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CHRONIC BENZODIAZEPINE ADMINISTRATION

IV. RAPID DEVELOPMENT OF TOLERANCE AND RECEPTOR DOWNREGULATION ASSOCIATED WITH ALPRAZOLAM ADMINISTRATION*

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Abstract—The triazolobenzodiazepine compound alprazolam may have unique clinical effects compared to other benzodiazepines, and both behavioral and neurochemical studies have indicated unusual results after acute doses of alprazolam. To determine the effects of chronic dosage in mice, alprazolam (2 mg/kg/day) was administered via osmotic pumps for 1–14 days, and open-field activity, plasma and brain concentrations, benzodiazepine receptor binding *in vivo* and *in vitro*, [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding, and muscimol-stimulated chloride uptake were determined. Alprazolam decreased motor activity after 1 and 2 days, but tolerance developed by day 4 and persisted to day 14. Plasma and brain concentrations remained constant during the 2-week period. Benzodiazepine receptor binding *in vivo* was decreased at day 4 compared to day 1 in cortex (CX) and hypothalamus (HYPO), and remained depressed to day 14 in CX but not HYPO. Benzodiazepine binding *in vitro* and [^{35}S]TBPS binding were decreased in CX at day 7. Muscimol-stimulated [$^{36}Cl^-$] uptake was decreased at days 4 and 7 compared to day 1, but at day 14 uptake was similar to day 1. These results indicate that behavioral tolerance and receptor downregulation develop rapidly during chronic alprazolam administration. Behavioral and neurochemical changes were similar to those associated with lorazepam administration, but occurred more rapidly and with different regional specificity.

The triazolobenzodiazepines are a relatively new class of benzodiazepines which have gained widespread clinical use. Included in this class are alprazolam, which is used primarily as an anxiolytic, and estazolam and triazolam which are used commonly as hypnotics [1–4]. The members of this class appear to exert their clinical effects by binding at the benzodiazepine site on the $GABA_A$‖ receptor complex [5]. It is possible that additional effects may occur through antagonism of platelet activating factor or interaction with other receptor systems [6–8], although evidence related to central nervous function is limited.

Alprazolam has been reported to have unusual clinical effects which distinguish it from other benzodiazepines, including antidepressant and enhanced antipanic activities [9]. In addition, there has been concern about the dependence potential of alprazolam, as well as discontinuation syndromes [10–13]. Alprazolam has also been reported to have anomalous effects on motor activity in mice [14] and on benzodiazepine receptor binding [15] after acute administration of low doses. Whether alprazolam exhibits unusual neurochemical or behavioral effects during chronic administration remains uncertain. In addition, the development of tolerance to alprazolam during chronic dosage has not been well characterized. We and others have demonstrated behavioral tolerance and decreases in benzodiazepine binding and $GABA_A$ receptor function during chronic administration of benzodiazepines such as lorazepam and flurazepam [16–18].

To assess the behavioral and neurochemical effects of chronic alprazolam administration, we treated mice with alprazolam for up to 14 days and examined motor activity, plasma and brain alprazolam concentrations, benzodiazepine and chloride channel binding, and $GABA_A$ receptor function as determined by chloride uptake.

MATERIALS AND METHODS

Materials. Male CD-1 mice, 6- to 8-weeks old, were obtained from Charles River Laboratories (Wilmington, MA) and maintained on a 12-hr light–dark cycle with laboratory chow and water *ad lib*. Osmotic pumps were obtained from Alza (Palo Alto, CA). [^{3}H]Ro15-1788 (sp. act. 82 Ci/mmol), [^{3}H]flunitrazepam (sp. act. 78 Ci/mmol), [^{35}S]TBPS (sp. act. 81 Ci/mmol), and [$^{36}Cl^-$] (sp. act. 12.5 mmol/g), unlabeled TBPS, and Protosol were obtained from New England Nuclear (Boston, MA). Alprazolam and the internal standard U-31485 were provided by Upjohn (Kalamazoo, MI). Other

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‖ Abbreviations: $GABA_A$, γ-aminobutyric $acid_A$; and [^{35}S]TBPS, *t*-butylbicyclophosphorothionate.

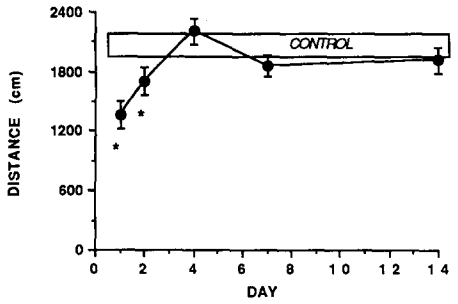

Fig. 1. Open-field activity during chronic alprazolam administration. Results are distance traveled in cm during a 5-min period (mean ± SE, N = 8 for each group). The box represents mean ± SE for vehicle-treated controls. Key: (*) $P < 0.05$ vs controls.

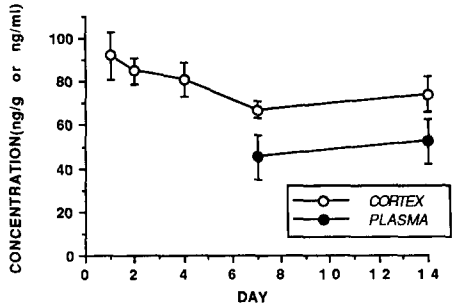

Fig. 2. Plasma and cortex concentrations of alprazolam during chronic administration. Results are means ± SE, N = 4 in cortex, N = 3 in plasma.

reagents were obtained from standard commercial sources.

Alprazolam administration. Alprazolam was dissolved in polyethylene glycol 400 and placed in osmotic pumps (Alzet 2001 or 2002). Pumps were implanted s.c. under brief ether anesthesia. Control mice received pumps containing vehicle alone.

Open-field activity. Open-field activity was determined using an Opto-Varimex (Columbus Instruments, Columbus, OH). Mice were evaluated for a 5-min period between 9:00 a.m. and noon, and distance traveled, ambulatory time, and resting time were determined.

Benzodiazepine concentrations. Alprazolam concentrations in plasma and brain were determined by gas–liquid chromatography as described previously [19].

Benzodiazepine receptor binding. Benzodiazepine receptor binding *in vivo* was determined as previously described using specific uptake of [^{3}H]Ro15-1788 [20]. Receptor binding to synaptosomal membranes (P_2) was determined as previously described using [^{3}H]flunitrazepam [21]. Proteins were determined by the method of Simpson and Sonne [22].

Chloride channel binding. Binding to the putative chloride channel site was determined in synaptosomal membrane preparations (P_2) using [^{35}S]TBPS as previously described [23].

Muscimol-stimulated [$^{36}Cl^-$] *uptake.* Chloride uptake was performed using cortical synaptoneurosome preparations as previously described [24].

Data analysis. Data from receptor-binding studies were analyzed using the EBDA programs [25]. Differences among groups were analyzed using analysis of variance with Dunnett's correction.

RESULTS

Open-field activity. After 1 day, alprazolam (ALPRZ, 2 mg/kg/day) significantly decreased open-field activity compared to vehicle-treated control mice (Fig. 1; distance traveled: controls 1976 ± 111 cm; ALPRZ 1358 ± 142 cm; mean ± SE, N = 8). At day 2, activity had increased slightly but remained significantly decreased compared to controls (1697 ± 139 cm). By day 4, activity had returned to control levels (2211 ± 133 cm) and it remained similar to controls at days 7 and 14 (day 7: 1865 ± 109 cm; day 14: 1914 ± 128 cm). Similar results were observed for ambulatory time.

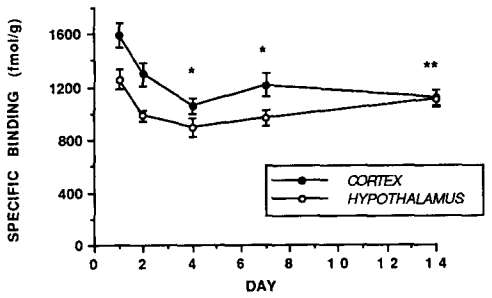

Fig. 3. Benzodiazepine receptor binding *in vivo* during chronic alprazolam administration. Binding was performed using [^{3}H]Ro15-1788. Nonspecific binding was unchanged among time points in both regions. Results are means ± SE, N = 14 at day 1, 13 at day 2, 11 at day 4, 7 at day 7, and 9 at day 14. Key: (*) $P < 0.05$ vs day 1 for both cortex and hypothalamus; (**) $P < 0.05$ for day 1 for cortex only.

Alprazolam concentrations. To ensure that pharmacokinetic effects did not account for neurochemical alterations and to validate the accuracy of osmotic pumps, concentrations of alprazolam in cerebral cortex were determined at days 1, 2, 4, 7, and 14, and concentrations in plasma at days 7 and 14 (Fig. 2). Cortical concentrations of ALPRZ remained constant throughout this period, and concentrations in plasma at days 7 and 14 were similar (day 7: 44 ± 10 ng/ml; day 14: 51 ± 9 ng/ml; mean ± SE, N = 3). The brain:plasma ratios for ALPRZ were similar at day 7 (1.42) and day 14 (1.48), indicating no change in brain uptake over time.

Benzodiazepine receptor binding in vivo. Benzodiazepine receptor binding in cortex was similar at days 1 and 2, but was decreased subsequently at day 4 (Fig. 3). Binding remained decreased compared to day 1 at days 7 and 14. In hypothalamus, binding was similar at days 1 and 2, but binding was decreased at days 4 and 7 compared to day 1. At day 14, binding again was similar to day 1. No significant changes in binding were observed in cerebellum, hippocampus,

Table 1. Benzodiazepine receptor binding *in vivo* during alprazolam administration

Day	Binding (fmol/g tissue)		
	Hippocampus	Cerebellum	Pons-medulla
1	1186 ± 93	536 ± 30	441 ± 38
2	1091 ± 83	509 ± 31	377 ± 31
4	1091 ± 91	495 ± 40	427 ± 22
7	1182 ± 104	504 ± 27	323 ± 14
14	1077 ± 98	445 ± 36	314 ± 53

Binding was performed using [^{3}H]Ro15-1788. Nonspecific binding was unchanged in each region throughout drug administration. Results are means ± SE, N = 14 for day 1, 13 for day 2, 11 for day 4, 7 for day 7, and 9 for day 14. No significant differences were observed in each region among time points.

Table 2. Benzodiazepine receptor binding in cortex during chronic alprazolam administration

Day	K_d (nM)	B_{max} (pmol/mg protein)
1	1.7 ± 0.2	2.20 ± 0.18
2	1.6 ± 0.4	1.96 ± 0.40
4	2.3 ± 0.4	2.32 ± 0.16
7	2.0 ± 0.2	1.64 ± 0.06*

Benzodiazepine receptor binding was performed in cortical synaptosomal membranes using [^{3}H]flunitrazepam. Results are means ± SE, N = 3 for each group.
* $P < 0.05$ compared to day 1.

Table 3. [^{35}S]TBPS binding in cortex during chronic alprazolam administration

Day	K_d (nM)	B_{max} (pmol/mg protein)
1	40.1 ± 4.5	1.85 ± 0.23
2	40.9 ± 6.2	1.82 ± 0.49
4	46.7 ± 3.3	2.04 ± 0.20
7	45.4 ± 10.0	0.72 ± 0.03*

[^{35}S]TBPS binding was performed in cortical synaptosomal membranes. Results are means ± SE, N = 3 for each group.
* $P < 0.05$ compared to day 1.

and pons-medulla during the treatment period (Table 1).

Benzodiazepine receptor binding in vitro. Receptor binding in cortical membrane preparations was determined at days 1, 2, 4, and 7 (Table 2). Rosenthal–Scatchard analysis indicated no change in receptor affinity, K_d, throughout. However, the number of binding sites, B_{max} was decreased at day 7 compared to day 1. These results, in part, corroborate data obtained from *in vivo* binding experiments, although binding was decreased *in vivo* by day 4 as compared to day 7 *in vitro*.

Chloride channel binding. Binding of the putative chloride channel ligand [^{35}S]TBPS to cortical membrane preparations was determined at days 1, 2, 4, and 7 (Table 3). There was no change in apparent affinity, K_d, throughout this period. The number of binding sites, B_{max}, was similar at days 1, 2, and 4, but was decreased significantly at day 7 compared to day 1.

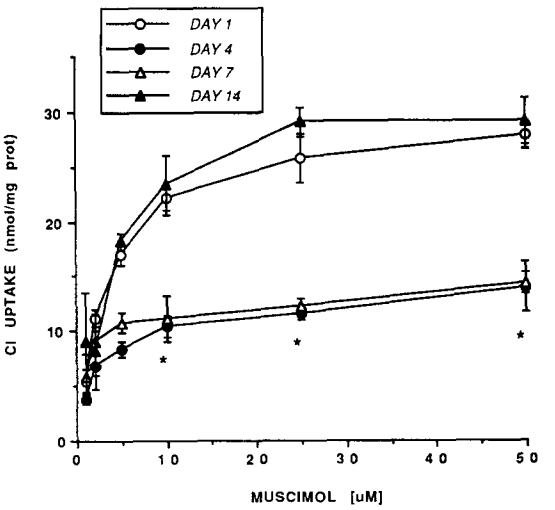

Fig. 4. Muscimol-stimulated chloride uptake in cortical synaptoneurosomes during chronic alprazolam administration. Uptake was performed using [$^{35}Cl^-$] and is corrected for non-muscimol-dependent chloride uptake. Results are means ± SE, N = 4. Key: (*) $P < 0.05$ vs day 1.

Muscimol-stimulated chloride uptake. Uptake of [$^{36}Cl^-$] into cortical synaptoneurosomes was compared at days 1, 4, 7, and 14 of alprazolam administration (Fig. 4). Uptake was decreased at days 4 and 7 at muscimol concentrations of 10, 25, and 50 μM compared to day 1, and maximal uptake was decreased at days 4 and 7 compared to day 1. There were no differences between uptake at days 4 and 7 at any muscimol dose evaluated. By day 14, uptake had returned to levels similar to those observed at day 1.

DISCUSSION

The development of tolerance to benzodiazepine effects has been reported to occur with a number of benzodiazepines and a variety of behaviors [3, 26]. The mechanism for the development of tolerance has remained uncertain. Although some data implicated pharmacokinetic changes [27], in general little change in plasma drug concentrations occurs in animals or humans during chronic administration [28, 29]. Limited data in animals do not indicate changes in brain drug concentrations during chronic benzodiazepine administration [16]. Data concerning a neurochemical basis for tolerance are conflicting. While some studies have shown no change or even increases in benzodiazepine binding during chronic drug administration [30–33], several recent studies have indicated decreased benzodiazepine receptor binding during chronic administration of flurazepam, diazepam, and lorazepam [16–18]. In addition, studies from several laboratories have indicated accompanying decreased $GABA_A$ receptor

function after chronic benzodiazepine administration [16, 34].

The triazolobenzodiazepines, and alprazolam in particular, have been reported to have unique clinical effects although this conclusion remains controversial [9]. Prior studies have also indicated that acute administration of alprazolam has unusual effects on open-field activity [14] and on benzodiazepine receptor binding in mice [15]. Specifically, low doses of alprazolam appear to increase open-field activity in mice, and to augment benzodiazepine receptor binding in a number of brain regions. Similar effects on behavior were not observed with other benzodiazepines, such as clonazepam, or with the triazolobenzodiazepine triazolam. Augmentation of receptor binding at low doses was not observed with lorazepam or clonazepam, or with the triazolobenzodiazepines triazolam or estazolam. However, the relation of acute effects on behavior and binding to chronic clinical usage of alprazolam was uncertain.

In the present study, tolerance to the effects of alprazolam on motor activity in mice occurred after 4 days of administration, and mice remained tolerant during 2 weeks of dosage. These results are similar to those observed with the same dose of lorazepam, although tolerance to lorazepam was not evident until 7 days of administration. Although tolerance to benzodiazepines may be dose dependent, alprazolam and lorazepam produce similar brain concentrations and have similar occupancy [5, 16], so that comparisons may be performed. For both alprazolam and lorazepam, the onset of tolerance correlated with alterations in benzodiazepine binding and muscimol-stimulated chloride uptake. Tolerance persisted throughout the 14 days of drug administration, as did the increase in benzodiazepine binding in cortex. However, chloride uptake at day 14 was similar to day 1, indicating that tolerance at this time point could be dissociated from changes in receptor function, and that benzodiazepine binding could also vary independently from receptor function. These results suggest that tolerance may be related to receptor downregulation at early time points, but at a later time point receptor function in chloride transport is not associated with tolerance.

The neurochemical alterations observed after chronic alprazolam administration were similar to those observed with lorazepam. Benzodiazepine receptor binding determined *in vivo* was decreased in cortex and hypothalamus after 4 days of alprazolam. With lorazepam, binding was not decreased until 7 days, and binding also declined in hippocampus. In addition, binding in hypothalamus remained depressed after 14 days of lorazepam. Similar results were obtained using alprazolam and lorazepam for *in vitro* benzodiazepine binding in cortex. However, binding of the putative chloride channel ligand [^{35}S]TBPS was decreased after 7 days of alprazolam but remained unchanged with lorazepam. Finally, decreased muscimol-stimulated chloride uptake was observed in cortex after 4 days of alprazolam, as compared to 7 days of lorazepam. Thus, chronic alprazolam showed similar overall neurochemical effects to lorazepam, except that downregulation of binding and receptor function appeared to occur more rapidly with alprazolam.

It should be pointed out that results obtained *in vitro* do not completely parallel *in vivo* data. That is, benzodiazepine binding in cortex was decreased *in vivo* at 4 days but *in vitro* at 7 days. We have discussed previously possible bases for discrepancies between *in vitro* and *in vivo* binding [35]. In the present study, alterations in both *in vivo* binding and chloride uptake at 4 days suggest that substantial receptor effects have occurred at this time.

The regional effects of alprazolam on *in vivo* benzodiazepine receptor binding differed somewhat from those of lorazepam. While both agents affected cortex and hypothalamus, lorazepam led to significant decreases in binding in hippocampus, whereas alprazolam was associated with no change in hippocampal binding. Similarly, binding remained depressed in hypothalamus at 14 days with lorazepam administration, while binding in hypothalamus had returned to control values after 14 days of alprazolam. It is possible that the different regional alterations associated with alprazolam contribute to its unique behavioral effects. In addition, the recovery of binding to control levels in hypothalamus may indicate differential regulation among regions with alprazolam. Finally, effects of alprazolam on the chloride channel site in cortex labeled by TBPS were greater than those of lorazepam, perhaps indicating a more robust effect on the $GABA_A$ receptor complex.

The mechanism for receptor downregulation associated with chronic benzodiazepine use remains uncertain. Recent data based on cloning and sequencing of the $GABA_A$ receptor indicate the presence of two subunits, and a proposed model suggests equal stoichiometry [36]. The benzodiazepine and $GABA_A$ sites appear to be located on different subunits, and the chloride channel site may span the subunits. In the present study, the decrease in chloride channel sites (about 60%) was substantially greater than benzodiazepine sites (30–40%). This difference may be due to imprecision of binding techniques or to a differential effect of alprazolam on the two subunits.

It is unlikely that the neurochemical alterations associated with chronic alprazolam dosage have a pharmacokinetic origin. Cortical concentrations of alprazolam remained constant over the 14-day period of administration. Plasma concentrations at 7 and 14 days were similar, and levels were consistent with concentrations attained in humans during administration of moderate therapeutic doses of alprazolam [9, 37]. In addition, brain : plasma ratios were similar at 7 and 14 days, making it unlikely that alterations in brain uptake contributed to receptor abnormalities.

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