in cerebral cortical slices, synaptosomes, primary cultures of neurons and astrocytes, as well as in glial cells [1, 2, 13], and consequently increases extracellular adenosine in brain as measured by [^{3}H]-adenosine efflux [20]. Our results suggest that this resulting increase in extracellular adenosine is sufficient to downregulate adenosine receptors in some brain areas. Interestingly, plasma concentrations of diazepam in rats and humans may reach levels between 1.0 and 10 μM, which are sufficient to inhibit adenosine uptake [2].

Of all the brain areas studied, we detected decreased [^{3}H]R-PIA binding only in the hippocampus. This regional difference of adenosine A_1 receptor binding suggests differential regulation of adenosine receptors. Autoradiographic studies show that the rat hippocampus contains high levels of adenosine A_1 receptors with low density of uptake sites [3]. Thus, regional variation of uptake sites could be involved in the effects of diazepam. If uptake sites control extracellular levels of endogenous adenosine, then the effect of diazepam on adenosine receptors could be amplified in those areas with low density of uptake sites.

The difference between [^{3}H]NECA and [^{3}H]R-PIA binding (A_2) to striatal membranes from rats pretreated with diazepam for 10 days was significantly reduced by 46% as compared to controls. Similarly, chronic administration of deoxycoformycin (adenosine deaminase inhibitor) or NECA decreases [^{3}H]NECA binding in the striatum without affecting [^{3}H]R-PIA binding [24]. Apparently, chronic stimulation of adenosine A_2 receptors by either increased levels of adenosine or by administration of an adenosine agonist decreases [^{3}H]NECA binding to these receptors.

To determine whether these changes in adenosine receptor binding were associated with changes in the GABA-benzodiazepine complex, we simultaneously studied [^{3}H]FNZ binding and GABA facilitation of [^{3}H]FNZ binding in the cortex and cerebellum of these animals. Ten days of chronic administration of diazepam failed to alter [^{3}H]FNZ binding or GABA facilitation of this binding. Twenty days of chronic administration also failed to alter [^{3}H]FNZ binding. However, the magnitude of the GABA enhancement of [^{3}H]FNZ binding in the cortex was decreased by 25%. These results are consistent with those of Gallager [9], where chronic treatment with diazepam (5 mg/kg/day, IP) during 21 days decreased the maximal facilitation of [^{3}H]FNZ binding caused by GABA with no changes in benzodiazepine binding site density or affinity. This is of interest since GABA facilitation of [^{3}H]FNZ binding provides information about the coupling between the benzodiazepine and the GABA components of the receptor complex. Gallager *et al.* [9] concluded that this subsensitivity to GABA was associated with tolerance and not with the withdrawal effects of benzodiazepines.

We report here that chronic administration of low doses of diazepam failed to downregulate benzodiazepine receptors in cortex and cerebellum. This finding does not rule out the possibility that specific changes occurred in other brain areas, i.e., hippocampus or striatum. In accordance, regional variation in the degree and rate of downregulation of benzodiazepine receptors may occur [29], suggesting a differential response of certain brain areas to chronic benzodiazepine treatment. However, chronic administration of low doses of diazepam in the present study as well as in the

studies of Gallager [9] and Mohler [14] did not affect benzodiazepine receptor density in other brain areas studied.

Administration of RO 15-1788 had no effect on adenosine and benzodiazepine receptors. A dose of 5 mg/kg was selected since both acute and chronic doses of 4–5 mg/kg have been shown to produce electrophysiological, biochemical and behavioral changes [8]. The lack of effect on adenosine receptors in this study could be due to the fact that 5 mg/kg of RO 15-1788: (a) failed to increase extracellular adenosine in vivo, (b) that higher doses of the compound are required to inhibit adenosine uptake in vivo, and (c) that a constant rate-regimen is required (pellet or minipumps).

A functional significance for the adenosine A_2 receptor has been implicated by the studies of Porter *et al.* [25]. The authors showed that decreased A_2 binding in the striatum following chronic treatment with adenosine agonists was correlated with changes in sleep during the first 48 hr of drug withdrawal. In accordance, a role for adenosine in the modulation of the sleep-wake cycle had previously been shown [26]. Intracerebroventricular administration of adenosine and intraperitoneal injections of adenosine analogs, R-PIA and cyclohexyladenosine, have increased deep slow-wave and REM sleep [26], suggesting that activation of adenosine receptors could be involved in the effects of adenosine on sleep. Furthermore, adenosine uptake inhibitors, such as nitrobenzylthioinosine and nitrobenzylthioguanosine, potentiated the sedative effects of adenosine in mice [6], whereas others, i.e., mioflazine, decreased wakefulness and increased slow-wave sleep in dogs [30].

Our data are in accordance with the postulate of Phillis and Wu [21] who proposed that the sedative effect of benzodiazepines could be related to their inhibition of adenosine uptake. Furthermore, Bruns *et al.* [4] concluded that of the four classic actions of benzodiazepines, the sedative and muscle relaxant actions could be mediated by adenosine. Although sedative effects of diazepam were not determined here, it is feasible that the sedative effect of diazepam may be related to changes in adenosine A_2 receptors. Tolerance develops to the sedative effects of benzodiazepines [27] and it may have developed in the present study 20 days following chronic administration of diazepam since no difference in adenosine receptor binding between control and experimental animals was seen after 20 days of drug treatment.

In conclusion, our results give new evidence for a role of adenosine in the central actions of benzodiazepines by showing that diazepam modifies adenosine receptor binding. Further studies are required to clarify the functional role of these changes.

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