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**Do subtype-selective GABA_A receptor modulators have a reduced
propensity to induce physical dependence in mice?**

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Running Title Page: Physical dependence and subtype-selective GABA_A modulators

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Number of text pages: 17

Number of tables: 2

Number of figures: 2

Number of references: 31

Number of words in *Abstract*: 283

Number of words in *Introduction*: 1019

Number of words in *Discussion*: 1518

Non-standard abbreviations: None

Preferred section assignment: Behavioural Pharmacology

Abstract

Recent evidence suggests that GABA_A receptors containing a α_1 subunit mediate the sedative effect of diazepam, whereas receptors with a α_2 subunit mediate this benzodiazepines anxiolytic effects. Thus, compounds selective for GABA_A- α_2 receptors may offer advantages, i.e. lack of sedation, over current benzodiazepines. Whether such compounds would offer additional advantages over benzodiazepines is unclear. Here we address the issue of physical dependence by comparing (i) the GABA_A- α_1 *affinity* selective drug zolpidem, (ii) the novel compounds L-838,417 and SL651498 with *functional* selectivity for certain non- α_1 GABA_A receptors, (iii) non-selective partial agonists (bretazenil, NS2710, NS2664), and (iv) non-selective full efficacy benzodiazepines, in a rapid precipitated withdrawal assay using the inverse agonist FG-7142. For all compounds we determined in-vitro IC₅₀ values to displace [³H]flunitrazepam from rat cortex and in-vivo ED₅₀ values for displacement of [³H]flunitrazepam from mouse forebrain (including length of in-vivo occupancy). In the precipitated withdrawal model compounds were administered at a dose giving ~80% receptor occupancy, obviating major differences in CNS bioavailability. Mice were administered compounds twice daily for 4 days and on day 5, 20h after the final dose, given a dose of FG-7142 (40 mg/kg, i.p.) that did not induce seizures in control animals. In mice treated with the three subtype selective compounds FG-7142 did not induce seizures. Moreover, there was a low propensity for FG-7142 to induce seizures in animals treated with the partial agonists, whereas seizures were clearly seen in animals treated with most benzodiazepines. Nonetheless, differences amongst the benzodiazepines themselves, similarities between the partial agonists and subtype-selective compounds, the in-vitro/in-vivo potency and in-vivo receptor exposure time data suggest a complex interaction between selectivity, efficacy, potency and receptor exposure in determining physical dependence liability of benzodiazepine site modulators in mice.

Introduction

Recent studies show that GABA_A receptors containing a α_1 subunit mediate diazepam's locomotor depressant action (Rudolph et al., 1999; McKernan et al., 2000), whereas GABA_A receptors containing a α_2 subunit mediate diazepam-induced anxiolysis in the elevated plus maze and light-dark box (Low et al., 2000). These studies raise the possibility that novel GABA_A subtype selective compounds may retain anxiolytic efficacy with sedative side-effects obviated. However, it is unclear what additional advantages such compounds may offer over current benzodiazepines.

This point is relevant because benzodiazepines interact with ethanol, impair cognition, induce physical dependence and abuse liability. Because of these safety issues antidepressants are first line drugs for treating anxiety disorders despite a slower onset of efficacy (Nutt, 2005). However, non-selective partial agonists that bind to the benzodiazepine site such as bretazenil do not fully potentiate the effect of GABA at GABA_A receptors (Haefely et al., 1990), and have reduced abuse, physical dependence, and memory impairment liabilities in animals and humans (Martin et al., 1995; Busto et al., 1994). Likewise, zolpidem, a drug that selectively binds to GABA_A- α_1 containing receptors, has a reduced propensity to induce physical dependence in rodents (Perrault et al., 1992; von Voigtlander and Lewis, 1991), baboons (Weerts et al., 1998), and humans (Hajak et al., 2003; Shaw et al., 1992).

Recently, two ligands, L-838,417 (McKernan et al., 2000) and SL651498 (Griebel et al., 2001), have been described that show selectivity for non- α_1 containing GABA_A receptors, i.e., a profile that is in many ways opposite to that of the GABA_A- α_1 selective drug zolpidem (see below). Therefore, it is relevant to ask whether this selectivity profile imparts a different side-effect profile for these compounds compared to benzodiazepines, in addition to their reduced sedative properties already described (McKernan et al., 2000; Griebel et al., 2001). Zolpidem's affinity selectivity for GABA_A- α_1 receptors means it selectively potentiates the effect of GABA at GABA_A- α_1 receptors; although this is concentration-dependent since it only shows 10-20 fold selectivity for GABA_A- α_1 receptors over GABA_A- α_2 or α_3 receptors. However, zolpidem does show >1000-fold selectivity

for GABA_A- α_1 over GABA_A- α_5 receptors (Sieghart, 1995; Faure-Halley et al., 1993). As described above the selectivity of zolpidem for GABA_A- α_1 receptors may be important in the reduced physical dependence properties of this drug. However, neither L-838,417 nor SL651498 have *selective affinity* for non- α_1 GABA_A receptors (Atack, 2003). Rather both compounds have been described as *functionally (or efficacy) selective* modulators of non- α_1 GABA_A receptors. That is, both compound's show no affinity difference in binding to GABA_A receptors containing α_1 , α_2 , α_3 or α_5 subunits, but differentially modulate these receptors when bound leading to *functional* selectivity. Thus, L-838,417 does not potentiate effects of GABA at GABA_A- α_1 receptors, but does so at GABA_A- α_2 , α_3 and α_5 receptors; L-838,417 is a partial agonist at these latter subtypes. SL651498 fully potentiates effects of GABA at GABA_A- α_2 receptors, has marginally lower efficacy at GABA_A- α_3 receptors, with least efficacy (partial agonist profile) at GABA_A- α_1 and α_5 receptors. SL651498 has reduced physical dependence liability compared to benzodiazepines in a mouse precipitated withdrawal model (Griebel et al., 2001). This compounds partial agonism at certain GABA_A receptor subtypes and/or its subtype selectivity may account for this profile. As described above, a non-selective partial agonist like bretazenil that does not fully potentiate the effect of GABA at GABA_A receptors, also has a reduced propensity to induce physical dependence in various species. Moreover, in the current study we introduce two novel non-selective partial agonists, NS2710 and NS2664, which also have a reduced physical dependence liability in mice (see Table 1 for structures of non-selective partial agonists and selective modulators described).

In addition to intrinsic efficacy and receptor selectivity, the liability of benzodiazepine site modulators to induce physical dependence may be dependent upon other factors including potency, half-life, length of treatment, and differences between continuous/intermittent treatment (Woods et al., 1995). For example, short-intermediate half-life benzodiazepines like midazolam, triazolam, and lorazepam result in more severe rebound night-time insomnia/daytime anxiety in man than longer-acting agents like diazepam and clonazepam. However, in rhesus monkeys Yanagita (1983) demonstrated a more severe withdrawal syndrome with diazepam rather than zopiclone, despite

diazepam's longer half-life. There is also evidence that high potency compounds such as triazolam are particularly noted for inducing withdrawal symptoms (Chouinard, 2004; Vgontzas et al., 1995).

We chose a precipitated withdrawal model of physical dependence and compared the propensity of the beta-carboline FG-7142, a partial inverse agonist at the benzodiazepine receptor, to precipitate seizures in mice (Martin et al. 1995; von Voigtlander and Lewis 1991) treated with non-selective full efficacy benzodiazepines that differed with respect to half-lives and potency. Thereafter we tested, (i) non-selective partial agonists: bretazenil, and two benzimidazoles: NS2710 (Mirza et al., 2003) and NS2664 (Mathiasen et al., 2003); (ii) the imidazopyridine zolpidem, a GABA_A- α 1 selective sedative-hypnotic; and (iii) SL651498 a pyridoindole, and the triazolopyridazine L-838,417, described above.

To ascertain if in-vitro potency correlated with propensity for physical dependence we determined IC₅₀ values for all compounds to displace [³H]flunitrazepam from rat cortex. Moreover, we determined ED₅₀'s for all compounds to displace [³H]flunitrazepam from mouse forebrain in-vivo and thereby selected doses giving ~80% receptor occupancy for the withdrawal studies. Thus we could compare the relative tendency of compounds to induce physical dependence based on their selectivity and intrinsic efficacy rather than CNS bioavailability. We also determined the time-course for in-vivo displacement of [³H]flunitrazepam to ascertain if length of receptor exposure was a factor in physical dependence liability. However, in-vivo receptor occupancy is potentially a combination of the parent compound administered and any active metabolite(s). Moreover, because of active metabolites, it is important to consider that receptor exposure time is not necessarily equivalent to either the plasma or brain half-life of the parent compound (Table 1). Since some benzodiazepines and active metabolites have a long plasma half-life (e.g., diazepam and desmethyldiazepam) that may contribute to prolonged receptor exposure, it is feasible that a challenge dose of FG-7142 may simply induce a seizure in an animal by competitively displacing the agonist (parent and/or metabolite) from its binding site. To circumvent this problem, to the extent possible, FG-7142 was administered 20 hours after the last administration of each compound.

Materials and methods

Animals

Female NMRI mice (Harlan Scandinavia, Allerød, Denmark) weighing 20-25 g were housed and habituated for at least 7 days before experiments in Macrolon III cages (20 x 40 x 18 cm) with 8 mice per cage. Food (Altromin[®]) and tap water were available ad libitum, with lights on at 6 a.m. and off at 6 p.m. All behavioural testing was conducted during the light phase. All experiments were performed according to the guidelines of the Danish Committee for Experiments on Animals.

Drugs and Solutions

Diazepam, chlordiazepoxide, clonazepam, lorazepam and FG-7142 were all purchased from Sigma-Aldrich (Vallensbæk Strand, Denmark), whereas alprazolam and triazolam were sourced from Cambrex (Charles City, Iowa), and midazolam was a gift from Roche (Basel, Switzerland). Bretazenil, zolpidem, SL651498, L-838,417, NS2710 and NS2664 were all synthesized at the medicinal chemistry department, NeuroSearch A/S. All compounds were administered i.p. at a dose volume of 10 ml/kg, dissolved in 5% cremophor. [³H]Flunitrazepam (88 Ci/mmol) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). All other chemicals for binding studies described below were purchased from regular commercial sources and were of the purest grade available.

Precipitated Withdrawal Procedure

Mice were administered vehicle or test substances (N=8-10) twice a day (8-9 a.m. and 2-3 p.m.) for four consecutive days (Monday-Thursday). Approximately 20 hrs after the last dose (Friday 10-11 a.m.) mice were administered FG-7142 (40 mg/kg, i.p.) and individually placed in separate observation cages for 30 min. Animals were continuously observed for any signs of seizure activity during this time. In the experiments reported here the seizures observed were always clonic seizures involving the forelegs and facial muscles. Full-blown whole body tonic-clonic seizures were never

observed and were not anticipated with FG-7142 (Little et al., 1988). In preliminary studies a dose response curve for FG-7142 (10-160 mg/kg) showed that this partial inverse agonist never engendered seizures in control animals (data not shown). FG-7142 was chosen for these experiments as partial inverse agonists appear to be more sensitive in detecting dependence liability of benzodiazepines site modulators compared to antagonists such as flumazenil (Martin et al., 1995).

The compounds tested in this study were administered at a dose that in most cases gave $\geq 80\%$ benzodiazepine receptor occupancy in mouse forebrain based on in-vivo binding experiments (see in-vivo binding Methodology below). Consequently the doses we have chosen for some compounds are generally lower than those utilised in previous studies assessing the liability of benzodiazepine site modulators to induce physical dependence (e.g., von Voigtlander and Lewis, 1991). In all experiments triazolam (1.4 mg/kg) was used as a positive control (N=6-10), consistently resulting in seizures in 80-100% of mice after challenge with FG-7142, whereas no seizures were seen in vehicle treated animals (N=8-10). See Table 2 (column 4) for doses of each compound used in the precipitated withdrawal model.

In-vitro [³H]flunitrazepam binding (rat)

Rat cerebral cortical membranes were prepared from male Wistar rats as described by Johansen et al., 1993. Cerebral cortices were removed rapidly after decapitation, homogenized for 5 to 10 s in 10 volumes of 30 mM Tris-HCl (pH 7.4), and centrifuged at 27,000g for 15 min. All procedures were performed at 0-4 °C unless otherwise indicated. After washing the pellet three times (resuspension in 10 volumes of ice-cold buffer and centrifugation at 27,000g for 10 min), the pellet was homogenized in Tris-HCl buffer, incubated on a water bath (37 °C) for 30 min, and then centrifuged at 27,000g for 10 min. The pellet was then homogenized in buffer and centrifuged for 10 min at 27,000g. After one more wash, the final pellet was resuspended in 10 volumes of buffer and the preparation was stored at - 20 °C. On the day of the experiment the membrane preparation

was thawed and centrifuged at 2 °C for 10 min at 27,000g. The pellet was washed twice with Tris-citrate (50 mM, pH 7.1) and centrifuged for 10 min at 27,000g. The final pellet was resuspended in Tris-citrate (500 ml buffer per g of original tissue), and then used for binding assays. Aliquots of 500 µl tissue were added to 25 µl compound at the indicated concentration and 25 µl [³H]flunitrazepam (1 nM, final concentration), mixed and incubated for 40 min at 2 °C. Non-specific binding was determined in the presence of 1 µM clonazepam. Compounds were tested at 7-10 concentrations ranging from 0.03 nM to 30 µM. Binding was terminated by rapid filtration over Whatman GF/C glass fiber filters.

In-vivo [³H]flunitrazepam binding (mouse)

The in vivo-binding studies were conducted as described by Jensen et al. (1983). Briefly, groups of three female NMRI mice (25-28 g) were injected i.p. with compound solutions prepared in 5% TWEEN 80. Ten min after compound administration the mice were injected i.v. via the tail vein with 5.0 µCi of [³H]flunitrazepam in 0.2 ml saline. Twenty min after injection of [³H]flunitrazepam (i.e. 30 min after administration of compound), the mice were killed by decapitation, the forebrains rapidly excised and homogenized in 12 ml of ice-cold Tris-citrate (50 mM, pH 7.1) using an Ultra-Turrax homogenizer. Three aliquots of 1 ml were immediately filtered through Whatman GF/C glass fiber filters and washed with 2 x 5 ml of ice-cold buffer. Groups of vehicle treated mice served as controls for estimation of total binding. To determine non-specific binding, groups of mice were injected with clonazepam (2.5 mg/kg i.p.) 10 min before [³H]flunitrazepam injection. The 30 min pre-treatment time was chosen to ensure an ED₅₀ value for all compounds regardless of differences in half-life. Four to five doses ranging from 0.03 to 100 mg were tested for determination of ED₅₀ values. In time course experiments, compounds were administered relative to the decapitation time to separate groups (N=3 at each time point) of female NMRI mice, and the dose tested was the same as used in the behavioural studies.

Although, it is possible that using a non-selective drug such as [³H]flunitrazepam may underestimate the receptor occupancy of ligands with *affinity selectivity* for subtypes of GABA_A receptors, in the current study we encountered no such problems with the GABA_A-α1 selective modulator zolpidem (Faure-Halley et al., 1993, see Results).

In all binding assays the amount of radioactivity on the filters was determined by conventional liquid scintillation counting.

Data analysis

Behavioural data is shown as the percentage of animals showing clonic seizures. In in-vitro binding studies IC₅₀ values were estimated from the equation $B = 100 - [100 * D^n / (IC_{50}^n + D^n)]$ by non-linear regression using GraphPad Prism version 2.01 where B is the specific binding in percentage of total specific binding, D is the drug concentration (μM) and n is the Hill coefficient. All results are given as mean ± SEM. ED₅₀ values for inhibition of in-vivo [³H]flunitrazepam binding were estimated from the equation $B = 100 - [100 * D^n / (ED_{50}^n + D^n)]$ where D is the administered dose of drug (mg/kg, i.p.) and n is the Hill coefficient. In vivo receptor occupancy was estimated from the equation $RO = D * 100 / (ED_{50} + D)$.

Results

Table 1 gives the structures of all compounds assessed in this study, along with available information on elimination half-lives of parent compound and any known active metabolites in man. Table 2 summarises the in-vitro and in-vivo potency of all compounds, and the dose chosen, based on in-vivo receptor binding studies described below, in the precipitated withdrawal test. All compounds completely inhibited in-vitro binding at the highest concentration tested, and in-vivo all benzodiazepines and partial agonists showed 90 - 100% inhibition at the highest dose tested. For the subtype-selective modulators the inhibition varied from ~80 - 90% at the highest dose tested. The Hill coefficients were not significantly different from 1.

1. Benzodiazepines

All benzodiazepines potently inhibited in-vitro [^3H]flunitrazepam binding with IC_{50} values ranging from 0.62 to 19 nM, except for chlordiazepoxide which had an IC_{50} of 930 nM (Table 2). Similarly, all benzodiazepines, with the exception of chlordiazepoxide, inhibited in-vivo [^3H]flunitrazepam binding with ED_{50} values in the range of 0.12 to 1.4 mg/kg, when measured 30 min after i.p. administration (Table 2). Chlordiazepoxide had an ED_{50} of 11 mg/kg. The calculated receptor occupancy at the doses tested in the behavioural studies described below for the benzodiazepines ranged from 74% (70-77%) for midazolam to 91% (87-95%) for alprazolam (Fig. 1A). Time course in-vivo binding studies using the doses of each benzodiazepine selected for behavioural studies described below showed that the benzodiazepines generally inhibited in-vivo [^3H]flunitrazepam binding >70% for at least 6 hr in the mouse brain (Fig. 2A). However, in-vivo inhibition of [^3H]flunitrazepam binding at 6 hr post-administration for triazolam, midazolam and clonazepam was 0%, 0% and 53%, respectively, suggesting a shorter receptor occupancy half-life compared to the other benzodiazepines tested. Moreover, for midazolam, very little in-vivo receptor occupancy (~20%) was still observed at 2 hrs. This short-lived receptor occupancy profile of midazolam is

similar to that observed with the majority of non-selective partial agonists and subtype selective modulators described below.

Sub-chronic treatment with the various benzodiazepines engendered a differential propensity to seizure response in mice after a challenge dose of FG-7142 (Fig. 1A). Thus, whereas 80-90% of mice treated with triazolam, clonazepam and diazepam had clonic seizures after challenge with FG-7142, no mice treated with the low potency benzodiazepine chlordiazepoxide seized after administration of this beta-carboline. Somewhat intermediate between these two extremes were the benzodiazepines lorazepam, midazolam and alprazolam where challenge with FG-7142 engendered seizures in 44-67% of mice.

2. Partial agonists

The three non-selective partial agonists, NS2664, bretazenil and NS2710, inhibited in-vitro [³H]flunitrazepam binding with IC₅₀ values ranging from 0.61 to 3.0 nM and potently inhibited in-vivo binding with ED₅₀ values between 0.15 to 1.7 mg/kg (Table 2). The calculated receptor occupancy at the doses tested in the behavioural study described below ranged from 83 to 89% (Fig. 1B). In-vivo [³H]flunitrazepam time course studies using doses of each compound selected for behavioural studies described below showed that NS2664 and bretazenil were short lasting in mouse brain with only 2% and 12% inhibition of [³H]flunitrazepam binding, respectively, 2 hr post-administration (Fig. 2B). However, NS2710 and/or metabolites, occupied the receptor for longer with 94% and 42% inhibition of [³H]flunitrazepam binding 2 and 6 hr post-administration, respectively (Fig. 2B).

Mice treated with the non-selective partial agonist bretazenil showed a very low propensity to clonic seizures after the challenge dose of FG-7142, with only 20% of mice showing clonic seizures (Fig. 1B). This study was replicated to ensure the reproducibility of this data, giving essentially the same percentage of mice with a seizure response after FG-7142 challenge (Table 2). Likewise, only 30% of mice treated with NS2710 a non-selective partial agonist at GABA_A

receptors had clonic seizures after challenge with FG-7142 (Fig. 1B). However, at the doses tested no mice treated with the non-selective highly potent weak partial agonist NS2664 seized after FG-7142 challenge, following treatment twice daily for 4 consecutive days (Fig. 1B).

3. Subtype selective agents

L-838,417 had subnanomolar potency ($IC_{50}=0.83$ nM) in inhibiting in-vitro [3H]flunitrazepam binding, whereas SL651498 and zolpidem had IC_{50} values of 34 and 160 nM, respectively (Table 2). However, L-838,417 and SL651498 showed similar potency in inhibiting in-vivo [3H]flunitrazepam binding ($ED_{50} = 6.3$ and 5.1 mg/kg, respectively, Table 2). Zolpidem was less potent with an ED_{50} of 19 mg/kg for inhibiting in-vivo [3H]flunitrazepam to mouse forebrain. All three compounds were short acting and no inhibition of in-vivo [3H]flunitrazepam binding in mouse forebrain was seen 2 hr post-administration at the doses of L-838,417 (10 mg/kg), SL651498 (10 mg/kg) or zolpidem (30 mg/kg) tested for receptor occupancy over time (Fig. 2B).

The selective GABA_A- α_1 receptor drug zolpidem at doses of 30 and 100 mg/kg resulted in 62% and 85% occupancy of [3H]flunitrazepam binding sites in mouse forebrain, respectively (Fig. 1B shows the occupancy attained after the 100 mg/kg dose only). In mice treated with these doses of zolpidem FG-7142 failed to engender any seizure response (Fig. 1B and Table 2). At a dose of 10 mg/kg the two functionally selective compounds, L-838,417 and SL651498, were calculated to displace 71% ($69-72\%$) and 75% ($70-80\%$) of [3H]flunitrazepam binding in mouse forebrain, respectively, when measured 30 min after administration (Fig. 1B). However, from the time course study it is clear that L-838,417 displaced 89% of [3H]flunitrazepam binding sites from mouse forebrain when binding was measured 15 min after administration (Fig. 2B), i.e., somewhat higher than seen when binding was measured 30 min after L-838,417 administration. In addition to testing both L-838,417 and SL651498 in the precipitated withdrawal study at a dose of 10 mg/kg giving $\sim 80\%$ benzodiazepine receptor occupancy, both compounds were also tested at higher doses (15 and 30 mg/kg) in the behavioural assay. However, mice treated sub-chronically for 4 days with

either agent at 10, 15 or 30 mg/kg, did not show any seizures after a challenge dose of FG-7142 on day 5 (Fig. 1B and Table 2).

Discussion

The current study shows that (i) mice sub-chronically administered a range of benzodiazepines, with the exception of chlordiazepoxide, have an increased propensity to clonic seizures after a challenge dose with the beta-carboline inverse agonist FG-7142, which at the dose tested (40 mg/kg) had no tendency to induce seizures in vehicle treated animals; (ii) after treatment with the partial agonists bretazenil, NS2710 and NS2664, the percentage of mice with clonic seizures after a challenge dose of FG-7142 was generally lower than the percentage of mice seizing after treatment with benzodiazepines; and (iii) no mice treated with the subtype selective agents zolpidem, L-838,417 and SL651498 and subsequently administered FG-7142 had seizures. One conclusion from this set of data may be that partial agonism and/or subtype selectivity lead to a reduced liability to physical dependence in mice. However, the in-vitro and in-vivo potency data, and in-vivo receptor occupancy time-curves for all the compounds suggests that additional factors may be important in the propensity for a given benzodiazepine site modulator to engender physical dependence.

Thus, even amongst the benzodiazepines there was a differential liability to induce physical dependence. For example, challenge with FG-7142 engendered no seizures in mice treated with chlordiazepoxide, whereas $\geq 80\%$ of mice treated with triazolam, clonazepam or diazepam seized after challenge with the beta-carboline. Somewhat intermediate between these extremes were midazolam, lorazepam and alprazolam with 60%, 44% and 67% of mice treated with these compounds seizing after FG-7142 challenge, respectively (Fig. 1A). Thus the overall rank order of propensity for seizures after FG-7142 challenge was: triazolam=clonazepam=diazepam>alprazolam=midazolam=lorazepam>>chlordiazepoxide.

Although it is reasonable to argue that the behavioural measure here may not be sensitive enough to truly rank compounds as such, the data nonetheless indicate obvious differences between chlordiazepoxide and other benzodiazepines. Since benzodiazepines are considered a homogenous group with respect to being full non-selective positive modulators at α_1 , α_2 , α_3 and α_5 containing GABA_A receptors (Sieghart et al., 1995), factors other than intrinsic efficacy and selectivity must

account for the differences we see in mice. Comparing the rank order for seizures above with in-vitro/in-vivo potency data suggests potency may play some role since chlordiazepoxide has the lowest in-vitro/in-vivo potency and engendered no physical dependence at the dose tested. Although this may be the most parsimonious conclusion, future studies should address the issue of treatment length (see Introduction). Moreover, dose is certainly a factor with chlordiazepoxide since others (von Voigtlander and Lewis, 1991) have demonstrated precipitated withdrawal with flumazenil challenge in mice treated with high (150 mg/kg/day) but not low (1.5-15 mg/kg) doses of chlordiazepoxide in a 3-day procedure.

By contrast, receptor occupancy was approximately matched between the benzodiazepines making CNS bioavailability of parent compound/metabolites an unlikely explanation for differences in physical dependence liability (Fig. 1A). Furthermore, receptor occupancy time-curves (Fig. 2A) show that ~70% in-vivo benzodiazepine receptor occupancy (parent compound/metabolite(s)) was maintained for up to 6 hr post-administration with most benzodiazepines, with the notable exceptions of midazolam and triazolam. Therefore differences in receptor exposure time do not explain why chlordiazepoxide differs from other benzodiazepines. However, although absolute receptor occupancy and exposure cannot explain the differences in withdrawal liability between the benzodiazepines, the nature of potential metabolites that contribute to this receptor occupancy for the different benzodiazepines and the rate of receptor occupancy may be important determinants. For example, chlordiazepoxide and diazepam have long acting active metabolites with pharmacological activity equivalent to the parent drug, whereas the metabolites of alprazolam, clonazepam and lorazepam are short lasting and essentially pharmacologically inactive (Table 1). Furthermore, the rate of receptor occupancy is a variable we have not fully explored. In the field of dopamine reuptake inhibitors, for example, it has been demonstrated that both the rate and absolute occupancy of the dopamine transporter are key variables in liability of such compounds to be abused (Volkow et al., 2005).

Notwithstanding the differences between the benzodiazepines per se, the partial agonists had a considerably reduced liability to engender seizure response in mice challenged with FG-7142 compared to benzodiazepines (Fig. 1B). Thus only ~20% and 30% of mice treated with bretazenil and NS2710, respectively, had seizures after FG-7142 challenge. The data with bretazenil is commensurate with prior literature clearly showing that this compound has less liability to induce physical dependence in rodents and primates compared to benzodiazepines (Martin et al., 1995; Busto et al., 1994; Haefely et al., 1990). The benzimidazole structure NS2710 has an in-vitro efficacy profile similar to bretazenil, i.e., a positive non-selective partial agonist at low GABA concentrations (Mirza et al., 2003). However, at high GABA concentrations NS2710 has an inhibitory modulatory effect that is independent of the α subunit and which, unlike its positive modulatory effect, is not antagonised by flumazenil (Mirza et al., 2003). However, the importance of this dual mechanism effect in the propensity to physical dependence is unclear as NS2710 and bretazenil appear equivalent in our model. By contrast, NS2664, another benzimidazole structure, differed in that it engendered no seizures in mice challenged with FG-7142. NS2664 in patch-clamp electrophysiology studies on cloned human GABA_A receptors expressed in HEK cells is classified as a weak partial agonist/antagonist since its positive modulatory effects are often difficult to detect (Mathiesen et al., 2003). It is possible that the reduced intrinsic efficacy of NS2664 relative to NS2710 and bretazenil leads to a very low physical dependence liability. Certainly the profile of NS2664 relative to NS2710 and bretazenil cannot be explained by major differences in in-vitro/in-vivo potency, in-vivo receptor occupancy or receptor exposure time (Table 2, Figs. 1B and 2B).

The functionally selective positive modulators, L-838,417 and SL651498 (10-30 mg/kg), did not engender seizures after FG-7142 challenge, at doses attaining the receptor occupancy equivalent to the benzodiazepines and partial agonists. The data was somewhat surprising with regard to SL651498 which has greater efficacy at α_1 , α_2 , α_3 and α_5 receptors compared to L-838,417 (Atack, 2003), although its efficacy values are generally intermediate between the full efficacy benzodiazepines and the partial agonists described above (Sieghart, 1995; Atack, 2003).

However, the data with SL651498 are in agreement with the lack of precipitated withdrawal after a challenge dose of flumazenil in mice administered 30 mg/kg SL651498 twice daily for 10 days (Griebel et al., 2001). The data appear to indicate that subtype selective compounds with selectivity for non- α_1 GABA_A receptors have reduced liability to engender physical dependence. However, it is worth noting that FG-7142 has a modest 3-5 fold affinity selectivity for GABA_A- α_1 over GABA_A- α_2 and α_3 receptors, and 20-fold selectivity over GABA_A- α_5 containing receptors. Since the partial agonists and the functionally selective compounds tested here have low or zero efficacy at GABA_A- α_1 receptors this may explain why FG-7142 is more liable to engender convulsions in mice treated with non-selective modulators with full efficacy at GABA_A- α_1 receptors. However, zolpidem has affinity selectivity for GABA_A- α_1 receptors and is a full agonist at this receptor like the benzodiazepines, but nonetheless has a considerably reduced seizure response after FG-7142 challenge as demonstrated in other species (Perrault et al., 1992; von Voigtlander and Lewis, 1991; Richards and Martin, 1998; Hajak et al., 2003; Voderholzer et al., 2001).

Thus subtype-selectivity per se, regardless of the specific receptor selectivity profile of a given compound, may be important in mitigating physical dependence. However, the partial agonist profiles of SL651498 and L-838,417 at select GABA_A receptors suggests intrinsic efficacy is also a factor. Likewise whilst zolpidem is a full agonist at GABA_A- α_1 receptors it has a considerably reduced affinity at α_5 receptors suggesting it is unlikely to activate GABA_A- α_5 receptors in-vivo (Richards and Martin, 1998; Sanger and Benavides, 1993). Therefore, at least in this mouse model, physical dependence is only induced by compounds with full efficacy at α_1 , α_2 , α_3 and α_5 receptors

Although it might be concluded from the precipitated withdrawal study that only non-selective full efficacy modulators like the benzodiazepines will induce physical dependence, it is worth noting that for the majority of partial agonists and subtype selective modulators receptor exposure time was shorter compared to the benzodiazepines (compare Figs. 2A and 2B). Therefore this factor may be important in explaining their reduced liability to engender physical dependence. However, the non-selective full efficacy benzodiazepine midazolam clearly engendered greater

physical dependence in mice compared to the partial agonists and subtype selective compounds, despite a similar receptor exposure time (Figs. 2A and 2B). Conversely, the partial agonist NS2710 had a receptor exposure time (Fig. 2B) roughly intermediate between that of clonazepam and triazolam but, nonetheless, had a lower tendency to engender physical dependence compared to these benzodiazepines.

In conclusion, we have considered in-vitro/in-vivo potency, receptor occupancy/receptor exposure time, selectivity and efficacy as potential factors in the predilection of positive benzodiazepine site modulators to engender physical dependence. Our data suggest that a combination of low intrinsic efficacy and selectivity are likely to lead to reduced physical dependence. However, future studies with novel, ideally, affinity selective modulators may give insight into the relative importance of different GABA_A receptors to dependence liability. Moreover, further consideration should be given to additional factors such as treatment length, metabolites, and the rate of receptor occupancy, when assessing current and novel benzodiazepine site positive modulators to better understand the underlying determinants of dependence.

Acknowledgements

We thank the technicians and academics in the Departments of Receptor Biochemistry and In-vivo Pharmacology for technical assistance.

References

- Atack JR (2003) Anxioregulatory compounds acting at the GABA(A) receptor benzodiazepine binding site. *Curr Drug Targets CNS Neurol Disord* **2**:213-232.
- Busto U, Kaplan HL, Zawertailo L and Sellers EM (1994) Pharmacologic effects and abuse liability of bretazenil, diazepam, and alprazolam in humans. *Clin Pharmacol Ther* **55**:451-463.
- Chouinard G (2004) Issues in the clinical use of benzodiazepines: potency, withdrawal, and rebound. *J Clin Psychiatry* **65 Suppl 5**:7-12.
- Faure-Halley C, Graham D, Arbilla S and Langer SZ (1993) Expression and properties of recombinant alpha 1 beta 2 gamma 2 and alpha 5 beta 2 gamma 2 forms of the rat GABAA receptor. *Eur J Pharmacol* **246**:283-287.
- Griebel G, Perrault G, Simiand J, Cohen C, Granger P, Decobert M, Francon D, Avenet P, Depoortere H, Tan S, Oblin A, Schoemaker H, Evanno Y, Sevrin M, George P and Scatton B (2001) SL651498: an anxioregulatory compound with functional selectivity for alpha2- and alpha3-containing gamma-aminobutyric acid(A) (GABA(A)) receptors. *J Pharmacol Exp Ther* **298**:753-768.
- Haefely W, Martin JR and Schoch P (1990) Novel anxiolytics that act as partial agonists at benzodiazepine receptors. *Trends Pharmacol Sci* **11**:452-456.
- Hajak G, Muller WE, Wittchen HU, Pittrow D and Kirch W (2003) Abuse and dependence potential for the non-benzodiazepine hypnotics zolpidem and zopiclone: a review of case reports and epidemiological data. *Addiction* **98**:1371-1378.
- Jensen LH, Petersen EN, and Braestrup C (1983) Audiogenic seizures in DBA/2 mice discriminate sensitively between low efficacy benzodiazepine receptor agonists and inverse agonists. *Life Sci* **33**:393-399.

Johansen TH, Drejer J, Wätjen F and Nielsen EØ (1993) A novel non-NMDA receptor antagonist shows selective displacement of low-affinity [³H]kainate binding. *Eur J Pharmacol* **246**:195-204.

Little HJ, Gale R, Sellars N, Nutt DJ and Taylor SC (1988) Chronic benzodiazepine treatment increases the effects of the inverse agonist FG7142. *Neuropharmacology* **27**:383-389.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H and Rudolph U (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* **290**:131-134.

Martin JR, Jenck F and Moreau JL (1995) Comparison of benzodiazepine receptor ligands with partial agonistic, antagonistic or partial inverse agonistic properties in precipitating withdrawal in squirrel monkeys. *J Pharmacol Exp Ther* **275**:405-411.

Mathiesen, C, Hartz, BP, Nielsen, EØ, Johansen, TH and Mirza NR (2003) NS2664: an atypical benzodiazepine site ligand: profile in animal models of epilepsy. *Behav. Pharmacol.* **14**: S47.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore KW, Carling RW, Street LJ, Castro JL, Ragan CI, Dawson GR and Whiting PJ (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha1 subtype. *Nat Neurosci* **3**:587-592.

Mirza NR, Watjen F, Hartz BP, Teuber L, Nielsen EØ, Mathiasen LI, Johansen TH and Jensen LH (2003) NS2710: A Novel Anxiolytic with a Dual Mechanism of Action. *Behav. Pharmacol.* **14**: S24.

Nutt DJ (2005) Overview of diagnosis and drug treatments of anxiety disorders. *CNS Spectr* **10**:49-56.

Perrault G, Morel E, Sanger DJ and Zivkovic B (1992) Lack of tolerance and physical dependence upon repeated treatment with the novel hypnotic zolpidem. *J Pharmacol Exp Ther* **263**:298-303.

Pieri L, Hunkeler W, Jauch R, Merz WA, Roncari G and Timm U (1988) Bretazenil. *Drugs Future* **13**:730-735.

Richards JG and Martin JR (1998) Binding profiles and physical dependence liabilities of selected benzodiazepine receptor ligands. *Brain Res Bull* **45**:381-387.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H and Mohler H (1999) Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. *Nature* **401**:796-800.

Sanger DJ and Benavides J (1993) Discriminative stimulus effects of omega (BZ) receptor ligands: correlation with in vivo inhibition of [3H]-flumazenil binding in different regions of the rat central nervous system. *Psychopharmacology (Berl)* **111**:315-322

Shaw SH, Curson H and Coquelin JP (1992) A double-blind, comparative study of zolpidem and placebo in the treatment of insomnia in elderly psychiatric in-patients. *J Int Med Res* **20(2)**:150-161.

Sieghart W (1995) Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol Rev* **47**:181-234.

Therapeutic Drugs, Second Edition (1999) Ed. Colin Dollery, Churchill Livingstone, Edinburgh.

Vgontzas AN, Kales A and Bixler EO (1995) Benzodiazepine side effects: role of pharmacokinetics and pharmacodynamics. *Pharmacology* **51**:205-223.

Voderholzer U, Riemann D, Hornyak M, Backhaus J, Feige B, Berger M and Hohagen F (2001) A double-blind, randomized and placebo-controlled study on the polysomnographic withdrawal

effects of zopiclone, zolpidem and triazolam in healthy subjects. *Eur Arch Psychiatry Clin Neurosci* **251**:117-123.

Volkow ND, Wang GJ, Fowler JS, Learned-Coughlin S, Yang J, Logan J, Schlyer D, Gatley JS, Wong C, Zhu W, Pappas N, Schueller M, Jayne M, Carter P, Warner D, Ding YS, Shea C, Xu Y (2005) The slow and long-lasting blockade of dopamine transporters in human brain induced by the new antidepressant drug radafaxine predict poor reinforcing effects. *Biol Psychiatry* **57**(6):640-646.

VonVoigtlander PF and Lewis RA (1991) A rapid screening method for the assessment of benzodiazepine receptor-related physical dependence in mice. Evaluation of benzodiazepine-related agonists and partial agonists. *J Pharmacol Methods* **26**:1-5.

Weerts EM, Ator NA, Grech DM and Griffiths RR (1998) Zolpidem physical dependence assessed across increasing doses under a once-daily dosing regimen in baboons. *J Pharmacol Exp Ther* **285**(1):41-53.

Woods JH, Katz JL and Winger G (1995) Abuse and therapeutic use of benzodiazepines and benzodiazepines-like drugs, in *Psychopharmacology: The Fourth Generation of Progress* (Bloom FE and Kupfer DJ eds), Raven Press Ltd., New York.

Yanagita T (1983) Dependence potential of zopiclone studied in monkeys. *Pharmacology* **51**: 216-227.

Legends for Figures

Figure 1

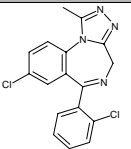
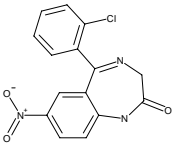
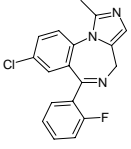
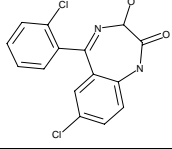
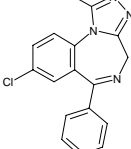
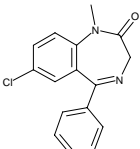
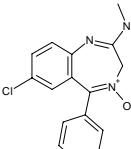
Propensity of FG-7142 (40 mg/kg i.p.) to induce clonic seizures in mice treated twice-daily for 4 days with the compounds shown (histograms indicate % mice with clonic seizures, left y-axis). The points above each histogram depict the in-vivo benzodiazepine receptor occupancy calculated using the individual in-vivo binding ED₅₀ values (right y-axis) attained at the dose of each compound tested in the behavioural study (see Table 1). (A) seizure propensity and receptor occupancy for benzodiazepines - TZM (triazolam), CLON (clonazepam), MID (midazolam), LZM (lorazepam), ALPZ (alprazolam), , DZM (diazepam), and CDP (chlordiazepoxide). (B) seizure propensity and receptor occupancy for non-selective partial agonists – NS2664, BRZ (bretazenil) and NS2710, and subtype selective modulators – L-838,417 (10 mg/kg), SL651498 (10 mg/kg) and ZPM (zolpidem, 100 mg/kg). Calculation of receptor occupancy was performed as described in Data Analysis.

Figure 2

Time course for inhibition of in-vivo [³H]-flunitrazepam binding in mouse forebrain for (A) benzodiazepines and (B) non-selective partial agonists and subtype-selective modulators at the doses used in the behavioural studies (see numbers in parenthesis). Time course for inhibition of in-vivo [³H]-flunitrazepam binding shown for L-838,417 and SL651498 is based on a 10 mg/kg, i.p. dose, and a 30 mg/kg i.p. dose of zolpidem (**note:** all these three subtype selective compounds were tested at additional higher doses in the precipitated withdrawal study – see Table 2). The in vivo [³H]-flunitrazepam binding assay was performed as described under Materials and Methods.

TABLES

Table 1 Structure of benzodiazepine site modulators assessed in the current study, their plasma elimination half-lives ($T_{1/2}$ in hr) in man and major active metabolites where known (Therapeutic Drugs, 2nd Ed., 1999; *Pieri et al., 1988; #in-house data). **Note:** see Introduction for explanation of compounds designated as having affinity or functional selectivity.

Compound	Structure & range of plasma elimination $T_{1/2}$ (hr)	Major active metabolites
Benzodiazepines		
Triazolam	 1.5 – 5.9	α -hydroxytriazolam 4-hydroxytriazolam
Clonazepam	 18 – 45	7-amino-clonazepam – <i>weak pharmacological activity relative to parent drug</i>
Midazolam	 2.0 – 3.0	α -hydroxymidazolam
Lorazepam	 8.0 – 25	No significant active metabolites
Alprazolam	 6.0 – 16	1-hydroxyalprazolam 4-hydroxyalprazolam - <i>both rapidly eliminated, hence little activity</i>
Diazepam	 30 – 200	<i>N</i> -desmethyldiazepam ($T_{1/2}$ range, 30-300 h) Oxazepam Temazepam
Chlordiazepoxide	 5.0 – 30	desmethylchlordiazepoxide ($T_{1/2}$ = 60 h) demoxepam ($T_{1/2}$ = 45 h) nordiazepam ($T_{1/2}$ = 50 h)

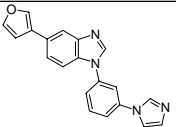
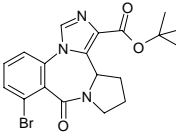
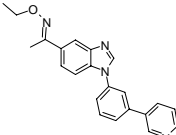
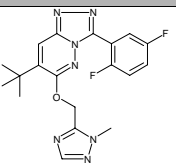
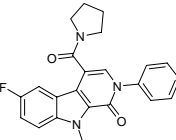
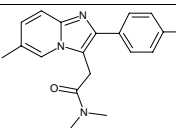
Partial agonists		
NS2664	 unknown	unknown
Bretazenil*	 1.8 – 3.8	Six metabolites identified
NS2710 [#]	 6.0 – 12	Five metabolites identified [#]
Subtype-selective agonists		
L-838,417 (functional-selectivity)	 unknown	unknown
SL651498 (functional-selectivity)	 unknown	unknown
Zolpidem (affinity-selectivity)	 2.0 – 4.0	No active metabolites

Table 2 Propensity for FG-7142 (40 mg/kg, i.p.) to induce clonic seizures in NMRI mice treated twice daily (i.p.) for 4 days with various benzodiazepine site modulators (column 1). The percentage of mice with clonic seizures is shown in column 5. FG-7142 was administered 20 hrs after the last dose of the test compound (see Methods for details). The dose of each compound administered for 4 days is shown in column 4, and represents a dose giving approximately $\geq 80\%$ benzodiazepine receptor occupancy for the majority of compounds (see Fig. 1). Also shown are the in-vitro (rat cortex, column 2, N=3-4, mean \pm SEM) and in-vivo (mouse forebrain, column 3, N=2 or 3*; the range is shown in paranthesis) potency of each compound for the benzodiazepine site as labelled with [3 H]Flunitrazepam ([3 H]FNM). The number of animals per group in behavioural studies is 8-10 in all cases. As highlighted in the Methods section: (i) vehicle treated animals (N=8-10) were used in each study with FG-7142 (40 mg/kg, i.p.) never inducing clonic seizures in these animals; (ii) in all experiments triazolam (1.4 mg/kg, i.p.) was run as a positive control (N=6-10), consistently resulting in seizures in 80-100% of mice after challenge with FG-7142. ^{\$}Bretazenil was tested twice at 1.1 mg/kg. [#]Both L-838,417 and SL651498 were run at three different doses (10, 15 and 30 mg/kg, i.p.) and zolpidem at 30 and 100 mg/kg, i.p.. **Note:** see Introduction for explanation of compounds designated as having affinity or functional selectivity.

Compound	In-vitro [³ H]FNM IC ₅₀ , nM	In-vivo [³ H]FNM ED ₅₀ mg/kg, i.p.	Dose mg/kg, i.p.	% Mice with clonic seizures
Benzodiazepines				
Triazolam	0.62 ± 0.15	0.13 (0.10-0.16)	1.4	83
Clonazepam	2.1 ± 0.5	0.12 (0.11-0.12)	0.8	80
Midazolam	2.4 ± 0.3	1.1 (0.9-1.3)	3.0	60
Lorazepam	4.1 ± 1.0	0.55 (0.52-0.58)	4.2	44
Alprazolam	10 ± 20	0.36 (0.18-0.54)	3.5	67
Diazepam	19 ± 3	1.4* (1.3-1.6)	10	90
Chlordiazepoxide	930 ± 250	11 (9.5-13)	40	0
Partial agonists				
NS2664	0.61 ± 0.20	0.61 (0.60-0.62)	3.5	0
Bretazenil	0.75 ± 0.15	0.15 (0.13-0.16)	1.1	20-22 ^{\$}
NS2710	3.0 ± 0.5	1.7* (1.5-2.0)	10	30
Subtype-selective agonists				
L-838,417 (functional-selectivity)	0.83 ± 0.24	6.3 (5.8-6.7)	10/15/30 [#]	0
SL651498 (functional-selectivity)	34 ± 8	5.1 (3.8-6.3)	10/15/30 [#]	0
Zolpidem (affinity-selectivity)	160 ± 20	19 (18-19)	30/100 [#]	0

Figure 1

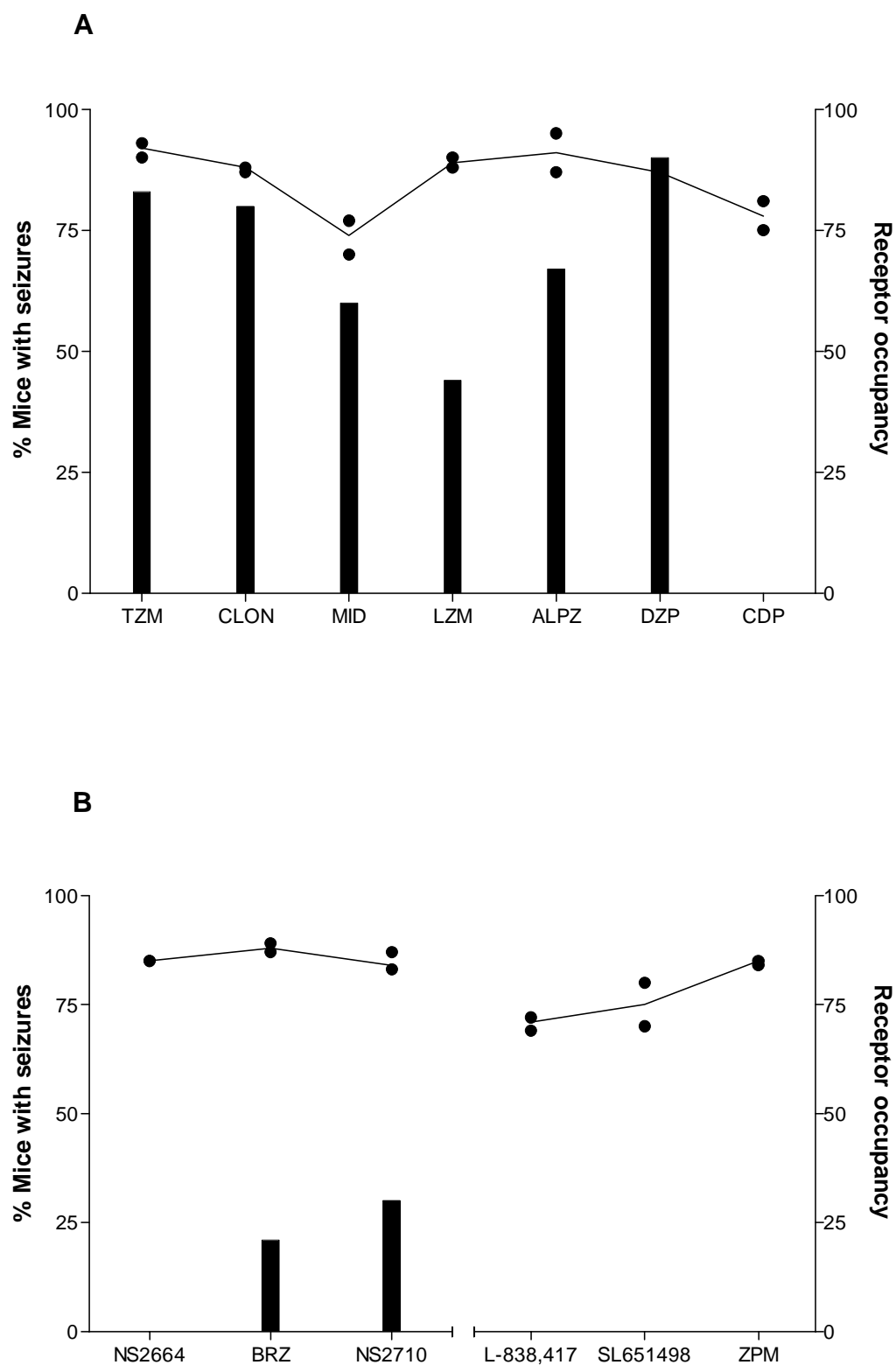


Figure 2

