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Changes of benzodiazepine receptors during chronic benzodiazepine administration in humans

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Abstract

Changes of central type $GABA_A$/benzodiazepine receptors during 24-day per-oral administration of alprazolam (2 mg/day) were measured with single photon emission computed tomography (SPECT) in nine healthy human subjects. Receptor densities were measured on days −4 (baseline), 3, 10, 17 and 24. Comparison of baseline and day 3 SPECT images was used to assess receptor occupancy; comparisons of the four scans on medication were used to assess alterations in receptor levels. Clinical effects were evaluated by subjective ratings of mood and the Hopkins verbal learning test. Alprazolam induced sedation associated with a 16% receptor occupancy. Unoccupied receptor levels decreased 10% from day 3 to day 10 but then normalized to baseline values by day 17. Clinical effects showed corresponding changes 1–2 weeks after the changes in the receptor. Thus, the decrease of benzodiazepine receptor densities may be one of the major mechanisms for tolerance development in humans.

Keywords: Benzodiazepine; $GABA_A$/benzodiazepine receptor; (Human); Down regulation; SPECT (single photon emission computed tomography)

1. Introduction

Benzodiazepines have been widely used as anxiolytics, hypnotics, sedatives and anticonvulsants. Since a large part of these drugs is consumed by long-term users suffering from chronic illnesses such as anxiety and panic disorders (Shader and Greenblatt, 1993; Michelini et al., 1996), problems in long-term usages, (e.g., tolerance and dependence) must be considered (Lucki et al., 1986; Shader and Greenblatt, 1993; Michelini et al., 1996). Development of tolerance and dependence can be due to changes in peripheral drug metabolism (pharmacokinetic changes) or functional changes within the central nervous system (pharmacodynamic changes). Studies in both animals (Miller et al., 1988a; Smith and Darlington, 1994) and humans (Greenblatt and Shader, 1986) showed that the tolerance developed by benzodiazepines is mainly due to pharmacodynamic changes because the development of tolerance was not correlated with the decrease of drug levels in plasma and brain.

To assess mechanisms for the pharmacodynamic changes, central type $GABA_A$/benzodiazepine receptors have been studied in animals and cell lines during chronic administration of benzodiazepine receptor agonists. Proposed mechanisms are: (1) desensitization, (2) sequestration, (3) decrease in the functional coupling between the GABA and benzodiazepine binding sites, (4) decrease in receptor densities on synaptic membrane and (5) post-translational regulation (Barnes, 1996; Hutchinson et al., 1996).

Among these mechanisms, correlations between receptor densities on synaptic membrane and the development of tolerance have been most widely studied using selective radiolabeled ligands. Several studies showed decrease in receptor densities and the concurrent development of tolerance (Rosenberg and Chiu, 1981; Miller et al., 1988a, 1989; Byrnes et al., 1993). However, many studies were performed without behavioral measurements. Some of these studies with only radioligand binding have shown de-

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creased receptor levels (Galpern et al., 1990; Byrnes et al., 1993) while others did not (Gallager et al., 1984; Mele et al., 1984; Brett and Pratt, 1992; Ramsey-Williams et al., 1994). Various benzodiazepines were used in these studies. In most reports which did not show decrease of receptor densities, diazepam was used. In contrast, studies using alprazolam showed decrease in receptor densities associated with the development of tolerance (Miller et al., 1989; Galpern et al., 1990; Byrnes et al., 1993).

More recently, mRNA levels in several subunits of the $GABA_A$ receptor have been reported to decrease during chronic benzodiazepine administration (Heninger et al., 1990; Kang and Miller, 1991; O'Donovan et al., 1992; Impagnatiello et al., 1996; Longone et al., 1996), which may explain the decrease in the binding of radiolabeled ligands. In addition, some studies have reported that chronic agonist treatment concurrently caused a decrease of some subunits and an increase in others (O'Donovan et al., 1992; Impagnatiello et al., 1996; Longone et al., 1996). Altering the subunit composition of the multimeric $GABA_A$ receptor may change the affinity of radioligands or decrease functional coupling between the GABA and benzodiazepine binding sites.

All studies referred above were done in cell lines expressing the receptors or in animals. Although the former is appropriate to study changes in protein levels, studies in animals or humans are necessary to correlate behavioral tolerance with neuronal changes. Furthermore, the generalization of results in animals to humans has several limitations. Most animal research has used rodents, which metabolize benzodiazepines very rapidly compared to humans (Klotz et al., 1976; Greenblatt et al., 1980; Friedman et al., 1986). To maintain drug levels in rodents, silastic implants have been used which release drug continuously over extended periods. This continuous administration prevents the fluctuations in plasma and brain benzodiazepine concentrations that occur in humans with intermittent oral administration. However, to our knowledge, no study has measured sequential changes in benzodiazepine receptor densities during chronic administration of benzodiazepines in humans.

In this study, single photon emission computed tomography (SPECT) using [^{123}I]iomazenil was performed five times on nine healthy human subjects, treated with per-oral administration of a common clinical dose of alprazolam (2 mg/day) for 24 days. The studies were done on days -4 (baseline; 4 days before the initiation of alprazolam treatment), 3, 10, 17 and 24. From day 3, plasma alprazolam levels were supposed to be the same at the time of SPECT scans in individual subjects. Because chronic administration of alprazolam did not change the affinity of benzodiazepine receptor (Miller et al., 1988a, 1989), changes in the binding of iomazenil from day 3 were presumed to be caused by the changes in receptor densities.

Among the proposed mechanisms shown above, changes in receptor densities were studied. However, binding to the receptors on synaptic membrane and in vesicles (i.e., sequestrated) cannot be differentiated with in vivo SPECT imaging. Iomazenil has adequate lipophilicity to easily cross membranes, as indicated by its substantial passage of the blood brain barrier with almost 10% of injected dose reaching the brain (Verhoeff et al., 1993; Dey et al., 1994). No published data are available on the affinity of iomazenil for various combinations of $GABA_A$ receptor subunits. However, analogy can be made to the in vitro radioligand [^{3}H]flumazenil. Flumazenil and iomazenil are halogen-substituted analogs, with fluorine in flumazenil and iodine in iomazenil. Flumazenil shows high affinity for several common forms of the $GABA_A$ receptor (Besnard et al., 1997). Therefore, changes of iomazenil binding do probably not indicate changes in the levels of particular subunits. Because some of the proposed mechanisms other than the decrease of receptors have also been sometimes called 'down regulation', decreases detected in iomazenil SPECT after day 3 will be described in this paper as decrease of receptor densities but not the more general term 'down regulation'.

In SPECT studies, the constant infusion/sustained equilibrium paradigm was applied to obtain equilibrium volumes of distribution ($= B_{max}/K_D$ in specific binding compartment) (Abi-Dargham et al., 1994; Laruelle et al., 1994). To study reproducibility of the SPECT measurements, a test/retest study was performed with a separate group of healthy subjects.

2. Materials and methods

2.1. Human subjects

Nine healthy subjects (3 males and 6 females; 27–46 (mean: 34) years of age) completed the study with alprazolam administration; and seven healthy subjects (5 males and 2 females; 25–59 (mean: 40) years of age) participated in the test/retest reproducibility study. Inclusion criteria were (a) the absence of any current medical condition and (b) the absence of present or past neuropsychiatric illnesses or substance abuse, based on history, physical examination, routine blood and urine tests, and electrocardiogram. Current usage of benzodiazepines, cannabinoids, cocaine, methadone, methaqualone, opiates, phencyclidine, propoxyphene and amphetamine was screened in urine samples and was an exclusion criterion. All subjects gave written informed consent. The studies were approved by the local human investigation and radioactive drug research committees. Subjects received 0.6 g potassium iodide (SSKI solution) 30–60 min prior to the administration of [^{123}I]iomazenil. Female subjects of child-bearing potential had negative urine pregnancy tests performed prior to tracer injection.

2.2. Alprazolam administration and clinical monitoring

The study with alprazolam administration lasted for 28 days. Each subject had SPECT scans five times at 1 week interval. Starting between 0700 and 0900 h on the fourth day after the baseline scan, 0.5 mg alprazolam was administered per-oral every 6 h (2 mg/day) for 24 days. SPECT studies were done on days −4 (baseline), 3, 10, 17 and 24 of alprazolam treatment. A timer was installed in a drug container so that subjects could know the time to take a dose. Compliance with the dose schedule was checked by a recorder installed in a drug container which noted each time the container was opened. From animal studies (Miller et al., 1989), we assumed that alprazolam did not change the affinity of benzodiazepine receptor for alprazolam or iomazenil. Therefore, the receptor occupancy by alprazolam was kept constant from the second to the fifth SPECT scans if plasma alprazolam levels were constant at the time of image acquisition. To satisfy this condition, the second dose of alprazolam on the day of SPECT studies and the start of iomazenil infusion were scheduled at the same time. Since alprazolam has an intermediate plasma elimination half-life of approximately 9–16 h (Woods and Charney, 1988; Friedman et al., 1991; Scavone et al., 1992; Greenblatt and Wright, 1993), steady state plasma alprazolam levels (more than 97% of the steady state levels) were expected by the time of the SPECT scan on day 3, which is approximately 5–9 plasma half lives. To schedule the second dose of alprazolam and the start of iomazenil infusion at the same time, on the days of SPECT scans, subjects had only three doses of alprazolam. To make sure that plasma alprazolam levels were stable throughout the five SPECT studies, 10 ml venous samples were obtained at 5 h 45 min after the first and the second doses of alprazolam on the days of SPECT studies. After the completion of 24 days alprazolam administration, doses were reduced gradually by the following schedule to avoid adverse reactions caused by an abrupt discontinuation; three times of 0.5 mg a day (1.5 mg/day) for 3 days, twice of 0.5 mg a day (1 mg/day) for 3 days and one 0.5 mg dose/day for 2 days.

Clinical effects of alprazolam were evaluated in 8 among 9 subjects in two ways; subjective rating for sedative effects by visual analog scales and tests for attention and memory. The questionnaire of visual analog scales contained the following 28 items which were arbitrarily selected: active, anxious, bushed, (often) close eyes, (difficulty to keep) concentration, conversation, drowsy, drunk, efficient, energetic, exhausted, (must keep) eyes open, drugged, fatigued, focusing, foggy, high, lie down, lively, (subjective rating for the impairment of) memory, moving, nervous, sedated, sleepy, tired, uncoordinated, vigorous, and worn out. Sedative effects were recorded on days −4, 0 (1 h after the first dose of alprazolam), 1, 2, 3, 10, 17 and 24. Except the baseline, ratings were recorded 1 h after the first dose of the day. Effects of alprazolam on attention and memory were examined by the Hopkins verbal learning test (Brandt, 1991) on days −4, 3, 10, 17 and 24, at 1 h after the first dose of the day.

2.3. Radiolabeling

Sodium [^{123}I]iodide (no carrier added in 0.1 M NaOH) was purchased from Nordion International, Vancouver, BC, Canada. [^{123}I]iomazenil was prepared by iododestannylation of its tributylstannyl precursor as previously described (Zoghbi et al., 1992; Zea-Ponce et al., 1993) in average yield of 64.6 ± 2.3% (with these and subsequent values expressed as mean ± S.E.M.) and radiochemical purity 97.3 ± 0.3%. Sterility was confirmed by lack of growth in two media, fluid thioglycollate at 35°C and soybean-casein digest at 25°C for 2 weeks (USP XXIII, 1995). Apyrogenicity was confirmed by the limulus amebocyte lysate test (Endosafe, Charleston, NC, USA).

2.4. Data acquisition

Subjects in alprazolam and test/retest studies received a priming bolus of [^{123}I]iomazenil (71 ± 0.7 MBq), followed by a continuous infusion at a constant rate (18.5 ± 0.2 MBq/h) using a computer-controlled pump (IMED pump, Gemini PC-1, San Diego, CA, USA). To study larger number of subjects on the same day, shorter duration of infusion and data acquisition were applied in the studies with alprazolam administration. Duration of infusion was 6 h in alprazolam and 6 h 40 min in test/retest study. Twenty-four minutes (alprazolam study) or 36 min (test/retest) SPECT imaging starting at 5 h 30 min (alprazolam) or 6 h (test/retest) of the infusion was acquired with a three headed camera with low energy high resolution fan-beam collimators (PRISM 3000XP, Picker, Cleveland, OH, USA), with a transaxial and axial resolution of 12.2 mm full width half-maximum measured with ^{123}I line sources and water in a cylindrical phantom with 20 cm diameter. Our previous study showed that at 5 h 30 min an equilibrium was achieved (Abi-Dargham et al., 1994). Three 10 ml venous samples were collected in the middle of the scanning session. The subjects in the test/retest study had two SPECT studies with interval of 72 ± 16 days.

To identify brain regions and spatially transform SPECT images into a standard stereotactic space (Talairach and Tournoux, 1988), magnetic resonance images of 3 mm contiguous slices were obtained with a 1.5 Tesla GE Signa device. Axial images were acquired with a spoiled GRASS (gradient recall acquisition in the steady state) sequence with TR = 25 ms, TE = 5 ms, NEX = 1, matrix = 256 × 256, field of view = 24 cm.

2.5. Image analysis

SPECT projection data were filtered with a two-dimensional Butterworth filter (order = 10, cutoff frequency = 0.24 cycles/pixel) and then transversely reconstructed with

a ramp backprojection filter on a 128×128 matrix. To minimize errors caused by inconsistency in the placement of ellipses for uniform attenuation correction, images of five (alprazolam study) or two (test/retest) SPECT studies of each subject were coregistered first and then uniform attenuation correction was performed with a single set of slice ellipses. Reconstructed SPECT images of a single subject were coregistered to each other using the 'realign' function in SPM96 (Statistical Parametric Mapping version 96) (Friston et al., 1995) using the SPECT image of the first study as the standard. An average image of coregistered SPECT was created in SPM96 and the subject's magnetic resonance images were resliced and coregistered to this average SPECT image using 'coregister' function in SPM96 (Friston et al., 1995). Attenuation correction was performed by assuming uniform attenuation equal to that of water ($\mu = 0.12$ cm^{-1}, determined from an ^{123}I-containing distributed source cylindrical phantom with 12 cm diameter) within an ellipse drawn around the skull of the coregistered magnetic resonance images.

Volumes of interest were placed on the frontal, parietal, temporal and occipital cortices and the cerebellum on the coregistered magnetic resonance image of each subject according to the anatomical criteria developed and validated by our group (Bremner et al., in press). Activities from right and left sides were averaged in these volumes of interest. For each volume of interest, data were obtained from three slices with 3.56 mm thickness each. Size of each volume of interest was, frontal cortex: 8.6 ± 0.5, parietal cortex: 22.3 ± 0.7, temporal cortex: 10.8 ± 0.6, occipital cortex: 10.7 ± 0.6 and the cerebellum: 17.7 ± 0.9 cm^3. In addition to the data obtained from the volumes of interest in individual cortical areas and the cerebellum, average activities in the whole brain areas were obtained in SPM96 after spatial normalization as described below. The voxels with activities less than 12.5% of the average in the whole image were assumed to be outside of brain and discarded. Then the average of the whole brain was calculated from remaining voxels.

Average regional activities (cpm/g) in each volume of interest and the whole brain were decay corrected for the time of the start of injection and expressed as Bq/g using a calibration factor of 45.4 Bq/cpm. This factor was calculated from 6 experiments using a 12 cm diameter cylindrical phantom containing uniformly distributed ^{123}I.

2.6. Iomazenil plasma analysis

Plasma samples were analyzed as previously described to measure iomazenil levels (Zoghbi et al., 1992). A 200 μl aliquot of plasma was counted to measure total plasma radioactivity. Extraction (ethyl acetate) was followed by reverse phase high performance liquid chromatography (HPLC) to measure the metabolite-corrected parent radioactivity (free plus protein bound). Plasma protein binding was measured by ultrafiltration through Centrifree membrane filters (Amicon Division, W.R. Grace, Danvers, MA, USA) (Gandelman et al., 1994). One aliquot from a pooled sample of plasma obtained from different healthy volunteers was processed with each experiment as a standard to control for day to day variability in the free fraction assay. The plasma free fraction (f_1) measured in the subject sample was corrected for interassay variability using the f_1 measured in the standard ($f_{1,\mathrm{std}}$) and the average of the standard measurement over the course of test/retest study ($f_{1,\mathrm{ave}}$, 40.8 ± 0.3) according to $[(f_1 f_{1,\mathrm{ave}})/f_{1,\mathrm{std}}]$. The total parent compound plasma concentration was multiplied by f_1 to yield the free plasma [^{123}I]iomazenil concentration.

2.7. Outcome measures

As summarized in Table 1, three distribution volume measures were evaluated in the test/retest study: V_T, V'_T, and $V_{\mathrm{T\text{-}p}}$ (Table 1). All three values are the equilibrium ratios of activities in brain to those in plasma. The measurements differ in the plasma measurement used in the denominator: V_T (free parent); V'_T (total parent); and $V_{\mathrm{T\text{-}p}}$ (total plasma activity—i.e., parent plus radiolabeled metabolites).

The distribution volume in the nondisplaceable compartment (V_2) in brain is only 10–15% of the total volume of distribution (V_T) ($V_\mathrm{T} = V_2 + V_3$; V_3: distribution volume in the specific binding compartment) (Abi-Dargham et al., 1994; Laruelle et al., 1994). The three outcome measures (V_T, V'_T, and $V_{\mathrm{T\text{-}p}}$) were based on the summation of radioactivities in specific and nondisplaceable compartments. Since V_3 is equal to $B_{\max}/K_\mathrm{D}^{\mathrm{IOM}}$ ($K_\mathrm{D}^{\mathrm{IOM}}$: K_D of iomazenil), if interstudy difference in the distribution vol-

Table 1
Outcome measures

	Calculation	Factors Reflected	
		Clearance	Binding to plasma proteins
V_T	Brain radioactivity/Free parent	+ + +	+ + +
V'_T	Brain radioactivity/Total parent	+ + +	−
$V_{\mathrm{T\text{-}p}}$	Brain radioactivity/Plasma radioactivity	+	−

Free parent = (Total plasma radioactivity) × (Fraction parent) × f_1.
Total parent = (Total plasma radioactivity) × (Fraction parent).
Fraction parent = (Radioactivity of total parent)/(Total plasma radioactivity).

ume in the nondisplaceable compartment can be neglected, V_T is proportional to B_{max}/K_D^{IOM} (Laruelle et al., 1994). Among the three outcome measures, V_T is theoretically the most accurate, since it uses the specific plasma variable (free parent concentration) which determines the equilibrium binding values in brain. However, V_T is subject to the most measurements errors, which include assessment of free fraction and metabolite correction. In contrast, metabolite correction and plasma protein binding need not be measured to obtain $V_{T\text{-}p}$.

2.8. Alprazolam plasma analysis

Plasma samples were analyzed with HPLC as described previously (McCormick et al., 1984) to measure alprazolam levels. Plasma was extracted with toluene/isoamyl alcohol (99/1 by volume), evaporated, and reconstituted in the mobile phase. The latter is wasted with hexane, then subjected to reversed-phase liquid chromatography and ultraviolet detection at 202 nm. Midazolam was used as internal standards.

2.9. Adjustment for the fluctuations of plasma alprazolam levels

As mentioned in the section of alprazolam administration and clinical monitoring, plasma alprazolam levels were expected to be stable in individual subjects from day 3 to day 24. However, changes of metabolism during alprazolam administration may cause fluctuations of alprazolam levels. Therefore, adjustments for these fluctuations were done in the calculation of $B_{max}(i)$, which equals the sum of benzodiazepine receptor densities which were both occupied and not occupied by alprazolam on day i. Most animal studies have shown statistically significant decreases in receptor densities which occurred after 4–14 days of benzodiazepine treatment, with a trend of nonsignificant decreases at earlier time points (Miller et al., 1988a, 1989). Therefore, we have assumed that differences between baseline and day 3 SPECT values reflected only receptor occupancy and not an alteration in the number of receptors. However, if receptor densities decreased by day 3, then the measurements of receptor occupancy were overestimated. The assumption that only receptor occupancy occurred on day 3 may overestimate receptor occupancy because it may have included a part of the decrease in receptor densities.

The relative distribution of brain activity in nondisplaceable and specific compartments was assumed to be the same in each subject throughout the study.

$$V_3(i) = kV_T(i) \tag{1}$$

Since [^{123}I]iomazenil was given at tracer doses, receptor occupancy by iomazenil was negligible. Therefore, as mentioned previously (Abi-Dargham et al., 1994; Laruelle et al., 1994),

$$V_3(-4) = \frac{B_{max}(-4)}{K_D^{IOM}} \tag{2}$$

$$V_3(i) = \frac{B_{max}(i) - B(i)}{K_D^{IOM}} \tag{3}$$

where $V_3(i)$ is the distribution volume in the specific compartment measured in SPECT, $B_{max}(i)$ is unoccupied plus occupied receptor density, and $B(i)$ is the receptor density occupied by alprazolam on day i. Note that $B_{max}(i)$ is the true receptor density and different from $V_3(i)$ which is affected by the fluctuations of alprazolam levels and its binding ($B(i)$) to benzodiazepine receptor. In addition to the study with alprazolam (Miller et al., 1989), the majority of animal studies showed that chronic benzodiazepine treatment did not alter benzodiazepine receptor binding affinity (K_D^{IOM}) (Miller et al., 1988a,b; Szczawinska et al., 1988; Tietz et al., 1989; Ramsey-Williams et al., 1994). Under the assumption that free fraction of alprazolam in plasma (f_1^A) and the affinity of alprazolam for benzodiazepine receptor (K_D^A) are constant in individual subject throughout the study, the following relationship is given by the classic mass action laws.

$$\frac{B(i)}{B_{max}(i)} = \frac{f_1^A A(i)}{K_D^A + f_1^A A(i)} \tag{4}$$

where $A(i)$ is the total (free plus protein bound) alprazolam level in plasma on day i. Under the above mentioned assumption that only receptor occupancy but not decrease in receptor densities occurred on day 3,

$$B_{max}(-4) = B_{max}(3) \tag{5}$$

Using equations shown above, and the equations given by $i = -4$ and 3 in Eq. (1), and $i = 3$ in Eqs. (3) and (4), the ratio of receptor densities at the baseline (day -4) and on day i ($i = 10$, 17 or 24) can be obtained by the following equation

$$\frac{B_{max}(i)}{B_{max}(-4)} = \left(1 + \frac{V_T(-4) - V_T(3)}{V_T(3)} \frac{A(i)}{A(3)}\right) \frac{V_T(i)}{V_T(-4)}. \tag{6}$$

As shown in Section 3, the reliability in the measurement of V_T was not good. Therefore, the adjustments for the fluctuations of alprazolam levels were done for $V_{T\text{-}p}$, under the assumption that f_1 and fraction parent were constant throughout the study (see Table 1). The mean of the two trough alprazolam levels on each day was used as $A(i)$.

2.10. Statistical analysis

In the study of alprazolam administration, comparisons of the data on the study days were done using repeated

measures analysis of variance (ANOVA) with Tukey's post hoc *t*-test. Correlations of receptor densities and the scores in visual analog scales or the Hopkins verbal learning test were studied by simple regression analysis and the determination of Pearson's product-moment correlation coefficient. The significance was defined as $P < 0.05$.

In the reproducibility study, the within subject variability (expressed as a percentage) between test and retest conditions, was calculated as the absolute value of the difference of the two measures divided by the mean. The reliability of outcome measures was determined relative to between subject variance by calculation of the intraclass correlation coefficient, ρ (Kirk, 1982). This coefficient is an estimate of the reliability of the measurement and varies from 0 (no reliability) to 1 (total reliability, when test = retest measures) and is expressed by:

$$\rho = \frac{\text{MSBS} - \text{MSWS}}{\text{MSBS} + (n-1)\text{MSWS}}$$

where MSBS and MSWS are the mean sum of squares between and within subjects, respectively, and n is the number of within subject measurement ($n = 2$ in test/retest study). Comparisons of variability and ρ were done with repeated measures ANOVA and Tukey's post hoc t-test.

2.11. Statistical parametric mapping (SPM) analysis

In addition to conventional volume of interest analysis, SPM96 was applied for the data of the alprazolam study. First, parametric images were created by dividing decay-corrected brain radioactivities by decay-corrected total plasma radioactivities to create voxel values equal to $V_{T\text{-}p}$. $V_{T\text{-}p}$ was selected because this was the most reliable outcome measure (see Section 3). Parametric images can easily be created by this simple division in an equilibrium study because radioactivities of both brain and parent tracer in plasma are stable. Coregistered magnetic resonance images were transformed into a standard stereotactic space (Talairach and Tournoux, 1988) using SPM96 (Friston et al., 1995). Then SPECT images were transformed into the standard space using the transformation parameters obtained from the coregistered magnetic resonance images. For statistical analysis in SPM96, the 3rd-order nonlinear regression method (Buchel et al., 1996) was used to detect a specific pattern in sequential changes in the five SPECT studies. Since the voxel values equaled to $V_{T\text{-}p}$, which is proportional to unoccupied receptor densities, global normalization was not applied to detect changes in absolute values of receptor densities. In addition, global normalization (subject specific analysis of covariance, ANCOVA) was applied to detect regional patterns of sequential changes in the five SPECT studies. Global normalization provides high sensitivity to detect regional changes which are different from those in mean brain values.

3. Results

3.1. Test / retest reproducibility of iomazenil SPECT

Three outcome measures, V_T, V'_T and $V_{T\text{-}p}$ were tested (Table 1). Although V_T is theoretically an ideal one among the three, it needs free parent levels for which the most extensive plasma metabolite analysis is required. Three levels of analyses are necessary to obtain free parent levels: (1) extraction of lipophilic compounds in plasma, (2) HPLC analysis to determine fraction of the parent tracer in lipophilic compounds, and (3) the determination of free fraction (f_1). The total plasma parent concentration is calculated by multiplying fractions of the parent tracer determined in (1) and (2) (the product is fraction parent). Because the composition of the parent tracer determined by HPLC was almost 100% ($99.6 \pm 0.4\%$) in all studies, HPLC analysis could not have been a major source of errors or true interstudy variability in plasma metabolite analyses. Therefore, there are two sources of errors or variability in the analysis: (1) extraction and (3) measurement of f_1. V_T contains both of these two while V'_T contains only one (extraction). $V_{T\text{-}p}$ does not require any metabolite analysis. If the true interstudy variability is greater than the errors in the analysis, extensive metabolite analyses are required. However, if the opposite is the case, extensive metabolite analysis decreases the accuracy of data. For example, if the true interstudy variability of the fraction of lipophilic compounds (determined by extraction) is greater than the errors in this analysis, V'_T is more appropriate than $V_{T\text{-}p}$. If errors are greater, $V_{T\text{-}p}$ is more appropriate. Because peripheral clearance is calculated from total parent levels, differences in clearance is fully taken into account in V'_T, and partially in $V_{T\text{-}p}$. Clearance is taken into account correctly in $V_{T\text{-}p}$ only when the interstudy variability in the fraction parent is negligible (Table 1).

The variability of $V_{T\text{-}p}$ was significantly different from that of the other two outcome measures (Table 2; $P < 0.01$). Intraclass correlation coefficient (ρ) of the three outcome measures was in the order of $V_{T\text{-}p}$, V'_T and V_T with significant differences in the following comparisons: V_T vs. V'_T, $P < 0.05$; V_T vs. $V_{T\text{-}p}$, $P < 0.01$; V'_T vs. $V_{T\text{-}p}$, $P < 0.01$. Very low ρ values of V_T and V'_T indicated that errors in these analyses were much greater than the true intersubject variability in the metabolism of iomazenil and $V_{T\text{-}p}$ was the clear choice for outcome measure. Variability in extraction, which was similar to those in V_T and V'_T, indicated that extraction was the major source of errors in plasma metabolite analysis. Therefore, $V_{T\text{-}p}$ was used as an outcome measure in the study of alprazolam treatment.

3.2. Chronic alprazolam administration

3.2.1. Plasma alprazolam levels

The compliance with the dosing schedule of alprazolam was excellent with average of 98% (range: 94–100%).

Table 2
Test retest reproducibility in [^{123}I]iomazenil equilibrium study

	Extraction	f_1	V_T	V'_T	$V_{T\text{-}p}$
Variability (%)	20.3 ± 15.4	5.4 ± 2.2	20.7 ± 12.0	20.3 ± 9.3	6.2 ± 4.0
Intraclass correlation coefficient (ρ)	0.34	0.79	0.34	0.44	0.87

Data are means of frontal, parietal, temporal and occipital cortices and cerebellum.
Variability (%) = abs(test − retest)/(mean test and retest) × 100.
Intraclass correlation coefficient = (MSBS − MSWS)/(MSBS + (n − 1)MSWS).
MSBS = mean sum of squares between subjects; MSWS = mean sum of squares within subjects.

Two trough levels of plasma alprazolam were measured on each day of SPECT studies (Fig. 1). Although there were small fluctuations, no significant differences were detected between study days. Therefore, under the assumption that K_D^A and f_1^A of each subject were constant throughout the study, there was no significant change in receptor occupancy.

3.2.2. Changes in benzodiazepine receptor densities

As described above, $V_{T\text{-}p}$ was used as an outcome measure. $V_{T\text{-}p}$ is proportional to V_T ($\approx B_{max}/K_D^{IOM}$) only when differences in fraction parent and f_1 were negligible among studies of individual subjects (Table 1). In this study, neither fraction parent nor f_1 showed significant differences among the five SPECT studies. Therefore, $V_{T\text{-}p}$ was assumed to be proportional to B_{max} under the assumption that K_D^{IOM} was constant throughout the studies of individual subjects. Changes in receptor densities were studied in three ways. First, changes of each voxel in images were analyzed with SPM96. Second, mean $V_{T\text{-}p}$ of the whole brain was analyzed because SPM96 did not show regional differences in the change of $V_{T\text{-}p}$ (see below). Third, the influence of fluctuations in plasma alprazolam levels (Fig. 1) was corrected by Eq. (6).

SPM analyses were done with the third-order nonlinear regression to assess changes in each voxel throughout the five SPECT studies. With global normalization, SPM did not show any region in which pattern of changes during the five SPECT studies was significantly different (corrected $P < 0.05$; data not shown). Without global normalization, SPM showed that almost the whole brain including the cerebellum showed a steep decrease between days −4 and 3, a shallow decrease between days 3 and 10, and an increase after day 10 (Fig. 2). The black area in Fig. 2 shows the area with this specific pattern of change with uncorrected $P < 0.0001$. Corrected P values were below 0.01 in almost all voxels in this black area with lower P values in darker areas.

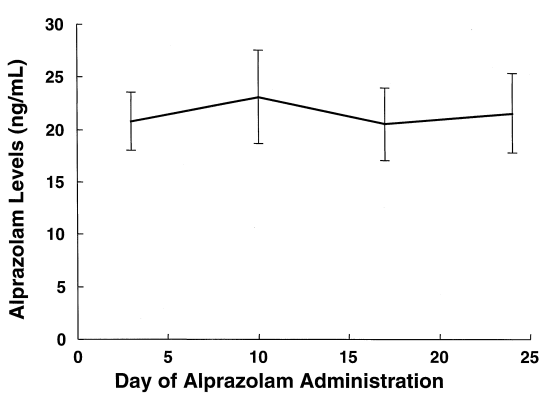

Fig. 1. Plasma alprazolam levels during chronic alprazolam administration. Results are means ± S.E.M. Levels were measured at 5 h 45 min after the first and the second doses of alprazolam on the days of SPECT studies. There were no significant differences in any comparison.

$V_{T\text{-}p}$ in frontal, parietal, temporal and occipital cortices and cerebellum obtained by volume of interest analysis showed very similar changes compatible with the results in SPM with and without global normalization. Results in the whole brain, temporal cortex and cerebellum are shown in Fig. 3. Since there was no regional difference and almost the whole brain showed the same change, further analyses primary used the mean $V_{T\text{-}p}$ of the whole brain.

$V_{T\text{-}p}$ of the whole brain showed a 16 ± 2% decrease on day 3 (days −4 vs. 3, $P < 0.01$); a further 10 ± 5% (of $V_{T\text{-}p}$ on day 3) decrease on day 10 (days 3 vs. 10, $P < 0.01$); and a 16 ± 4% (of $V_{T\text{-}p}$ on day 3) increase on day 17 (days 10 vs. 17, $P < 0.01$). Although $V_{T\text{-}p}$ on days 17 and 24 was slightly greater than that on day 3, there were no significant differences. Since plasma alprazolam levels were stable throughout the study (Fig. 1), receptor occupancy by alprazolam was constant from day 3 to day 24 as described above. Therefore, changes of $V_{T\text{-}p}$ after day 3 was the changes in receptor densities but not in receptor occupancy. Thus the results indicated that the decreases in receptor densities occurred between days 3 and 10 and normalization occurred between days 10 and 17. Since decrease of receptor densities might have occurred before day 10, receptor occupancy was 16% at maximum and decrease of receptor densities was 10% at minimum.

Because minor fluctuations of plasma alprazolam levels might have affected the results of $V_{T\text{-}p}$, adjustments were done under the assumptions described above. The adjustments could not be done for one subject because of a slight but unexplainable increase (0.1%) of $V_{T\text{-}p}$ between days −4 and 3. B_{max} (occupied plus unoccupied receptors) of the other 8 subjects showed 11 ± 6% decrease on day 10 (days 3 vs. 10, $P < 0.05$) and 20 ± 5% (of the baseline) increase on day 17 (days 10 vs. 17, $P < 0.01$) (Fig. 3).

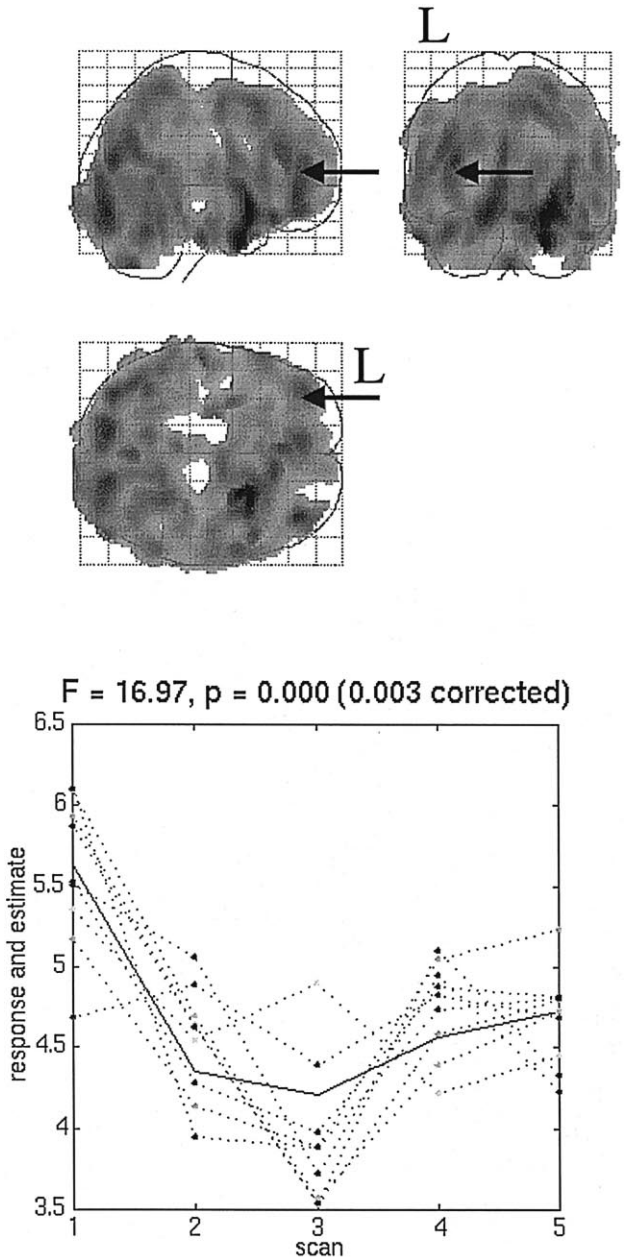

Fig. 2. The third-order nonlinear regression analysis in SPM96 (statistical parametric mapping version 96). Parametric images in which pixel values were equal to $V_{T\text{-}p}$ were analyzed without global normalization. Voxels below uncorrected $P = 0.0001$ are shown in black in the upper scheme where P values were lower in darker areas. The whole brain showed specific pattern of changes during chronic alprazolam administration. The graph below shows representative changes of $V_{T\text{-}p}$ in a voxel in the left (L) frontal cortex (arrows, $x = -34$, $y = 48$ and $z = 8$ in Talairach space). Dashed lines show changes from individual subjects, and the solid line shows regression to the data of all subjects. Scan 1: day -4; scan 2: day 3; scan 3: day 10; scan 4: day 17; and scan 5: day 24.

Although B_{max} on days 17 and 24 was slightly greater than that on days -4 and 3, there was no significant difference. Therefore, data with and without correction for the fluctuations in plasma alprazolam levels consistently showed decrease of receptor densities between days 3 and 10, and increase of receptor densities between days 10 and 17. Note that a part of the decrease in receptor densities might have occurred before day 3. If so, receptor occupancy was overestimated in this correction and the influence by the fluctuations of plasma alprazolam levels (Fig. 1) was exaggerated.

3.2.3. Clinical effects of alprazolam

Clinical effects of alprazolam were evaluated in two ways: subjective ratings of sedation and the Hopkins verbal learning test. Because some of 28 items showed sensitive changes but others did not, repeated measures ANOVA without post hoc test was performed for each item to select the ones most sensitive to the effects of alprazolam. The following 11 items showed differences among 8 days (days -4, 0, 1, 2, 3, 10, 17 and 24) with $P < 0.1$: active, concentration, drowsy, drugged, efficient, eyes open, focusing, foggy, memory, sedated, and uncoordinated. To minimize noise in these 11 subjective ratings, scores in each item were averaged. Because 'active' and 'efficient' decreased and all the others increased with alprazolam administration, scores in these items were multiplied by -1 before calculating means. Sedative effects showed a sharp increase soon after the start of alprazolam administration (Fig. 4; day -3: -5.6, day 0: 5.8, day 1: 27; day -3 vs. 1: $P < 0.01$). Then the scores quickly decreased from day 1 to day 3 (score: 16), stayed at the same level until day 10 (score: 16), and then showed further decrease

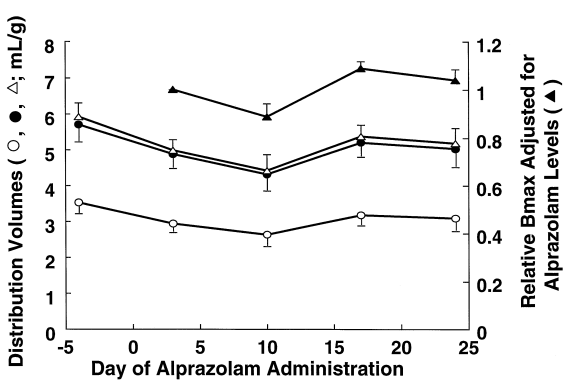

Fig. 3. Benzodiazepine receptor densities during chronic alprazolam administration. Distribution volumes of [^{123}I]iomazenil ($V_{T\text{-}p}$; brain/plasma total radioactivity) in whole brain (○), temporal cortex (●), and cerebellum (△). To calculate B_{max} (unoccupied + occupied receptor densities) of the whole brain (▲), changes of distribution volumes between days -4 and 3 were assumed to reflect receptor occupancy by alprazolam and errors caused by the fluctuations of alprazolam levels on each day were adjusted (see text). B_{max} relative to the baseline is shown (B_{max} on days -4 and $3 = 1$). Repeated measures ANOVA with Tukey's post hoc t-test showed significant differences in the following comparisons; $V_{T\text{-}p}$ of the whole brain: days -4 vs. 3, $P < 0.01$; days -4 vs. 10, $P < 0.01$; days -4 vs. 17, $P < 0.01$; days -4 vs. 24, $P < 0.01$; days 3 vs. 10, $P < 0.05$; days 10 vs. 17, $P < 0.01$; days 10 vs. 24, $P < 0.01$; B_{max} of the whole brain adjusted for the fluctuations of alprazolam levels: days 3 vs. 10, $P < 0.05$; days 10 vs. 17, $P < 0.01$; days 10 vs. 24, $P < 0.01$.

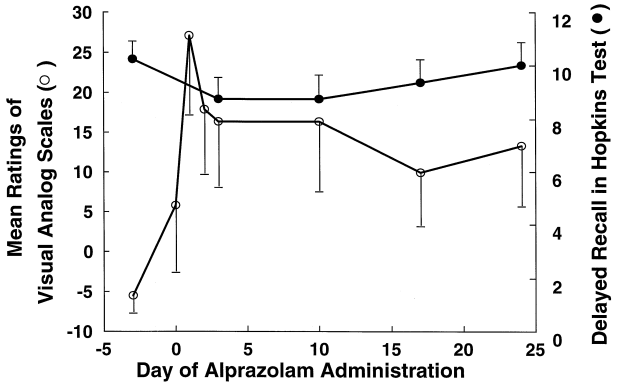

Fig. 4. Ratings of visual analog scales (○) and delayed recall in the Hopkins verbal learning test (●). Ratings of visual analog scales are the mean of 11 items, each of which showed changes with $P < 0.1$. Results of eight subjects who did these tests are shown. Repeated measures ANOVA with Tukey's post hoc *t*-test showed significant differences in the following comparisons; visual analog scales: days −3 vs. 1, $P < 0.01$; days −3 vs. 2, $P < 0.01$; days −3 vs. 3, $P < 0.01$; days −3 vs. 10, $P < 0.01$; days −3 vs. 17, $P < 0.05$; days −3 vs. 24, $P < 0.05$; days 0 vs. 1, $P < 0.01$; days 1 vs. 17, $P < 0.05$; Hopkins: days −4 vs. 3, $P < 0.05$; days −4 vs. 10, $P < 0.05$; days 3 vs. 24, $P < 0.05$; days 10 vs. 24, $P < 0.05$.

on day 17 (score: 10). Although the score showed a decrease as early as day 3, it did not reach a significant level until day 17 (day 1 vs. 17, $P < 0.05$). The scores on day 1 and 24 were not significantly different. Therefore, the results of sedative effects can be interpreted that alprazolam showed maximum effects on day 1, tolerance was developed on day 17. However, the tolerance did not continue until day 24.

Delayed recall but not immediate recall in the Hopkins verbal learning test showed significant changes by alprazolam administration. Scores of delayed recall significantly decreased on day 3 (days −4 vs. 3, $P < 0.05$) and 10 (days −4 vs. 10, $P < 0.05$) and returned to the baseline level on day 24 (days 10 vs. 24, $P < 0.05$ and days −4 vs. 24, n.s.) (Fig. 4). Therefore, the effects of alprazolam were detected on days 3 and 10 and tolerance developed by day 24. Thus, the time-course of sedative effects detected by subjective ratings (tolerance development on day 17 and loss of tolerance on day 24) and that of the Hopkins test (tolerance on day 24) was different (Fig. 4).

Although the time course in the changes of $V_{T\text{-}p}$, sedative effects by visual analog scales and delayed recall in the Hopkins test did not match well, these results indicated that the decrease of receptor densities between days 3 and 10 may be the cause of the tolerance detected by the visual analog scales between days 10 and 17 and the Hopkins test between days 10 and 24, and the normalization of receptor densities between days 10 and 17 may be the cause for the loss of tolerance detected by the visual analog scales between days 17 and 24. Therefore, correlations were studied between $V_{T\text{-}p}(10)/V_{T\text{-}p}(3)$ and VAS(17) − VAS(10), $V_{T\text{-}p}(17)/V_{T\text{-}p}(10)$ and VAS(24) − VAS(17), and $V_{T\text{-}p}(10)/V_{T\text{-}p}(3)$ and Hop(24)—(mean of Hop(3) and Hop(10)) where $V_{T\text{-}p}(i)$, VAS(i) and Hop(i) are the scores of $V_{T\text{-}p}$, visual analog scales and delayed recall of the Hopkins test on day i, respectively. Results showed that there were no significant correlations.

4. Discussion

Sequential changes of benzodiazepine receptor densities were studied during 24-day administration of alprazolam to healthy human subjects. $V_{T\text{-}p}$ was used as the outcome measure, because the test/retest study showed that $V_{T\text{-}p}$ was more reliable than V_T or V'_T and since there were no significant changes in fraction parent and f_1. Receptor occupancy on day 3 was 16% (maximum estimate) and the decrease of receptor densities between days 3 and 10 was 10% (minimum estimate). Between days 10 and 17, receptor densities showed 16% increase. Analyses with SPM showed that no regional difference in these changes occurred (Fig. 2). Subjective rating of sedative effects showed development of tolerance and loss of tolerance, and delayed recall in the Hopkins test showed development of tolerance. However, changes in both of these clinical ratings occurred 1–2 weeks after the changes in receptor densities.

4.1. Outcome measure in iomazenil equilibrium SPECT

Among three outcome measures tested (Table 1), $V_{T\text{-}p}$ was the only one which was appropriate to detect small changes of receptor densities (Fig. 3) in this study. Variability in the extraction of radioactivity from plasma was the likely source of the poor reproducibility in V_T and V'_T. Improvement in the accuracy of extraction is required to be able to use V_T, which is theoretically an ideal outcome measure.

4.2. Chronic administration of alprazolam

The results showed three phases of changes in the benzodiazepine receptor: occupancy, decrease of receptor densities, and normalization (Fig. 3).

4.2.1. Occupancy

The results of the current study showed that oral administration of 2 mg/day alprazolam occupied only 16% of benzodiazepine receptor. However, this low occupancy caused significant clinical effects (Fig. 4). These results are compatible with previous reports showing that clinically effective dose of benzodiazepine receptor agonists occupy only 10–40% of the receptor (Innis et al., 1991; Videbæk et al., 1993). The remaining receptors were considered as 'receptor reserve' which could be inactivated without diminishing efficacy of agonists (Innis et al., 1991).

Because there is a widely accepted consensus that, at equilibrium, concentration of free ligand in plasma is equal to that in fluid in brain which can interact with receptor (Carson et al., 1993), free plasma alprazolam levels from day 3 can be assumed to be equal to the concentrations in free-compartment in brain. Therefore, receptor occupancy at day 3 can be calculated by Eq. (4) using $f_1^A = 0.29$ (human data) (Greenblatt and Wright, 1993), $K_D^A = 11$ nM (human prefrontal cortex, measured in vitro at 37°C) (Richelson et al., 1991), and mean $A(3) = 21$ ng/ml obtained in this study. Calculated receptor occupancy is 64%. Although SPECT data are the mixture of the data from gray and white matter due to its low resolution, V_2 is only 10–15% of V_T. Therefore, low resolution of SPECT does not explain the discrepancy between the receptor occupancy measured in SPECT (16%) and the calculated value (64%). This discrepancy indicated that K_D^A in vivo was greater than that in vitro, which is compatible with a report showing that K_D of a benzodiazepine receptor agonist, midazolam measured in vivo was greater than that in vitro (Videbæk et al., 1993).

4.2.2. Decrease of benzodiazepine receptor densities

One of major purposes in this study was to know if there was a decrease of receptor densities during chronic alprazolam administration. The results showed that there was a small (10%) but significant decrease of receptor densities (Figs. 2 and 3). This decrease was significant even after the correction for the small fluctuations of plasma alprazolam levels (Fig. 1). As mentioned above, this correction assumed that only receptor occupancy occurred between days −4 and 3. However, a small part of the decrease of receptor densities might have occurred before day 3. Therefore, the correction might have exaggerated the influence of the fluctuations of alprazolam levels, which decreased significance in the comparison of the receptor densities between days 3 and 10. As described earlier, receptors on synaptic membrane and in vesicles cannot be differentiated with SPECT. Therefore, if there was a transfer of benzodiazepine receptors from synaptic membrane to vesicles (i.e., sequestration), decrease of receptor densities measured in this study underestimated the decrease of the density on cell membrane.

Both subjective ratings for sedation and delayed recall showed development of tolerance. However, time-course of these two ratings were different. The former and the latter showed development of tolerance on days 17 and 24, respectively (Fig. 4), showing that mechanisms of tolerance may be different for these two types of tests. Although the decrease of receptor densities and the development of tolerance were detected, the time-courses were different, and the correlations between changes in the receptor and tolerance in individual subjects were poor. These discrepancies indicated that the decrease of receptor densities may be one but not the sole mechanism for the development of tolerance. Other mechanisms (i.e., desensitization, sequestration, decrease in the functional coupling, and post-translational regulation) may be involved. For example, benzodiazepine receptor on synaptic membrane and in vesicles cannot be measured separately in SPECT study because of relatively high lipophilicity of iomazenil, which is required to pass blood–brain barrier and enable brain imaging. Therefore, involvement of sequestration in the development of tolerance cannot be studied in vivo in humans.

4.2.3. Normalization

The most striking result in this study was normalization of receptor densities on day 17 (Fig. 3). Both $V_{T\text{-}p}$ and B_{max} corrected by the fluctuations of alprazolam levels showed that this change was significant. Although V_T', in which nonsignificant differences in fraction parent were taken into account (Table 1), was not a reliable outcome measure (Table 2), this outcome measure also showed significant differences between days 10 and 17 ($P < 0.01$) and days 10 and 24 ($P < 0.01$). Although there was a trend of up-regulation ($V_{T\text{-}p}$ on day 17 was greater than that on day 3 and B_{max} on day 17 was greater than the baseline), this trend did not reach significance. Similar to the changes between days 3 and 10, results in this study do not differentiate receptors on synaptic membrane and vesicles. If there was a normalization in the distribution of benzodiazepine receptors on synaptic membrane and vesicles (translocation of vesicles to the synaptic membrane), results of this study underestimated the increase of receptor densities on the outer cell membrane.

Although an animal study with chronic alprazolam administration showed a trend of normalization, it did not reach a significant level (Miller et al., 1989). In addition, adjustment for nonsignificant fluctuations of alprazolam levels in that study was not done. It is not clear if the normalization shown in the current study is peculiar to alprazolam administration in humans. However, among benzodiazepines, alprazolam may exert unusual clinical effects, including antidepressant, anxiolytic and antipanic activity (Feighner et al., 1983; Ciraulo et al., 1986; Ballenger et al., 1988). In addition, animal studies showed that low-dose alprazolam increased receptor densities (Miller et al., 1987a) and motor activities (Lopez et al., 1988) which were not detected with other benzodiazepines (Miller et al., 1987a,b).

In conclusion, chronic administration of alprazolam to healthy human subjects caused three phases of changes in the benzodiazepine receptor: occupancy, decrease, and normalization of receptor densities. Development and loss of tolerance were detected 1–2 weeks after the changes in the receptor. Discrepancy in the time-course and poor correlations between receptor densities and clinical effects may indicate that the changes in the receptor density may be one but not the sole mechanism for the development and loss of tolerance.

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