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Differential effects of chronic lorazepam and alprazolam on benzodiazepine binding and $GABA_A$-receptor function

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1 Chronic benzodiazepine administration has been associated with tolerance and with downregulation of γ-aminobutyric $acid_A$ ($GABA_A$)-receptor binding and function. However, effects of individual benzodiazepines on brain regions have varied.

2 To compare the effects of chronic lorazepam and alprazolam, we have administered these drugs to mice for 1 and 7 days ($2\,mg\,kg^{-1}\,day^{-1}$) and determined benzodiazepine receptor binding *in vivo* with and without administration of CL 218,872, $25\,mg\,kg^{-1}$ i.p., and GABA-dependent chloride uptake in 3 brain regions at these time points.

3 Benzodiazepine binding was decreased in the cortex and hippocampus at day 7 compared to day 1 of lorazepam, with an increase in CL 218,872-resistant (Type 2) sites in both regions. Maximal GABA-dependent chloride uptake was also decreased in the cortex and hippocampus at day 7.

4 Binding was decreased only in the cortex after 7 days of alprazolam, with no significant change in Type 2 binding. Maximal GABA-dependent chloride uptake was also decreased only in the cortex.

5 These data suggest that the effects of chronic benzodiazepine administration on the $GABA_A$-receptor may be both region-specific and receptor subtype-specific.

Introduction

In clinical use, chronic benzodiazepine administration is associated with the development of tolerance to anticonvulsant and hypnotic effects (e.g., Greenblatt & Shader, 1978). Tolerance has also been observed in a number of animal models with a variety of benzodiazepines, including 'classical' benzodiazepines and the newer triazolobenzodiazepines (Garratt *et al.*, 1989). In previous studies, we demonstrated the development of tolerance during chronic administration of the classical benzodiazepine lorazepam (Miller *et al.*, 1988a) and the triazolobenzodiazepine alprazolam (Miller *et al.*, 1989c). In both cases, tolerance was associated temporally with benzodiazepine receptor downregulation and decreased $GABA_A$-receptor function. Similar results have been obtained by other investigators for flurazepam (Tietz *et al.*, 1986) and diazepam (Marley & Gallager, 1989).

However, our results indicated that receptor downregulation produced by alprazolam and lorazepam had differing regional specificity. Receptor alterations induced by lorazepam occurred in the cortex, hypothalamus, and hippocampus, whereas those associated with alprazolam occurred in the cortex and hypothalamus only. A possible mechanism for this discrepancy is differential effects of the two drugs on benzodiazepine receptor subtypes (Sieghart, 1989), although binding studies do not indicate a substantial difference in this regard (Haefely *et al.*, 1985). We did not assess regional specificity for γ-aminobutyric acid (GABA)-dependent chloride uptake, although other investigators have demonstrated effects in the cortex but not the cerebellum after chronic diazepam (Marley & Gallager, 1989).

To assess possible region-specific effects of lorazepam and alprazolam during chronic administration, we evaluated benzodiazepine binding *in vivo* and GABA-dependent chloride uptake in several brain regions both before (day 1) and after (day 7) the development of tolerance to both compounds. In addition, we evaluated the relative proportion of benzodiazepine subtype binding by use of the subtype-specific ligand CL 218,872 (Sato & Neale, 1989).

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Methods

Male CD1 mice, 6–8 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA), given food and water *ad libitum*, and maintained on a 12 h light/dark cycle.

Lorazepam and alprazolam ($2\,mg\,kg^{-1}\,day^{-1}$) were dissolved in PEG 400 and administered by subcutaneously implanted osmotic pumps as previously described (Miller *et al.*, 1988a). Day 1 was chosen as a point before the development of tolerance and receptor alterations, and day 7 as a point associated with tolerance and receptor changes (Miller *et al.*, 1988a). Benzodiazepine receptor subtypes were distinguished by use of CL 218,872, which appears to bind preferentially to Type 1 sites (Sieghart, 1989). CL 218,872 was dissolved in ethanol and diluted with saline to a final ethanol concentration of 0.1%. Vehicle contained 0.1% ethanol diluted with saline.

Benzodiazepine binding *in vivo* was performed as previously described (Miller *et al.*, 1988a). Briefly, mice were injected i.v. with 3 μCi [^{3}H]-Ro15-1788. After 20 min, animals were killed and brains rapidly removed and dissected on ice. After the brain regions had been weighed they were dissolved in Protosol (40°C for 24 h) and then counted by scintillation spectrometry. For subtype specific binding mice were injected with CL 218,872, $25\,mg\,kg^{-1}$ i.p., 30 min before the radioligand.

GABA-dependent chloride uptake was performed as previously described (Miller *et al.*, 1988a). Briefly, cortical synaptoneurosomes were prepared and resuspended in assay buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4). After incubation for 10 min at 30°C, 100 μl of membrane suspension mixed with 100 μl of a solution containing muscimol (1–50 μM) and $^{36}Cl^-$, $0.2\,\mu Ci\,ml^{-1}$ assay buffer. After 6 s the incubation was terminated by addition of 0.5 ml cold assay buffer containing 6 μM picrotoxin and filtration on Whatman GF/C filters by a Brandel M24 apparatus. Filters were washed twice with cold buffer and counted by scintillation spectrometry.

[^{3}H]-Ro15-1788 (flumazenil, spec. act. 80 Ci mmol^{-1}) and $^{36}Cl^-$ (spec. act. 25 Ci mg^{-1}) were obtained from New England Nuclear (Boston, MA). Muscimol was obtained from Sigma and polyethylene glycol 400 (PEG 400) from J.T. Baker (St. Louis, MO). Osmotic pumps were obtained from Alza (Palo Alto, CA). Alprazolam was a gift from Upjohn (Kalamazoo, MI), lorazepam from Wyeth (Philadelphia, PA) and CL 218,872 from Dr Joseph Moerschbaecher.

Data were analysed by Student's t test, the Mann-Whitney test, or analysis of variance with Dunnett's test.

Results

Benzodiazepine receptor binding *in vivo* was significantly decreased in cortex and hippocampus at day 7 compared to day 1 of lorazepam (cortex, day 1: 1609 ± 191 fmol g^{-1}; day 7: 1182 ± 150 fmol g^{-1}; $n = 6$–9; $P < 0.05$; hippocampus, day 1: 2509 ± 254 fmol g^{-1}; day 7: 1718 ± 145 fmol g^{-1}; mean ± s.e., $n = 6$–9, $P < 0.05$; Figure 1). These results are similar to those previously obtained (Miller *et al.*, 1988a), except that decreased binding in the hypothalamus did not reach significance ($P < 0.10$). Differences in the cerebellum were not significant (day 1: 668 ± 18 fmol g^{-1}, day 7: 277 ± 27 fmol g^{-1}; $n = 6$–9; $P > 0.15$). In contrast, Type 2 benzodiazepine receptor binding (binding remaining after administration of 25 mg kg^{-1} CL 218,872) was increased in the cortex and hippocampus at day 7 compared to day 1. Type 2 binding was increased both in absolute terms (cortex, day 1: 254 ± 14 fmol g^{-1}; day 7, 405 ± 45 fmol g^{-1}; $P < 0.05$; hippocampus, day 1: 554 ± 23 fmol g^{-1}; day 7: 754 ± 91 fmol g^{-1}; $P < 0.05$) and even more dramatically as a percentage of total specific binding (Figure 1 inset). Changes in Type 2 binding in the cerebellum (day 1: 241 ± 18 fmol g^{-1}; day 7: 277 ± 27 fmol g^{-1}; $P > 0.15$) and other regions were not significant.

For alprazolam, benzodiazepine receptor binding *in vivo* was significantly decreased in the cortex at day 7 compared to day 1 (day 1: 1918 ± 127 fmol g^{-1}; day 7: 1595 ± 127 fmol g^{-1}; $n = 7$–9; $P < 0.05$; Figure 2). No alterations in other brain regions were observed (hippocampus, day 1: 2522 ± 214 fmol g^{-1}; day 7: 2436 ± 145 fmol g^{-1}; $n = 7$–9; $P > 0.40$; cerebellum, day 1: 914 ± 82 fmol g^{-1}; day 7: 818 ± 77 fmol g^{-1}; $n = 7$–9, $P > 0.30$). Unlike in previous studies (Miller *et al.*, 1989c), alterations in the hypothalamus did not achieve significance (day 1: 2141 ± 214 fmol g^{-1}; day 7: 1809 ± 214 fmol g^{-1}; $n = 7$–9; $P < 0.15$). Type 2 benzodiazepine receptor binding was slightly but not significantly increased in the cortex at day 7 compared to day 1, either as specific binding (day 1, 295 ± 20 fmol g^{-1}; day 7, 332 ± 25 fmol g^{-1}; $P < 0.15$) or as a percentage of total specific binding (Figure 2 inset). Changes in Type 2 binding in the hippocampus (day 1: 527 ± 36 fmol g^{-1}; day 7: 618 ± 41 fmol g^{-1}; $P > 0.10$), cerebellum (day 1: 223 ± 14 fmol g^{-1}; day 7: 241 ± 18 fmol g^{-1}; $P > 0.20$) and other brain regions were not significant.

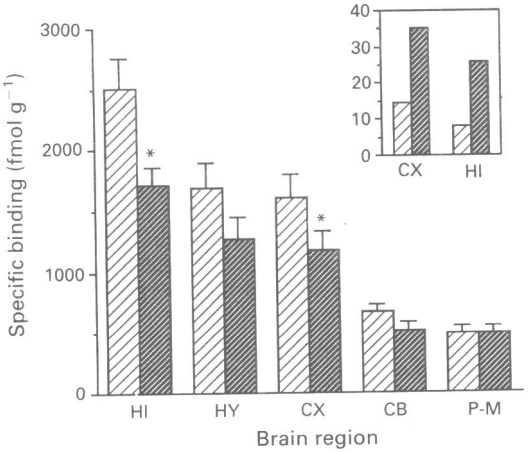

Figure 1 Effects of chronic lorazepam on benzodiazepine binding *in vivo*. Binding was determined by specific uptake of [^{3}H]-Ro15-1788. Results are mean and vertical bars show s.e.mean, $n = 7$–10. CB = cerebellum, CX = cortex, HI = hippocampus, HY = hypothalamus, and P-M = pons-medulla. *$P < 0.05$ vs. day 1. Inset: effects of chronic lorazepam on Type 2 benzodiazepine binding. Binding was determined as above after administration of CL 218,872, 25 mg kg^{-1} i.p. Results are means expressed as a percentage of total binding, $n = 6$–9. Results in cortex and hippocampus for lorazepam are significant ($P < 0.05$). (▨) Day 1; (▨) day 7.

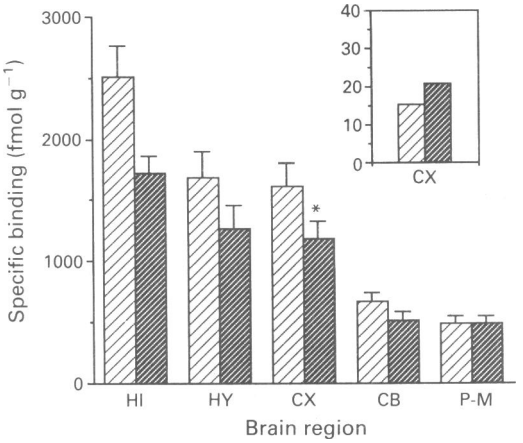

Figure 2 Effects of chronic alprazolam on benzodiazepine binding *in vivo*. Binding was determined by specific uptake of [^{3}H]-Ro15-1788. Results are mean and vertical bars show s.e.mean, $n = 7$–10. CB = cerebellum, CX = cortex, HI = hippocampus, HY = hypothalamus, and P-M = pons-medulla. *$P < 0.05$ vs. day 1. Inset: effects of chronic alprazolam on Type 2 benzodiazepine binding. Binding was determined as above after administration of CL 218,872, 25 mg kg^{-1} i.p. Results are means expressed as a percentage of total binding, $n = 6$–9. Results in the cortex are not significant. (▨) Day 1; (▨) day 7.

GABA-dependent chloride uptake in the cortex was decreased at day 7 compared to day 1, as previously found (Miller *et al.*, 1988a; Figure 3). Maximal chloride uptake was decreased, but the EC_{50} for muscimol was not altered (day 1: 3.2 μM; day 7; 3.8 μM). A similar decrement in maximal uptake without a change in the EC_{50} was observed in the hippocampus (EC_{50}: day 1, 4.2 μM; day 7, 3.4 μM), but no changes in either maximal uptake or EC_{50} for muscimol were observed in the cerebellum.

For alprazolam, maximal GABA-dependent chloride uptake was decreased at day 7 compared to day 1 (Figure 4) with no change in the EC_{50} (day 1: 3.9 μM; day 7: 3.1 μM), as previously demonstrated (Miller *et al.*, 1989c). A small, non significant decrease in uptake was observed in the hippocampus, and no changes in the EC_{50} for the maximal uptake of muscimol were observed in the cerebellum.

Discussion

These data corroborate previous studies indicating down-regulation of $GABA_A$-receptor binding and function after chronic lorazepam and alprazolam administration (Miller *et al.*, 1988a; 1989c). For both lorazepam and alprazolam, decreases in benzodiazepine binding and chloride uptake were observed in the cortex at day 7 compared to day 1. Binding was decreased in the hippocampus after lorazepam but not alprazolam, as previously found. For both drugs decreases in binding in the hypothalamus did not achieve significance, in contrast to previous findings (Miller *et al.*, 1988a; 1989c).

The present study extends our previous data in two respects. First regional alterations observed in

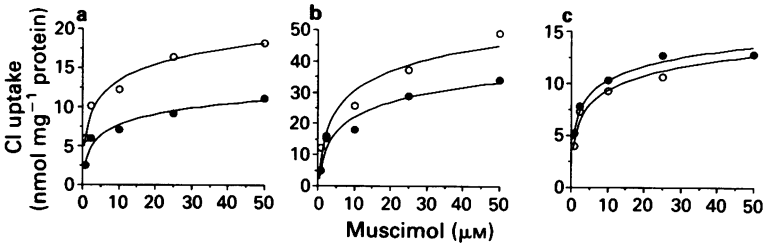

Figure 3 Effects of chronic lorazepam on $GABA_A$-receptor function in (a) cortex, (b) hippocampus and (c) cerebellum. Chloride uptake was determined in the presence (1–50 μM) and absence of muscimol. Maximal uptake was decreased at day 7 (●) compared to day 1 (○) for lorazepam in the cortex and hippocampus ($P < 0.05$). Results are means of 3–5 determinations at each point.

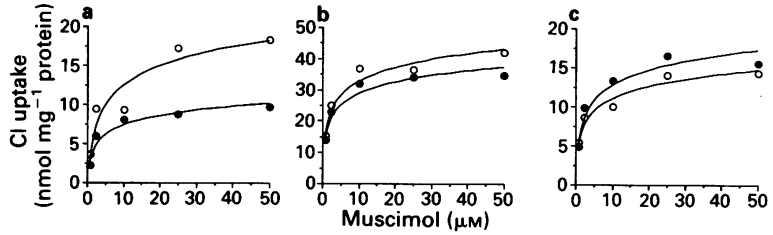

Figure 4 Effects of chronic alprazolam on $GABA_A$-receptor function in (a) cortex, (b) hippocampus and (c) cerebellum. Chloride uptake was determined in the presence (1–50 μM) and absence of muscimol. Maximal uptake was decreased at day 7 (●) compared to day 1 (○) for alprazolam in the cortex ($P < 0.05$). Results are means of 3–5 determinations at each point.

$GABA_A$-receptor function were analogous to those previously observed in binding studies. That is, lorazepam effects on chloride uptake were observed in cortex and hippocampus but not cerebellum, and alprazolam in cortex alone. Alterations in cortex but not cerebellum are similar to results obtained for diazepam by other investigators (Marley & Gallager, 1989). In addition, the association of changes in benzodiazepine binding and GABA-dependent chloride uptake observed in this study has been observed in most (Lopez *et al.*, 1990a,b; Miller *et al.*, 1989a,b), but not all (Lopez *et al.*, 1989), previous studies.

Second, alterations in binding after chronic lorazepam appear to have a greater effect on Type 2 benzodiazepine receptors compared to Type 1 sites. Not only did the binding in the cortex and hippocampus resistant to CL 218,872 (Type 2) increase after 7 days of lorazepam, but the percentage of Type 2 sites increased substantially. A non-significant increase in Type 2 binding was observed with alprazolam. These data suggest that chronic benzodiazepine treatment may preferentially affect one subclass of benzodiazepine receptors, despite the use of a ligand (lorazepam) which does not appear to bind differentially at the two sites. An alternative explanation, although it remains speculative, is that chronic lorazepam administration leads to altered receptor structure so that the ligand used, Ro15-1788, bound preferentially to one site.

For both binding and functional studies, results of chronic (7 days) treatment were compared to short-term treatment (1 day) as in previous studies. Short-term treatment rather than vehicle is the appropriate comparison for chronic treatment, since the presence of a benzodiazepine in the tissue would be expected to alter the results of both binding and functional assays. We have previously demonstrated that brain concentrations achieved by implanted pumps are unchanged at days 1 and 7 for both lorazepam and alprazolam (Miller *et al.*, 1988a; 1989c), indicating that the comparison of these time points is appropriate.

Substantial neurochemical evidence (e.g., Sato & Neale, 1989; Sieghart, 1989), and recently molecular biological evidence (e.g., Shivers *et al.*, 1989; Olsen & Tobin, 1990), support the existence of multiple benzodiazepine receptors. A number of benzodiazepine, β-carboline, and non-benzodiazepine ligands have been demonstrated to distinguish between receptor subtypes. The identification of multiple variants of the α, β and γ subunit mRNAs, together with *in situ* hybridization studies demonstrating region-specific localization of several subunits (Shivers *et al.*, 1989), strongly support the existence of receptor subtypes with different structural and perhaps functional characteristics. Recent transfection studies confirm differences in benzodiazepine binding related to the α_1 versus α_3 subunits (Pritchett *et al.*, 1989). Thus, it is likely that several $GABA_A$-receptors exist with differing benzodiazepine binding characteristics and these subtypes are likely to have differing regional specificity.

Several mechanisms might account for the alteration in the percentage of Type 1 and Type 2 sites in cortex associated with chronic lorazepam. It is possible that lorazepam might mediate interconversion of the two receptor states, either at the genome or post-translationally. Alternatively, the lack of alteration in subtype binding in cerebellum, where Type 1 sites predominate, may indicate a specific effect of chronic lorazepam on Type 2 sites. Finally, chronic lorazepam might alter receptors such that the radioligand used, Ro15-1788, binds preferentially to one site.

Our results suggest region-specific effects for chronic lorazepam and alprazolam on binding and function at the $GABA_A$-receptor. It is unlikely that these effects are related to dose or drug concentration, since the same doses were used and the regimen used maintains similar chronic drug concentrations in brain. However, it remains possible that different concentrations of individual benzodiazepines are required to alter receptors in different regions. Our results over a broad dose range for lorazepam ($1–10\,mg\,kg^{-1}\,day^{-1}$) argue against this hypothesis (Miller *et al.*, 1988b). That is, results are similar with different lorazepam doses but are distinct from alprazolam at each dose evaluated. Rather, it appears more likely that chronic lorazepam downregulates benzodiazepine sites in the cortex and hippocampus, with a preference for Type 2 sites. In contrast, alprazolam downregulates receptors in the cortex only, without a significant effect on either receptor subtype. Effects on $GABA_A$-receptor function exhibit similar regional specificity. It is possible that these differential effects are due to the binding characteristics of lorazepam and alprazolam. It is also possible that either compound might effect another neurotransmitter system, with indirect effects on the $GABA_A$ complex. Finally, the two drugs might differentially affect the $GABA_A$-receptor subunit gene expression. Additional studies examining the effects of these compounds on the $GABA_A$-receptor gene regulation may shed light on this hypothesis.

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