RAT PHARMACOKINETICS AND PHARMACODYNAMICS OF A SUSTAINED RELEASE FORMULATION OF THE GABA_A α5-SELECTIVE COMPOUND L-655,708

John R. Atack, Andy Pike, Ashley Clarke, Susan M. Cook, Bindi Sohal, Ruth M. McKernan, and Gerard R. Dawson

Departments of in Vivo Neuroscience (J.R.A., S.M.C., R.M.M., G.R.D.) and Drug Metabolism and Pharmacokinetics (A.P., B.S.), Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, United Kingdom; and Devlab, Merck Sharp & Dohme Research Laboratories, Hoddesdon, Hertfordshire, United Kingdom (A.C.)

ABSTRACT:

The pharmacokinetic and pharmacodynamic (i.e., receptor occupancy) properties of L-655,708, a compound with selectivity for α5- over α1-, α2-, and α3-containing GABA_A receptors, were examined in rats with the aim of developing a formulation that would give sustained (up to 6 h) and selective occupancy of α5-containing GABA_A receptors suitable for behavioral studies. Standard rat pharmacokinetic analyses showed that L-655,708 has a relatively short half-life with kinetics in the brain mirroring those in the plasma. In vivo binding experiments showed that plasma concentrations of around 100 ng/ml gave relatively selective in vivo occupancy of rat brain α5- versus α1-, α2-, and α3-containing GABA_A receptors. Therefore, this plasma concentration was chosen as a target to achieve relatively selective occupancy of α5-containing receptors using s.c. implantations of L-655,708 (0.4, 1.5, or 2.0 mg) formulated into tablets of various size (20 or 60 mg) containing different amounts of L-655,708 and combinations of low and high viscosity hydroxypropyl methylcellulose (LV- and HV-HPMC). The optimum formulation, 1.5 mg of L-655,708 compressed into a 60-mg tablet with 100% HV-HPMC, resulted in relatively constant plasma concentrations being maintained for at least 6 h with very little difference between C_{max} concentrations (125–150 ng/ml) and plateau concentrations (100–125 ng/ml). In vivo binding experiments confirmed the selective occupancy of rat brain α5- over α1-, α2-, and α3-containing GABA_A receptors.

GABA is the major inhibitory neurotransmitter within the brain, and its actions are mediated primarily by the GABA_A receptor chloride channels and the G-protein-coupled metabotropic GABA_B receptors. GABA_A receptors have attracted particular attention because, in addition to their binding site for GABA, they have recognition sites for clinically relevant drugs such as benzodiazepines, barbiturates, neurosteroids, ethanol, and certain anesthetics (Sieghart, 1995).

GABA_A receptors are pentamers made up of subunits assembled from the 16 members (α1-6, β1-3, γ1-3, δ, ε, π, and θ) of this gene family (Barnard et al., 1998; Simon et al., 2004). The majority (ca. 75%) of GABA_A receptors in the brain contain a benzodiazepine binding site, and such receptors contain β and γ2 subunits along with an α1, α2, α3, or α5 subunit (McKernan and Whiting, 1996). The benzodiazepine binding site occurs at the interface of the α and γ2 subunits, with the α subunit being the major contributor to differences in the benzodiazepine pharmacology between the various GABA_A receptor subtypes (McKernan and Whiting, 1996).

Classical benzodiazepines, typified by diazepam, have a number of pharmacological actions, including anxiolytic, anticonvulsant, sedative, and cognition-impairing activities (Lüddens et al., 1995). Such compounds have equivalent affinity for the benzodiazepine binding site of GABA_A receptors containing an α1, α2, α3, or α5 subunit, and

the heterogeneity in behavioral effects is probably a consequence of modulation of these different receptors subtypes (Lüddens et al., 1995). As a corollary, particular behavioral properties may be associated with modulation of specific GABA_A receptor subtypes. In this regard, the use of α subunit-deleted or point-mutated mice has begun to delineate which GABA_A subtypes are associated with which pharmacological property of nonselective benzodiazepines such as diazepam (Rudolph et al., 1999; Löw et al., 2000; McKernan et al., 2000; Rudolph and Möhler, 2004).

A complimentary approach has been to use subtype-selective compounds to characterize the behavior consequences of affecting only certain GABA_A subtypes (McKernan et al., 2000; Atack et al., 2005b). In this regard, a number of compounds with higher affinity for the α5-containing compared with α1-, α2-, or α3-containing GABA_A receptors have been described. These include L-655,708 (Quirk et al., 1996), RY 80 (Skolnick et al., 1997), RY 023 (June et al., 2001), and RY 024 (Bailey et al., 2002). Clearly, such compounds should help further delineate the functions of the α5 subtype. However, because these compounds bind to the other subtypes, albeit with lower affinity, it is often not possible to unequivocally state that the observed in vivo effects are mediated via the α5 subtype or the much more abundant α1, α2, and/or α3 subtypes (Bailey et al., 2002). Thus, it is important to characterize the effects of such compounds at doses that selectively occupy the α5 subtype. For example, if plasma concentrations are too high, then there will be appreciable occupancy of α1-, α2-, and α3-containing, as well as α5-containing, GABA_A receptors, which would confound the interpretation of behavioral data. On the other

---

ABBREVIATIONS: HV-HPMC, high viscosity hydroxypropyl methylcellulose; HPLC, high-performance liquid chromatography; LV-HPMC, low viscosity hydroxypropyl methylcellulose.
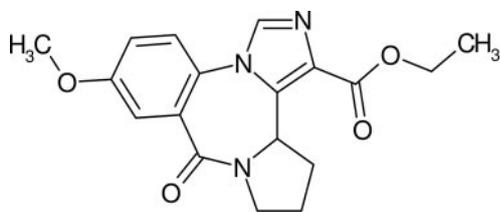
FIG. 1. Structure of L-655,708.

hand, if the plasma concentrations are too low, although occupancy of $\alpha 1$-, $\alpha 2$-, and $\alpha 3$-containing GABA$_A$ receptors would be minimal, occupancy of $\alpha 5$-containing GABA$_A$ receptors would also be relatively low and might not be sufficient to elicit a behavioral response.

In the present study, we examined the pharmacokinetics and GABA$_A$ receptor occupancy of L-655,708, a compound which because of its $\alpha 5$-binding selectivity and inverse agonist profile (Street et al., 2004) offers the potential to be an $\alpha 5$-selective cognition enhancer (Maubach, 2003). However, L-655,708 was found to have poor pharmacokinetic properties after bolus injections; therefore, we developed a slow-release formulation that would give relatively constant plasma concentrations, and thus preferential occupancy of $\alpha 5$- versus $\alpha 1$-, $\alpha 2$-, and $\alpha 3$-containing GABA$_A$ receptors, over a period appropriate for subsequent behavioral evaluation.

Materials and Methods

Bolus Dosing Pharmacokinetic Properties. After overnight food removal, six male Sprague-Dawley rats (approximate weight, 280 g) were anesthetized with isoflurane, and their tail arteries were cannulated. Animals were pretreated with heparin and allowed to recover for at least 30 min before dosing with L-655,708 (Fig. 1). The compound was administered i.v. as a solution (3 mg/ml) in water/1-methyl-2-pyrrolidinone/propylene glycol (56:22:22 v/v/v) and p.o. as a suspension (0.8 mg/ml) in aqueous methylcellulose [0.5% w/v high viscosity hydroxypropyl methylcellulose (HV-HPMC); Colorcon, Dartford, UK]. Blood samples (approximately 600 μl) were taken from each rat via the tail artery cannula at 0.017, 0.167, 0.5, 1, 2, 3, and 4 h after the i.v. dose and at 0.083, 0.25, 0.5, 1, 2, 3, and 4 h after the p.o. dose. After each sample an equivalent volume of heparinized saline was injected into the rat via the cannula. Plasma samples were frozen ($-20°C$) before being analyzed by high-performance liquid chromatography (HPLC) with UV detection as described below. Standard methods were used to estimate plasma pharmacokinetic parameters.

Receptor Occupancy. *Occupancy after bolus i.p. dosing of L-655,708.* L-655,708 was dosed to male Sprague-Dawley rats (0.3–3 mg/kg i.p., 0.5% HV-HPMC). Fifteen minutes later (a time point chosen based on the time course of occupancy in the mouse) (Atack et al., 2005a), animals were dosed i.v. with either [^{3}H]L-655,708 (synthesized in-house; also commercially available from American Radiolabeled Chemicals, St. Louis, MO) (Quirk et al., 1996) or [^{3}H]Ro 15-1788 (Perkin-Elmer Life Sciences, Boston, MA) to measure in vivo binding of either $\alpha 5$- or the combined $\alpha 1$-, $\alpha 2$-, plus $\alpha 3$-containing GABA$_A$ receptor populations, respectively, as described in more detail elsewhere (Atack et al., 1999, 2005a). Strictly speaking, [^{3}H]Ro 15-1788 also labels $\alpha 5$-containing receptors, but because these constitute a minority of brain GABA$_A$ receptors (McKernan and Whiting, 1996), the binding of [^{3}H]Ro 15-1788 in vivo is effectively measured binding to the combined population of $\alpha 1$-, $\alpha 2$-, and $\alpha 3$-containing receptors with a negligible contribution from $\alpha 5$-containing receptors. Briefly, rats were killed by decapitation either 1 min after [^{3}H]L-655,708 or 3 min after [^{3}H]Ro 15-1788 injections. Trunk blood was collected, and brains were then removed and homogenized in 10 volumes of phosphate buffer. Aliquots of homogenate (300 μl) were filtered and washed (10 ml of phosphate buffer) over Whatman GF/B glass fiber filters, which were then placed in scintillation vials. Scintillation fluid was added, and the filters were counted (membrane-bound radioactivity). For both [^{3}H]L-655,708 and [^{3}H]Ro 15-1788, nonspecific binding was defined in rats dosed with bretazenil, which at a dose of 5 mg/kg i.p. (in PEG 300 vehicle) occupies essentially all the benzodiazepine binding sites. Blood samples were centrifuged, and plasma was removed and analyzed for L-655,708 concentrations as described below.

Sustained Release Formulation. Initially, L-655,708 was administered s.c. in either an aqueous (0.5% HV-HPMC) or oil-based (sesame oil) 2.5-mg/ml suspension. Because neither of the s.c. formulations proved to give adequate, prolonged exposure, we next focused on developing a sustained release formulation, the most common method of which is to formulate compound into a tablet containing a hydrophobic polymer matrix, such as HPMC (Lordi, 1987). These tablets are usually administered p.o., but given the short gastrointestinal transit time in rats (approximately 90 min) (Davies and Morris, 1993), it was decided to administer the tablets s.c. Hence, L-655,708 was formulated as a solid in tablets (20 or 60 mg) containing various compositions or either low viscosity HPMC (LV-HPMC) (Pharmacoat 606, Shin-Etsu, Japan) and/or HV-HPMC (Colorcon). The rationale for tablet formulation was that when placed in an aqueous environment, water diffuses into the solid tablet and the HPMC forms a gelatinous layer on the tablet surface, followed by a progressive swelling and gelation of the tablet matrix. The rate of release of L-655,708 from the tablet is related to its rate of diffusion through the hydrated HPMC and the erosion of the hydrated polymer. Tablets were made by grinding the appropriate proportions of L-655,708 and HPMC in a pestle and mortar and then compressing the mixture with a hand press.

Sample Preparation and Analysis. *Plasma samples.* Aliquots of plasma were applied to C_2 solid-phase extraction cartridges preconditioned with 2 ml of methanol and 2 ml of water. Cartridges were washed with water, and samples were eluted with 1 ml of acetonitrile/0.1% acetic acid (90:10 v/v) + 0.4% triethylamine. Eluates were evaporated to dryness ($60°C$, N$_2$) and then dissolved in 300 μl of 25 mM triethylamine phosphate (pH 3.2)/acetonitrile (75:25 v/v). For calibration, 1-ml aliquots of control rat plasma were spiked to give standards within the range of 5 to 5000 ng/ml of L-655,708. These were extracted as for the samples above. Calibration curves obtained were linear ($r = 0.99$ or greater), with a limit of detection in the region of 5 ng/ml.

Brain samples. Two milliliters of acetonitrile was added to half of the brain tissue and then homogenized with a sonic probe, shaken, and centrifuged for 10 min. The supernatant was removed and evaporated to dryness ($60°C$, N$_2$), and the residue was dissolved in 300 μl of 25 mM triethylamine phosphate (pH 3.2)/acetonitrile (75:25 v/v) and filtered through a 0.2-μm liquid chromatography filter. For calibration purposes, control brain tissue was spiked to give standards equivalent to 5 to 500 ng/sample of L-655,708. These were extracted using the same methods as for the samples. The calibration curves were linear ($r > 0.99$) with a limit of detection of 5 ng/sample.

HPLC analysis. HPLC analysis was performed using a Gilson binary gradient system with 231/401 autosampler and UV detection. Samples were run on a Hichrom RPB 100 $\times$ 4.6 mm column at $45°C$ using a mobile phase of 25 mM triethylamine phosphate (pH 3.2)/acetonitrile (75:25 v/v) with a flow rate of 1.5 ml/min. The injection volume was 100 μl, and detection was performed at a wavelength of 250 nm using an ABI 759A UV detector. Signals were processed using a VG Multichrom system assuming a retention time for L-655,708 defined by the use of calibration standards (generally in the region of 5.0–6.5 min).

Results and Discussion

Bolus Dose Pharmacokinetic Parameters. The plasma concentrations of L-655,708 after bolus dosing L-655,708 i.v. or p.o. are illustrated in Fig. 2. The pharmacokinetic parameters calculated from the mean data are summarized in Table 1, which shows that despite the relatively low plasma clearance rate (19 ml/min/kg), L-655,708 had a very short half-life (0.5 h), presumably as a consequence of cleavage of the ester by blood esterases and the fact that it has a relatively low steady-state volume of distribution of 0.7 l/kg, which, for example, compares with a volume of distribution of 19.3 l/kg for diazepam in rats (Friedman et al., 1986). After p.o. dosing C_{max} was achieved rapidly (t = 0.25 h), after which plasma concentrations decreased rapidly. These data suggest that p.o. absorption is rapid but is not sustained. Because the animals used for this study were cannulated, brain samples were only available at completion of the study; therefore, in this experiment it was not possible to assess the pharmacokinetics of L-655,708 in rat brain.

Receptor Occupancy as a Function of L-655,708 Dose. When dosed i.p. as a bolus in an HV-HPMC suspension, L-655,708 gave greater occupancy of $\alpha 5$- versus $\alpha 1$-, $\alpha 2$-, and $\alpha 3$-containing GABA$_A$

A. i.v. kinetics
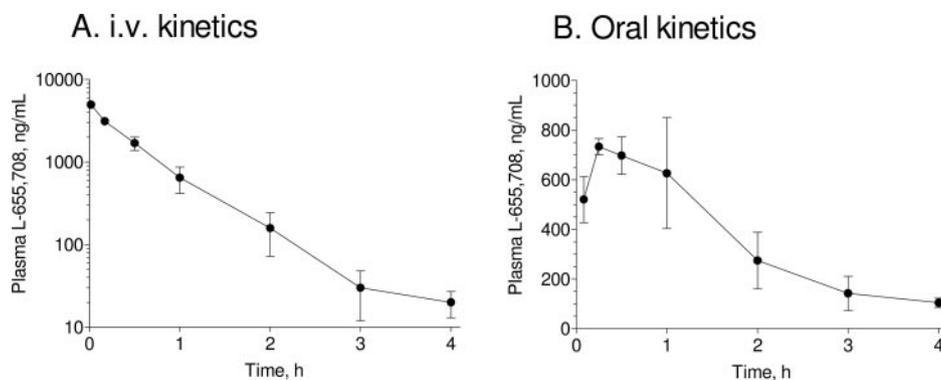
B. Oral kinetics

FIG. 2. Concentrations of L-655,708 in plasma obtained via tail artery cannulation after bolus injection of 3 mg/kg i.v. (in water/1-methyl-2-pyrrolidinone/propylene glycol, 56:22:22 v/v; $n = 3$) (A) and 4 mg/kg p.o. (in 0.5% HV-HPMC; $n = 3$) (B). Values shown are mean ± S.E.M. ($n = 3$/group).

TABLE 1

Plasma pharmacokinetic parameters of L-655,708 after dosing with 3 mg/kg i.v. and 4 mg/kg p.o. in rat

Parameter	Value
Plasma clearance	19 ml/min/kg
Plasma half-life (0.5–4 h)	0.5 h
Steady-state volume of distribution	0.7 l/kg
Oral bioavailability	45%
Oral C_{max}	733 ng/ml
Oral T_{max}	0.25 h

receptors (Fig. 3A). For example, at a dose of 1 mg/kg L-655,708 gave 64% occupancy of $\alpha5$ receptors versus 18% occupancy at $\alpha1$, $\alpha2$, and $\alpha3$ receptors, whereas at 3 mg/kg, occupancy of $\alpha5$ receptors was 75% and at $\alpha1$, $\alpha2$, and $\alpha3$ receptors it was 33%.

Trunk blood samples were collected from each animal; the plasma concentrations of L-655,708 were determined; and the relationship between plasma drug concentrations and occupancy is shown in Fig. 3B. The $\alpha5$ selectivity of L-655,708 observed in vitro was also reflected in rat brain in vivo as the occupancy at $\alpha5$-containing receptors was greater than at those containing $\alpha1$, $\alpha2$, and $\alpha3$ subunits. Hence, the plasma EC_{50} for $\alpha5$ occupancy (35 ng/ml) was much lower than that for $\alpha1$, $\alpha2$, and $\alpha3$ receptors (346 ng/ml). These data are consistent with observations in mice, which showed that the ID_{50} for [^{3}H]L-655,708 binding was much lower than that for [^{3}H]Ro 15-1788 binding to $\alpha1$ or $\alpha2/\alpha3$ receptors (respective ID_{50} values = 0.2, 1.1, and 4 mg/kg) (Atack et al., 2005a). Based on these data, it was estimated that a plasma drug concentration of 100 ng/ml should result in an inhibition of [^{3}H]L-655,708 and [^{3}H]Ro 15-1788 binding by around 75% and 20%, respectively, thereby producing relatively selective occupancy of $\alpha5$-containing receptors.

Compounds that bind with higher affinity to $\alpha5$- compared with $\alpha1$-, $\alpha2$-, and $\alpha3$-containing $GABA_A$ receptors, such as L-655,708 (Quirk et al., 1996), RY 80 (Skolnick et al., 1997), RY 023 (June et al., 2001), and RY 024 (Bailey et al., 2002), are valuable tools for characterizing the roles of the $GABA_A$ $\alpha5$ subtype. However, to ascribe particular functions of an $\alpha5$-selective compound to the $\alpha5$-containing $GABA_A$ receptors requires careful selection of the dose. Thus, although at relatively high doses there will be substantial occupancy of $\alpha5$-containing receptors, there will also be significant occupancy of the lower affinity $\alpha1$-, $\alpha2$-, and $\alpha3$-containing $GABA_A$ receptors. Moreover, although compared with the $\alpha5$ subtype the occupancy of $\alpha1$, $\alpha2$, and $\alpha3$ receptors will be less, the much greater abundance of these latter subtypes in the brain as a whole (McKernan and Whiting, 1996) will complicate the interpretation of behavioral responses mediated by the $\alpha5$ subtype. On the other hand, if a lower dose is used such that there is only modest occupancy of $\alpha5$-

containing $GABA_A$ receptors, this lower level of occupancy of the $\alpha5$ subtype may be insufficient to elicit a behavioral response. Consequently, to ascribe particular L-655,708-mediated effects to the $\alpha5$ subtype, it is necessary to select a dose of compound that gives preferential, sustained, and appreciable occupancy at $\alpha5$- compared with $\alpha1$-, $\alpha2$-, and $\alpha3$-containing $GABA_A$ receptors.

Brain and Plasma Concentrations of L-655,708 after s.c. Dosing. Although the plasma kinetics of L-655,708 after p.o. dosing are relatively rapid, it is possible that plasma concentrations may be more sustained if the compound is dosed s.c. as a suspension. Furthermore, L-655,708 may become sequestered within the brain such that brain concentrations do not reflect plasma kinetics (Jack et al., 1983). Therefore, the kinetics of brain and plasma L-655,708 were measured in rats dosed s.c. in either an aqueous (0.5% HV-HPMC) or oil-based (sesame oil) vehicle. Rats were killed at various times after dosing, and the brain and plasma profiles of these experiments are presented in Fig. 4. The plasma profile of L-655,708 is comparable whether compound is dosed (5 mg/kg s.c.) as an aqueous (0.5% HV-HPMC) or oil-based (sesame oil) suspension (Fig. 4, A and B). Moreover, the kinetics in either vehicle are rapid, with a C_{max} at around 0.5 h, after which the compound is rapidly cleared. Thus, s.c. dosing of L-655,708 in a lipophilic rather than aqueous suspension does not produce a prolonged absorption phase. Nevertheless, both these formulations do give a degree of sustained absorption because at lower doses, 1 and 0.6 mg/kg s.c. in 0.5% HV-HPMC, the kinetics of plasma L-655,708 were even more rapid, with C_{max} (around 325 and 250 ng/ml at 1 and 0.6 mg/kg, respectively) at 0.25 h and thereafter a rapid clearance of compound such that by 2 h postdosing, plasma concentrations were in the region of 25 to 20 ng/ml. Thus, compared with p.o. administration, bolus dosing in an aqueous suspension via the s.c. route did not appreciably prolong or flatten the pharmacokinetic profile of L-655,708.

For all the s.c. bolus doses the time course of brain concentrations of L-655,708 mirrored the corresponding plasma concentrations. Thus, there was no tendency for L-655,708 to be retained in the brain because compound is cleared from the plasma. The comparable kinetics of L-655,708 in brain and plasma are similar to those seen in rodents for other benzodiazepines, such as lorazepam, clonazepam, and diazepam and its metabolites desmethyldiazepam and oxazepam (Friedman et al., 1986; Miller et al., 1987; Barnhill et al., 1990; Greenblatt and Sethy, 1990). Similarly, in cats the kinetics of a variety of benzodiazepines (including diazepam, desmethyldiazepam, midazolam, lorazepam, alprazolam, triazolam, flunitrazepam, and clobazam) were similar in plasma and cerebrospinal fluid (Arendt et al., 1983). These data suggest that, like other benzodiazepines, L-655,708 does not become sequestered in the brain.

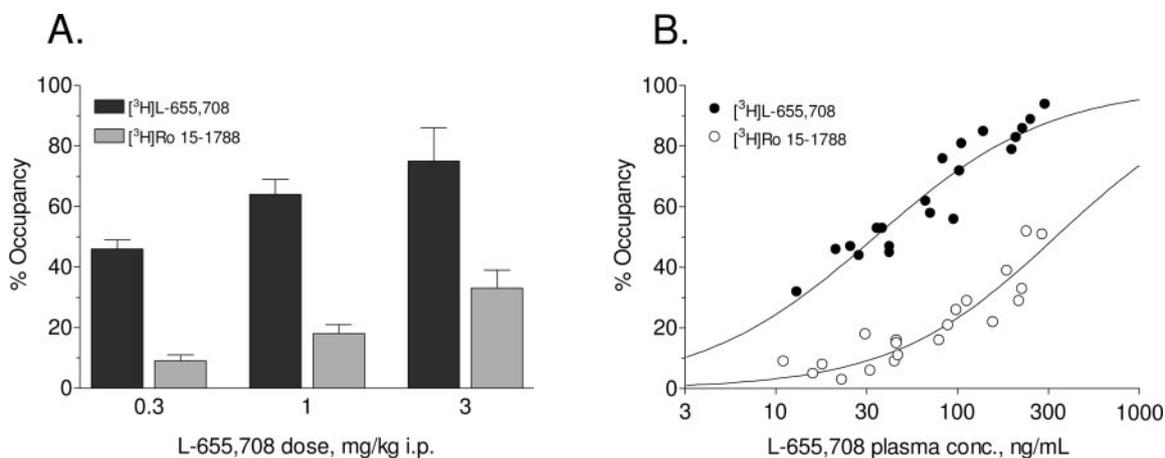However, the brain/plasma ratio for L-655,708, which was around

FIG. 3. Rat brain benzodiazepine binding site occupancy as a function of L-655,708 dose and plasma concentration. A, receptor occupancy of α5- and the combined population of α1-, α2-, plus α3-containing GABA$_A$ receptors was measured using in vivo binding of [^{3}H]L-655,708 and [^{3}H]Ro 15-1788, respectively, 15 min after administration of various doses of L-655,708 given i.p. in a 0.5% HV-HPMC suspension. Values shown are mean $\pm$ S.E.M. ($n = 7$/group). B, occupancy was plotted as a function of plasma concentration of L-655,708 for each animal. Respective EC$_{50}$ values for the inhibition of [^{3}H]L-655,708 and [^{3}H]Ro 15-1788 binding were 35 ng/ml and 346 ng/ml.
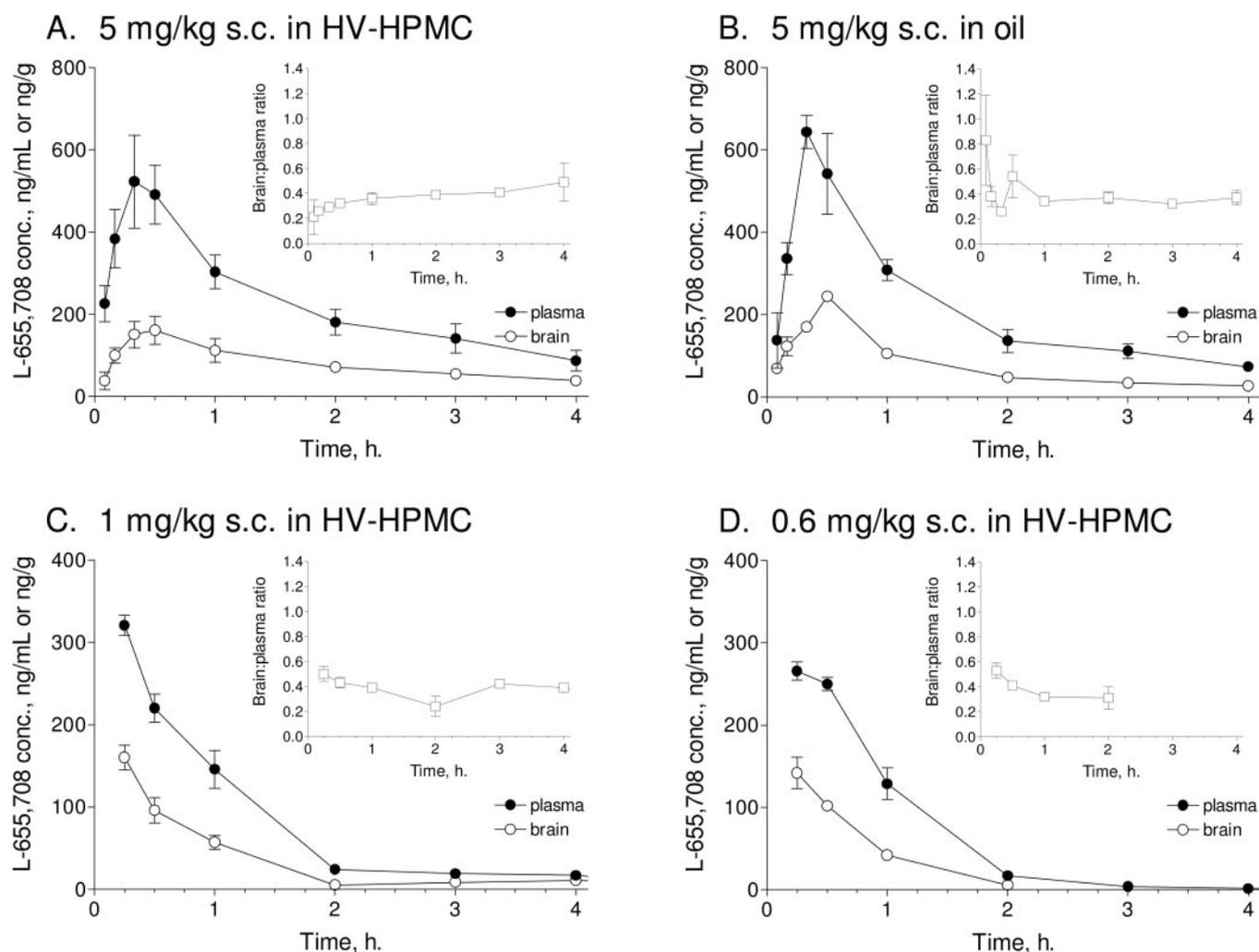

FIG. 4. Plasma concentration versus time profiles of L-655,708 dosed at 5 mg/kg s.c. in either HV-HPMC or sesame oil (A and B, respectively) or dosed at 1 or 0.6 mg/kg s.c. in HV-HPMC (C and D, respectively). Insets show the corresponding brain/plasma ratios. Values shown are mean $\pm$ S.E.M. ($n = 3$/time point).

0.4, is in marked contrast to other benzodiazepines, such as diazepam, desmethyldiazepam, clonazepam, lorazepam, midazolam, triazolam, and oxazepam, which have brain/plasma ratios in the region of 2 to 5 (Friedman et al., 1986; Arendt et al., 1987; Barnhill et al., 1990; Green-

blatt and Sethy, 1990). Moreover, although flunitrazepam and alprazolam differ from this group of benzodiazepines by virtue of their lower brain/plasma ratios (0.9–1.1) (Arendt et al., 1987), they nevertheless have brain/plasma ratios considerably greater than the value of 0.4 for
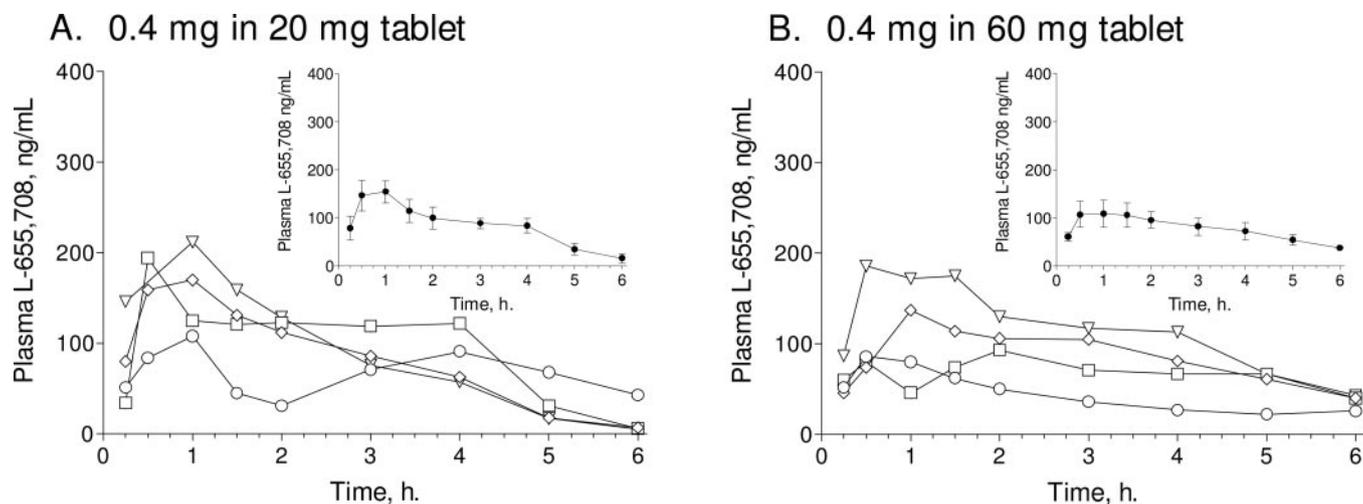
FIG. 5. Concentration versus time profiles of 0.4 mg of L-655,708 administered by s.c. implantation of L-655,708 contained within an LV-HPMC tablet. A, 0.4 mg of L-655,708 in a 20-mg LV-HPMC tablet. B, 0.4 mg of L-655,708 in a 60-mg LV-HPMC tablet. Values shown are plasma concentrations of each of three individual, cannulated rats, and the inset shows the mean ± S.E.M. of plasma concentrations for each tablet formulation ($n = 3$).

L-655,708. The physicochemical properties that render L-655,708 less brain penetrant than other benzodiazepines (e.g., log D for L-655,708 of 1.6 versus 2.8 for diazepam) (Worboys et al., 1997) may also contribute to the relatively low volume of distribution (0.7 l/kg) of this compound, although lipophilicity per se does not seem to be the major determinant of benzodiazepine brain/plasma ratios (Arendt et al., 1987).

Obviously, the plasma pharmacokinetics of L-655,708 are unsuitable to provide sustained and selective occupancy of $\alpha 5$-containing GABA$_A$ receptors (i.e., relatively constant plasma concentrations of around 100 ng/ml). Hence, although plasma concentrations of L-655,708 in the region of 100 ng/ml can be obtained 4 h after dosing with 5 mg/kg (ca. 80 ng/ml), the high peak concentrations required to achieve such sustained concentrations (around 600 ng/ml) would produce appreciable occupancy of $\alpha 1$-, $\alpha 2$-, and $\alpha 3$-containing GABA$_A$ receptors, as well as those containing an $\alpha 5$ subunit, thereby confounding the interpretation of behavioral results.

Pharmacokinetics of HPMC Tablets. The plasma versus time profiles for 0.4 mg of L-655,708 administered via s.c. implantation in an HPMC tablet are shown in Fig. 5. The equivalent dose of compound (0.4 mg) gave less intraindividual variability when dosed in a 60-mg rather than a 20-mg tablet. However, there remained considerable interindividual variability, especially at the earlier time points, and plasma concentrations in each animal decreased progressively from the time of C_{max} (t = 0.5–1 h), suggesting that a tablet containing 0.4 mg of compound was insufficient to produce a continual release from the tablet. In addition, the relatively early C_{max} suggests that compound leaves the tablet relatively rapidly such that plasma concentrations at 6 h were much lower than peak concentrations obtained at around 1 h. Therefore, if dissolution of the tablet is slower, such as by the inclusion of HV-HPMC, then the plasma profile of L-655,708 may be more prolonged. Furthermore, the plasma profile for the 60-mg tablet was flatter than the 20-mg tablet, presumably because of the greater HPMC/L-655,708 ratio of the former, resulting in slower drug release, and accordingly a 60-mg tablet size was used for subsequent formulations. In the next series of experiments, 60-mg tablets were made containing 2 mg of L-655,708 and varying ratios of LV- and HV-HPMC.

Pharmacokinetics of HPMC Tablets Containing HV-HPMC. Increasing the proportion of HV-HPMC resulted not only in a flattening of individual rat plasma L-655,708 versus time profiles but also greatly reduced interindividual variability. The effect of the HV-HPMC on tablet dissolution is reflected in the lower mean peak

plasma concentrations observed in tablets containing 100% compared with 5% HV-HPMC (peak mean plasma concentrations = ~150 and ~450 ng/ml, respectively).

Two milligrams of L-655,708 was administered to rats in s.c. tablets with varying ratios of LV-HPMC and HV-HPMC, and the corresponding plasma versus time curves for individual animals are shown in Fig. 6. The tablet containing the lowest proportion of HV-HPMC (5%) gave marked interindividual variability (Fig. 6A), especially at later time points (4–6 h). Moreover, there was a distinct peak in plasma concentrations around 1 to 2 h. However, increasing the proportion of HV-HPMC in the tablet to 20, 40, 60, 80, or 100% reduced both of these features, such that each of these formulations gave relatively reproducible plasma kinetic profiles between animals, and there was not a pronounced peak of L-655,708 concentrations at early time points. The sustained plasma concentrations achieved ranged from 250 to 300 ng/ml to 100 to 150 ng/ml for the 20% and 100% HV-HPMC tablets, respectively (Fig. 6, B and F), indicating that increasing the proportion of HV-HPMC results in reduced absolute plasma concentrations. With the 80% HV-HPMC tablet (Fig. 6E), there was some interindividual variability, with one of four of the rats having plasma concentrations of around one-half those of the other three. However, using the 100% HV-HPMC tablet, all four rats had very similar plasma L-655,708 pharmacokinetic profiles (Fig. 6F).

Optimum Tablet Formulation. As a final iteration, 1.5 mg of L-655,708 was compressed into a 60-mg tablet containing 100% HV-HPMC as excipient. The plasma L-655,708 versus time profiles for four rats implanted with this type of tablet are shown in Fig. 7. As with the HV-HPMC tablet containing 2 mg of L-655,708 (Fig. 6F), 1.5 mg of L-655,708 in a 60-mg HV-HPMC tablet gave very similar plasma pharmacokinetics in the different animals with little variation in the absolute plasma concentrations (Fig. 7) and minimal difference between peak concentrations at 1 h and concentrations at 6 h (~150 and ~100 ng/ml, respectively). Thus, mean plasma concentrations were around 125 ng/ml at 1 to 2 h and thereafter achieved relatively constant concentrations of around 100 ng/ml for the duration of plasma sampling (6 h).

In a separate group of rats implanted with these tablets, receptor occupancy was measured. As shown in Fig. 8, 1.5 mg of L-655,708 in a 60-mg HV-HPMC tablet gave relatively selective occupancy at $\alpha 5$ versus $\alpha 1$, $\alpha 2$, and $\alpha 3$ subunit-containing GABA$_A$ receptors. Thus, occupancy of $\alpha 5$-containing receptors measured using in vivo [^{3}H]L-
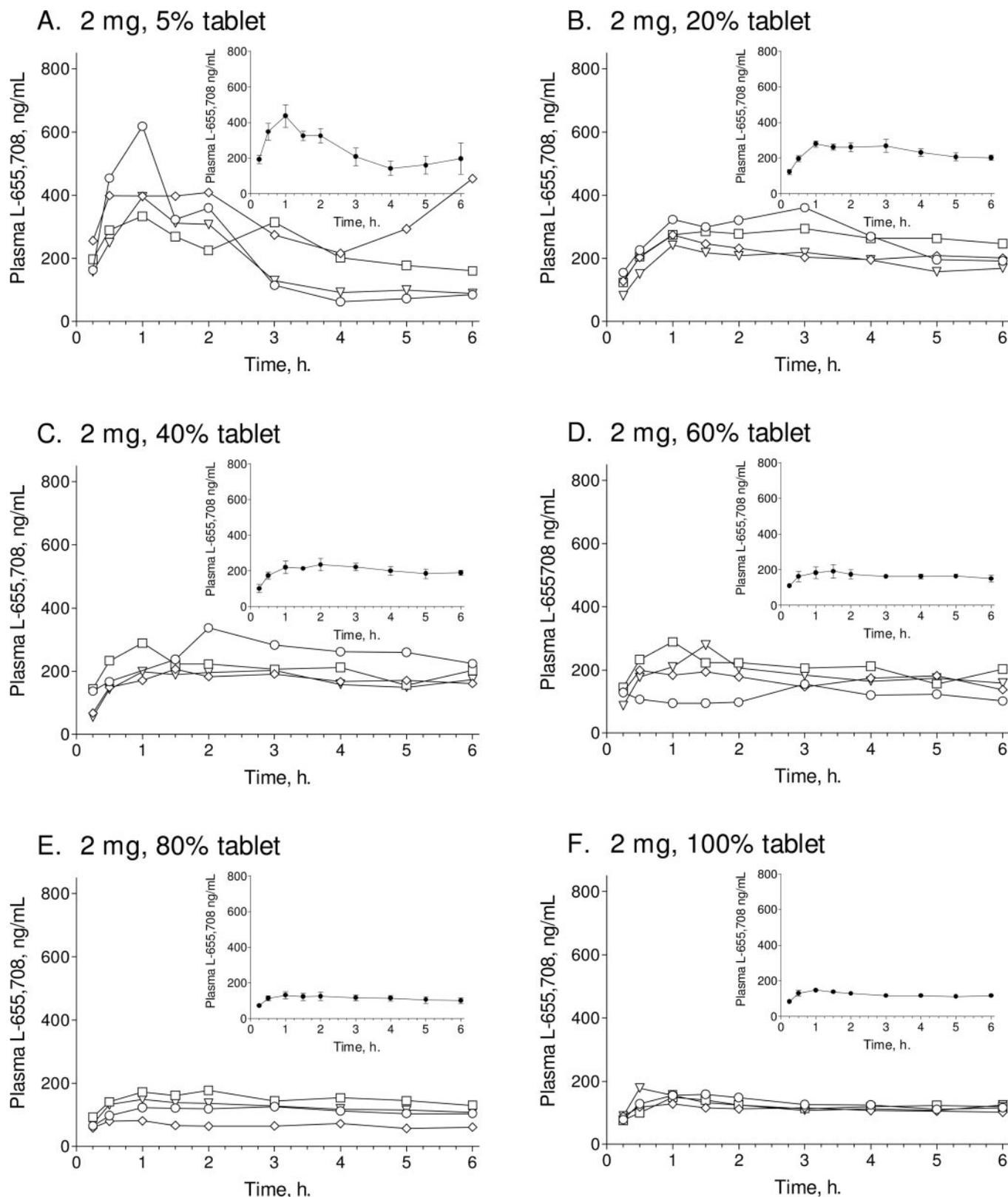
FIG. 6. Concentration versus time profiles of 2 mg of L-655,708 administered by s.c. implantation of 60-mg tablets containing 5% (A), 20% (B), 40% (C), 60% (D), 80% (E), and 100% (F) HV-HPMC. Values shown are plasma concentrations of each of four individual, cannulated rats, and the inset shows the mean ± S.E.M. of plasma concentrations for each tablet formulation ($n = 4$).

655,708 binding was 83%, whereas occupancy at $\alpha1$, $\alpha2$, and $\alpha3$ receptors was 18%.

Functions of $\alpha5$ Subtype Selective Compounds. The in vivo prop-

erties of a number of compounds that have higher affinity for the $\alpha5$ compared with $\alpha1$, $\alpha2$, and $\alpha3$ GABA$_A$ subtypes have been described (Liu et al., 1996; June et al., 2001; Bailey et al., 2002; Navarro et al.,
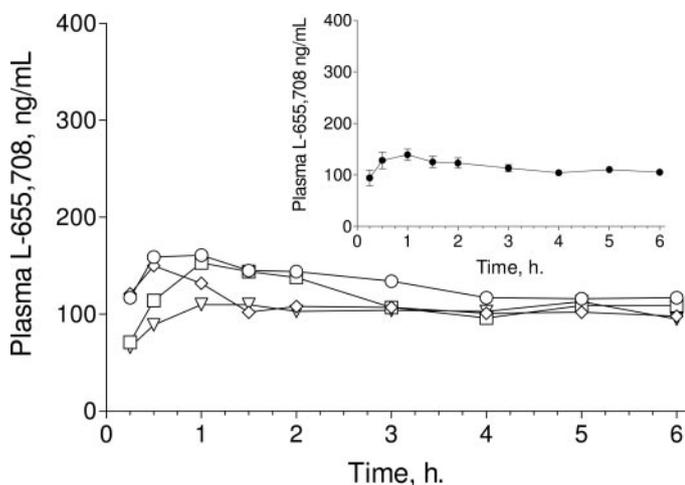
FIG. 7. Plasma concentration versus time profiles of L-655,708 administered by s.c. implantation in a 60-g tablet containing 1.5 mg of L-655,708 and 100% HV-HPMC. Values shown are plasma concentrations of each of four individual, cannulated rats, and the inset shows the mean ± S.E.M. of plasma concentrations ($n = 4$).
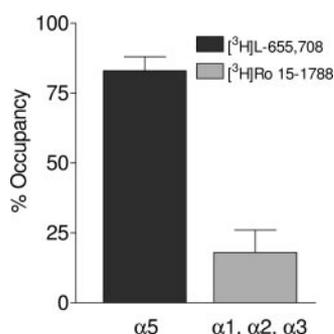

FIG. 8. Four hours after s.c. implantation of rats with 60-mg tablets containing 1.5 mg of L-655,708 and 100% HV-HPMC, occupancy of $\alpha 5$- and the combined population of $\alpha 1$-, $\alpha 2$-, and $\alpha 3$-containing GABA$_A$ receptors was measured using in vivo binding of [^{3}H]L-655,708 and [^{3}H]Ro 15-1788, respectively. Values shown are mean ± S.E.M. ($n = 4$–5).

2002). For example, such compounds have been described as being convulsant and producing anxiogenic and fear-like behaviors (Liu et al., 1996; Bailey et al., 2002, Navarro et al., 2002). However, none of these studies have measured levels of receptor occupancy; therefore, it remains unknown to what extent these effects may be mediated via the $\alpha 5$ subtype (Bailey et al., 2002). Clearly, this issue is of considerable importance in defining the suitability of this GABA$_A$ receptor subtype as a potential target for novel therapeutics (Maubach, 2003). In the present studies, we have achieved a formulation of L-655,708 that gives sustained and constant plasma concentrations, which gives high and relatively selective occupancy of the $\alpha 5$ subtype (83% occupancy) with minimal occupancy at the $\alpha 1$, $\alpha 2$, and $\alpha 3$ subtypes (18%). In future studies, this formulation may be used to characterize the pharmacological properties of L-655,708 and thereby help define the behavioral consequences of selective modulation of the GABA$_A$ receptor $\alpha 5$ subtype.

References

Arendt RM, Greenblatt DJ, DeJong RH, Bonin JD, Abernethy DR, Ehrenberg BL, Giles HG, Sellers EM, and Shader RI (1983) In vitro correlates of benzodiazepine cerebrospinal fluid uptake, pharmacodynamic action and peripheral distribution. *J Pharmacol Exp Ther* 227:98–106.

Arendt RM, Greenblatt DJ, Liebisch DC, Luu MD, and Paul SM (1987) Determinants of benzodiazepine brain uptake: lipophilicity versus binding affinity. *Psychopharmacology* 93:72–76.

Atack JR, Smith AJ, Emms F, and McKernan RM (1999) Regional differences in the inhibition of mouse in vivo [^{3}H]Ro 15-1788 binding reflect selectivity for $\alpha 1$ versus $\alpha 2$ and $\alpha 3$ subunit-containing GABA$_A$ receptors. *Neuropsychopharmacology* 20:255–262.

Atack JR, Alder L, Cook SM, Smith AJ, and McKernan RM (2005a) In vivo labelling of $\alpha 5$ subunit-containing GABA$_A$ receptors using the selective radioligand [^{3}H]L-655,708. *Neuropsychopharmacology* 49:220–229.

Atack JR, Hutson PH, Collinson N, Marshall G, Bentley G, Moyes C, Cook SM, Collins I, Wafford K, McKernan RM, et al. (2005b) Anxiogenic properties of an inverse agonist selective for $\alpha 3$ subunit-containing GABA$_A$ receptors. *Br J Pharmacol* 144:357–366.

Bailey DJ, Tetzlaff JE, Cook JM, He X, and Helmstetter FJ (2002) Effects of hippocampal injections of a novel ligand selective for the $\alpha 5\beta 2\gamma 2$ subunits of the GABA/benzodiazepine receptor on Pavlovian conditioning. *Neurobiol Learn Mem* 78:1–10.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, and Langer SZ (1998) International Union of Pharmacology. XV. Subtypes of γ-aminobutyric acid$_A$ receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* 50:291–313.

Barnhill JG, Greenblatt DJ, Miller LG, Gaver A, Harmatz JS, and Shader RI (1990) Kinetic and dynamic components of increased benzodiazepine sensitivity in aging animals. *J Pharmacol Exp Ther* 253:1153–1161.

Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. *Pharm Res (NY)* 10:1093–1095.

Friedman H, Abernethy DR, Greenblatt DJ, and Shader RI (1986) The pharmacokinetics of diazepam and desmethyldiazepam in rat brain and plasma. *Psychopharmacology* 88:267–270.

Greenblatt DJ and Sethy VH (1990) Benzodiazepine concentrations in brain directly reflect receptor occupancy: studies with diazepam, lorazepam and oxazepam. *Psychopharmacology* 102:373–378.

Jack ML, Coburn WA, Spirt NM, Bautz G, Zanko M, Horst WD, and O'Brien RA (1983) A pharmacokinetic/pharmacodynamic/receptor binding model to predict the onset and duration of pharmacological activity of the benzodiazepines. *Prog Neuropsychopharmacol Biol Psychiatry* 7:629–635.

June HL, Harvey SC, Foster KL, McKay PF, Cummings R, Garcia M, Mason D, Grey C, McCane S, Williams LS, et al. (2001) GABA$_A$ receptors containing $\alpha 5$ subunits in the CA1 and CA3 hippocampal fields regulate ethanol-motivated behaviors: an extended ethanol reward circuitry. *J Neurosci* 21:2166–2177.

Liu R, Hu RJ, Zhang P, Skolnick P, and Cook JM (1996) Synthesis and pharmacological properties of novel 8-substituted imidazobenzodiazepines: high-affinity, selective probes for $\alpha 5$-containing GABA$_A$ receptors. *J Med Chem* 39:1928–1934.

Lordi GN (1987) Sustained release dosage forms, in *The Theory and Practice of Industrial Pharmacy* (Lachman L, Liberman HA, Kanig JL, eds) pp 430–456, Varghese Publishing House, Mumbai, India.

Löw K, Crestani F, Keist R, Benke D, Brünig I, Benson JA, Fritschy J-M, Rülicke T, Bluethmann H, Möhler H, et al. (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. *Science (Wash DC)* 290:131–134.

Lüddens H, Korpi ER, and Seeburg PH (1995) GABA$_A$/benzodiazepine receptor heterogeneity: neurophysiological implications. *Neuropharmacology* 34:245–254.

Maubach K (2003) GABA$_A$ receptor subtype selective cognition enhancers. *Curr Drug Targets CNS Neurol Disord* 2:233–239.

McKernan RM and Whiting PJ (1996) Which GABA$_A$-receptor subtypes really occur in the brain? *Trends Neurosci* 19:139–143.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, et al. (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA$_A$ receptor $\alpha 1$ subtype. *Nat Neurosci* 3:587–592.

Miller LG, Greenblatt DJ, Paul SM, and Shader RI (1987) Benzodiazepine receptor occupancy in vivo: correlation with brain concentrations and pharmacodynamic actions. *J Pharmacol Exp Ther* 240:516–522.

Navarro JF, Buron E, and Martin-Lopez M (2002) Anxiogenic-like activity of L-655,708, a selective ligand for the benzodiazepine site of GABA$_A$ receptors which contain the alpha-5 subunit, in the elevated plus-maze test. *Prog Neuropsychopharmacol Biol Psychiatry* 26:1389–1392.

Quirk K, Blurton P, Fletcher S, Leeson P, Tang F, Mellilo D, Ragan CI, and McKernan RM (1996) [^{3}H]L-655,708, a novel ligand selective for the benzodiazepine site of GABA$_A$ receptors which contain the $\alpha 5$-subunit. *Neuropharmacology* 35:1331–1335.

Rudolph U, Crestani F, Benke D, Brünig I, Benson JA, Fritschy J-M, Martin JR, Bluethmann H, and Möhler H (1999) Benzodiazepine actions mediated by specific γ-aminobutyric acidA receptor subtypes. *Nature (Lond)* 401:796–800.

Rudolph U and Möhler H (2004) Analysis of GABA$_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. *Annu Rev Pharmacol Toxicol* 44:475–498.

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

Simon J, Wakimoto H, Fujita N, Lalande M, and Barnard EA (2004) Analysis of the set of GABA$_A$ receptor genes in the human genome. *J Biol Chem* 279:41422–41435.

Skolnick P, Hu RJ, Cook CM, Hurt SD, Trometer JD, Liu R, Huang Q, and Cook JM (1997) [^{3}H]RY 80: a high-affinity, selective ligand for γ-aminobutyric acid$_A$ receptors containing alpha-5 subunits. *J Pharmacol Exp Ther* 283:488–493.

Street LJ, Sternfeld F, Jelley RA, Reeve AJ, Carling RW, Moore KW, McKernan RM, Sohal B, Cook S, Pike A, et al. (2004) Synthesis and biological evaluation of 3-heterocyclyl-7,8,9,10-tetrahydro-(7,10-ethano)-1,2,4-triazolo[3,4-a]phthalazines and analogues as subtype-selective inverse agonists for the GABA$_A\alpha 5$ benzodiazepine binding site. *J Med Chem* 47:3642–3657.

Worboys PD, Bradbury A, and Houston JB (1997) Kinetics of drug metabolism in rat liver slices. III. Relationship between metabolic clearance and slice uptake rate. *Drug Metab Dispos* 25:460–467.

Address correspondence to: John R. Atack, Neuroscience Research Centre, Merck Sharp & Dohme Research Laboratories, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, England. E-mail: john_r_atack@yahoo.co.uk