Pharmacology Biochemistry and Behavior, Vol. 59, No. 4, pp. 925–934, 1998

0091-3057/98 $19.00 + .00

ELSEVIER

PII S0091-3057(97)00539-X

Benzodiazepine Dependence: From Neural Circuits to Gene Expression

JUDITH A. PRATT, ROS R. BRETT[1] AND DAVID J. LAURIE[2]

Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW, Scotland, UK

Received 17 July 1997; Revised 9 October 1997; Accepted 9 October 1997

PRATT, J. A., R. R. BRETT AND D. J. LAURIE. *Benzodiazepine dependence: From neural circuits to gene expression*. PHARMACOL BIOCHEM BEHAV **59**(4) 925–934, 1998.—The neural mechanisms underlying benzodiazepine dependence remain equivocal. The present studies tested the hypothesis that similar neural systems are recruited during diazepam tolerance and withdrawal, and that these are associated with changes in $GABA_A$ receptor properties. 2-Deoxyglucose quantitative autoradiography was employed to map the brain structures affected during chronic treatment and withdrawal from diazepam (5 mg/kg IP daily) in rats. Acute administration of diazepam evoked widespread reductions in local rates of cerebral glucose (LCGU) utilization throughout the brain. Brain structures associated with sensory processing developed tolerance to these depressant effects of diazepam after 3 days of treatment, whereas tolerance occurred in the Papez circuit of emotion after 28 days of treatment. These data suggest that adaptive changes in different neuroanatomical circuits may underlie tolerance to the various effects of diazepam. During flumazenil-precipitated withdrawal from diazepam there were marked increases in glucose use in structures of the Papez circuit, the nucleus accumbens, and the basolateral amygdala. These data suggest that the Papez circuit features strongly in diazepam tolerance and withdrawal and supports a common adaptive process being involved in these phenomena. While GABA enhancement of benzodiazepine binding was reduced in the nucleus accumbens after repeated diazepam treatment, there was little evidence to support adapative changes in $GABA_A$ receptors or $GABA_A$ subunit gene expression (γ_2, α_1, or α_4) as underlying the functional changes in the identified circuits. Alternative neurochemical mechanisms, such as changes in glutamatergic function should be considered. © 1998 Elsevier Science Inc.

Benzodiazepine Tolerance Withdrawal Dependence Diazepam Rat $GABA_A$ receptor Autoradiography 2-Deoxyglucose autoradiography Gene expression Papez circuit

BENZODIAZEPINES are commonly prescribed for the treatment of anxiety and sleep disorders. However, prolonged treatment can lead to dependence with a recognized withdrawal syndrome (17). In addition, tolerance occurs at different rates to different aspects of benzodiazepine action. Tolerance to the sedative effects occurs rapidly, followed by tolerance to the anticonvulsant actions, and last, but less clearly, tolerance to anxiolysis (4,24).

One hypothesis to explain the processes underlying diazepam tolerance and withdrawal is that chronic drug treatment leads to adaptive processes that counter the effects of the drug at the $GABA_A$ receptor and that these processes persist after the drug has been cleared from the brain, thereby leaving the opposing processes unopposed and the resultant emergence of withdrawal symptoms.

Indeed, many studies have focussed on investigating changes at the level of the $GABA_A$ receptor after chronic benzodiazepine treatment. Results from in vitro approaches have in general found support for the view that changes in the $GABA_A$ receptor occur after continuous drug exposure to cells in culture (5). However, the results from ex vivo approaches have been less convincing. For example, radioligand binding studies have shown no change, downregulation, or even upregulation of benzodiazepine binding (7). In general, downregulation appears to occur when large doses of benzodiazepines are administered chronically (28). There is some

Requests for reprints should be addressed to Dr. J. A. Pratt, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow Gl lXW, Scotland.
[1]Present address: Department of Biological Sciences, University of Paisley, Paisley, PA1 2BE.
[2]Present address: Sandoz AG, CH 4002, Basle 61, Switzerland.

evidence for a reduction in allosteric coupling between the GABA binding site and benzodiazepine binding site (7) following chronic treatment with doses in the therapeutic range but results from other groups do not support this (24). During withdrawal from benzodiazepines, there is again no clear consensus on the mechanisms involved. For example, one group reports subsensitivity to GABA for several weeks after withdrawal (8), whereas others report an increase in $GABA_A$ receptor function (16).

The interpretation of these data is difficult because many of these studies are not directly comparable because of differences in treatment regimes and methodologies employed. Furthermore, there have been few attempts to correlate neurochemical changes with behavioral events. In addition, the majority of studies have used tissue from grossly dissected brain regions that may have masked any relevant regional differences.

We have adopted a different approach to study benzodiazepine tolerance and dependence. We are of the opinion that before an understanding of the neurochemical bases of these phenomena can be elucidated, it is first necessary to know which neuronal systems are recruited during these processes. We have developed a treatment protocol in which rats exhibit behavioral tolerance and withdrawal symptoms after repeated administration of low doses of diazepam (akin to the therapeutic doses employed in humans). Using these treatment protocols we have then employed the quantitative 2-deoxyglucose autoradiographic technique in conscious rats to map the brain structures recruited (22). This technique measures local rates of cerebral glucose use (LCGU) throughout the brain, and so decisions do not have to be made beforehand of the regions to be investigated. Because altered rates of LCGU are believed to predominantly reflect altered ion pump activity in the nerve terminal, the technique provides information on changes in the functional activity of brain structures consequent to receptor occupation.

Following identification of the brain structures, and hence, neuroanatomical circuits, recruited in diazepam tolerance and withdrawal, we have then examined whether there are alterations in the characteristics of the $GABA_A$ receptor and $GABA_A$ receptor subunit gene expression using receptor autoradiography and in situ hybridization respectively.

The present article reviews the results of these studies and attempts to address the question as to whether common neuroanatomical circuits and neurochemical processes are involved in benzodiazepine tolerance and withdrawal.

METHOD

Experiment 1: 2-DG Tolerance Study

The first study examined the effects of the acute and repeated administration of diazepam upon local rates of cerebral glucose use using the quantitative 2-deoxyglucose technique. We hypothesized that tolerance would occur in different neural circuits after differing periods of drug treatment.

Methods. Experiments were performed on male Long–Evans hooded rats (Charles River, Kent, UK) maintained on a natural day/night light cycle and allowed food and water ad lib. Rats were randomly assigned to four treatment groups.

The control group (group 1) received daily injections of vehicle (1% Tween 20 in saline IP) for 28 days. The acute diazepam group (group 2) received daily injections of vehicle for 28 days followed by an acute dose of diazepam (0.3 mg/kg IV) on the day of LCGU measurements (day 29). The subacute group (group 3) received vehicle for 25 days, followed by three daily injections of diazepam (5 mg/kg IP). The chronic group (group 4) received diazepam (5 mg/kg IP) daily for 28 days. A challenge dose of diazepam (0.3 mg/kg IV) was administered to groups 3 and 4 on the day of the experiment (day 29) when LCGU were determined.

The dose of diazepam selected is one that has been widely employed in animal behavioral studies of anxiety (4,24). While a systematic evaluation of tolerance to the behavioral effects of diazepam was not conducted in the present study, previous reports have shown that the treatment regimen employed produces tolerance to the sedative effects and to the anxiolytic effects of benzodiazepines after about 3 days and 3 weeks, respectively (4). On the day of the experiment (day 29), rats were lightly anesthetized with halothane and nitrous oxide and polythene cannulae inserted into both femoral arteries and one femoral vein. The lightly restrained animal was allowed to recover from the effects of anesthesia for at least 2 h. LCGU was measured in the conscious rat using the quantitative [^{14}C]2-DG technique of Sokoloff et al. (22). Vehicle (1 ml/kg IV) (group 1, control group) or diazepam (0.3 mg/kg IV) to groups 2, 3, and 4 (referred to subsequently as the "acute," "subacute," and "chronic" groups, respectively) was injected 10 min before administration of [^{14}C]2-DG. The period of LCGU measurement was initiated by an IV injection of [^{14}C]2-DG (125 μCi/kg) (Sigma, Dorset, UK) into the femoral vein over 30 s. Fourteen timed arterial blood samples were withdrawn over the subsequent 45 min. Samples were centrifuged and aliquots of plasma taken for the determination of ^{14}C and glucose levels by liquid scintillation analysis and by glucose oxidation assay (Beckman Glucose Analyzer, Beckman Instruments Ltd, High Wycombe, UK), respectively. The animals were killed 45 min after the radioisotope injection and the brains removed and frozen in isopentane precooled to −42°C. The frozen brains were coated with embedding medium (Lipshaw Embedding Matrix from Life Sciences International, Cheshire, UK), and coronal sections (20 μm thick) cut in a cryostat maintaned at −22°C. Throughout the length of the brain three consecutive sections every 200 μm were thaw mounted onto glass coverslips and rapidly dried on a hotplate. To prepare autoradiograms the sections, together with a set of 10 plastic standards of known ^{14}C concentration (44–1475 nCi/g tissue equivalent for 20 μm brain sections), were exposed to X-ray film (Kodak GRL) in cassettes for 14–21 days. The films were developed according to the manufacturer's instructions.

Optical density measurements were performed using a computer-based densitometer. For each of the 66 brain structures analyzed, bilateral readings were made in six consecutive sections and the average reading calculated for each structure. The optical density values of the tissue images were compared to those of the standards to derive local tissue concentrations of ^{14}C. Local rates of cerebral glucose utilization were calculated from the final ^{14}C, local tissue concentrations, the histories of ^{14}C, and of glucose in arterial plasma over the measurement period, and the appropriate rate constants for the rat using the operational equation established by Sokoloff et al. (22).

Data analysis. LCGU values were compared using a one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple range test to assess statistical differences between individual means.

Experiment 2: 2-DG Withdrawal Study

In this study we examined the effects of diazepam withdrawal upon local rates of cerebral glucose utilization, to de-

termine whether similar or distinct neural circuits were recruited during withdrawal and tolerance.

Chronic treatment regimen. Male Long–Evans rats (bred at the University of Strathclyde), were randomly assigned to six treatment groups (n = 6–8 per group). Two groups of rats received an IP injection daily for 28 days of vehicle (1% Tween 20 in saline) and the other four groups received daily IP injections of diazepam (5 mg/kg), suspended by ultrasound in vehicle. Twenty-four hours after the last IP injection, each rat was prepared for the [^{14}C]2-DG procedure as outlined in the Method section of Experiment 1. Thus, cannulae were implanted under halothane anesthesia into both femoral arteries and one femoral vein. The animals were then allowed to recover for at least 2 h prior to the administration of drugs and [^{14}C]2-DG.

Of the two groups that had received chronic vehicle, one group was administered vehicle (1 ml/kg IV) 10 min and 1 min, respectively, prior to the injection of [^{14}C]2-DG (vehicle control group). The other group received vehicle and flumazenil (5 mg/kg IV) 10 min and 1 min, respectively, prior to the injection of [^{14}C]2-DG (acute flumazenil group). Of the four groups that received chronic diazepam, one group received diazepam (0.3 mg/kg IV) and vehicle (1 ml/kg IV) 10 min and 1 min, respectively, prior to the injection of [^{14}C]2-DG (chronic diazepam group). Similarly, the "flumazenil-precipitated withdrawal group" was injected with diazepam (0.3 mg/kg IV) and flumazenil (5 mg/kg IV).

To compare the effects of spontaneous withdrawal from diazepam with the effects of flumazenil in rats withdrawn from diazepam 1 day previously, the remaining two groups were administered vehicle (1 ml/kg IV) 10 min and 1 min, respectively, prior to the injection of [^{14}C]2-DG (spontaneous withdrawal group) or vehicle (1 ml/kg IV) and flumazenil (5 mg/kg IV) 10 min and 1 min prior to the isotope (spontaneous withdrawal plus flumazenil group). Data for these last two groups are not shown here but reported in Laurie and Pratt (12).

Flumazenil was injected just prior to the [^{14}C]2-DG because of its very short half-life in the rat. The IV dose of diazepam chosen on the experimental day (day 29) is one that is broadly equivalent to the 5 mg/kg IP chronic pretreatment dose and that produced a significant depression of LCGU after acute administration (Experiment 1).

Behavioral signs of withdrawal. There were no obvious gross behavioral symptoms in the control group or the chronic diazepam group after administration of vehicle and diazepam, respectively. Similarly, the acute administration of flumazenil produced minimal behavioral effects in the group chronically treated with vehicle. However, immediately after the flumazenil injection was begun, all rats in the chronic diazepam-treated groups became agitated and struggled. They also showed heightened responsiveness to environmental stimuli such as operator movement and background noise. This behavior continued for approximately 7–10 min after the [^{14}C]2-DG injection before subsiding, and reappeared periodically throughout the experimental session. Ocasionally arching of the back and "poker" tail was noted.

Data analysis. LCGU results for each brain structure were analyzed by two-way ANOVA (pretreatment $\times$ final IV injection). Comparisons between individual means were made using the Bonferroni method for multiple comparisons where appropriate.

Experiment 3: Receptor Autoradiography Study

The aim of this experiment was to determine whether changes in the activity of neural circuits identified in the 2-deoxyglucose autoradiography studies following chronic diazepam treatment, were accompanied by regional changes in the properties of the $GABA_A$ receptor.

Methods: treatment regime. In these experiments the effects of subacute and chronic diazepam treatment on $GABA_A$ receptor properties were investigated in rats receiving the drug by the IP route and by continuous subcutaneous release. The treatment groups were as follows: three groups of male Long–Evans rats (bred at the University of Strathclyde) (n = 8–11 per group) received once daily IP injections, either 28 days vehicle (control IP), 25 days vehicle, and 3 days DZP 5 mg kg^{-1} (subacute IP), or 28 days DZP 5 mg kg^{-1} (chronic IP). Further groups (n = 8–11) were each implanted subcutaneously under anesthesia, with two silastic capsules. These were either empty (control implanted, and subacute implanted) or filled with DZP (chronic implanted). On days 26–28, these implanted groups received IP vehicle (control implanted and chronic implanted) or diazepam (5 mg/kg IP) (subacute implanted). All capsules were removed on day 28. Animals were tested in the elevated plus-maze on day 29 [see (3) for behavioral results]. All animals were killed on day 30 and the brains dissected and frozen for autoradiography.

Preparation of capsules. Capsules were prepared and implanted according to the method of Gallager et al. (6) with minor modifications (3). To maintain consistent release over the 28-day period, two capsules containing diazepam were implanted on day 1 and an additional capsule on day 15. This treatment protocol is similar to that employed by Gallager et al. (6), who demonstrated that rats implanted with silastic capsules containing DZP displayed relatively constant levels of DZP in the blood over the treatment period.

Receptor autoradiography. The animals were killed and the brains removed and frozen in isopentane prechilled to −42°C. Sections were cut at −20°C and stored at −70°C until required for binding. On the day of the experiment, the slides were allowed to reach room temperature in the boxes. Circles were marked round the sections with a wax pencil to permit a "bubble" of ligand solution to be applied to the section. The brain sections were subjected to "osmotic shock" to eliminate endogenous GABA (15) by a 3-min wash in room temperature distilled deionized water, followed by two 5-min rinses in ice-cold buffer, then dried for 1 h in a stream of air. The slides were then placed in humidified trays and cooled to 4°C in a refrigerator. Incubations were started by application of a bubble of the appropriate ligand solution.

Flunitrazepam and GABA enhancements of flunitrazepam binding. Sections were incubated for 90 min with 1 nM [^{3}H]-FNZP (87 Ci mmol^{-1}; Dupont, UK, New England Nuclear Division, Stevenage, UK) in 50 mM Tris citrate buffer pH 7.1 containing 200 mM NaCl in the absence of (total binding) or the presence (nonspecific binding) of 2 μM clonazepam (Roche, Welwyn Garden City, UK). For GABA enhancement of FNZP binding, both the total and the nonspecific binding solutions also contained 100 μM GABA (Sigma, Dorset, UK). Sections from the same brain were incubated in parallel with both ligands. Following a brief dip in ice-cold buffer, the slides were washed with two 1-min rinses in ice-cold buffer, followed by two 10 s washes in ice-cold distilled deionized water to remove buffer salts [adapted from (29)].

Slides were dried for 1 h in a stream of air, packed into boxes containing silica gel, and left at least overnight before exposure to tritium-sensitive film in light-tight cassettes, together with a set of precalibrated ^{3}H standards (Amersham International, Little Chalfont, UK) for 8–9 days. The films were developed according to the manufacturer's instructions.

Optical density measurements were determined with a computer-based image analyzer (MCID Imaging Research Inc., Ontario, Canada) and converted to pmol g^{-1} tissue by reference to ^3H standards.

The nonspecific binding of [^{3}H]-FNZP was very low (less than 2%) and difficult to measure accurately, as it was indistinguishable from background. The analysis was therefore performed on total [^{3}H]-FNZP binding, because the objective was to compare the binding at a single ligand concentration in different pretreatments, and not to provide a detailed kinetic analysis. GABA enhancement of [^{3}H]-FNZP binding was determined by calculating the percentage increase in total [^{3}H]-FNZP binding in the presence of GABA over that in the absence of GABA, the incubations with and without GABA being carried out in immediately adjacent sections. Nonspecific [^{3}H]-GABA binding varied between 20 and 50% according to the brain structure, and was subtracted from the total binding.

Statistical analysis. Results from all the groups except the chronic capsules plus IP injection group were analyzed by two-way analysis of variance with drug treatment and treatment route as the factors. Analysis of variance was followed by Newman–Keuls multiple range test where appropriate.

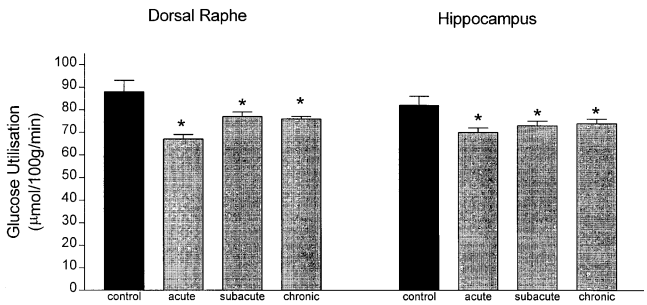

FIG. 1. Depressant effects of diazepam upon LCGU in the dorsal raphe and hippocampus. Data are presented as mean glucose use ±SEM for $n = 7$–8 rats. Control rats received chronic vehicle treatment and an IV injection of vehicle on the 2-DG experimental day. The acute group received chronic vehicle followed by 0.3 mg/kg diazepam (IV). The subacute and chronic groups received daily injections of diazepam (5 mg/kg IP) for 3 and 28 days, respectively, followed by a challenge dose of diazepam (0.3 mg/kg IV) on the day of the LCGU measurements. *$p < 0.05$ compared to the control group.

Experiment 4

The aim of these experiments was to determine whether or not there were regional changes in the expression of $GABA_A$ receptor subunit mRNA levels after chronic diazepam treatment, and if these could be related to alterations in $GABA_A$ receptor protein.

Methods. Groups of rats received diazepam either for one day or 21 days via subcutaneous capsules containing diazepam (90 mg) according to the procedure outlined in Experiment 3. Control rats received empty capsules. In situ hybridization on the rat brains was performed according to Laurie et al. (13). Briefly, 14 μm brain sections were incubated overnight at 42°C in hybridization buffer containing ^{35}S-labeled 45-base antisense oligonucleotides. The oligonucleotides were constructed complementary to rat cDNA encoding subunit residues 342–356 for α_1 and 338–352 for γ_2. Sections were then washed in standard saline citrate at 60°C, dried, and apposed to Kodak X-OMAT film together with ^{14}C standards for 3–4 weeks.

The optical densities of the hybridization signals on the resulting autoradiograms were quantified by computer-digitized image analysis; amounts of mRNA were expressed as nCi/g relative to the ^{14}C standards.

Samples of forebrain tissue not used for in situ hybridization were assayed for tissue diazepam levels using the method of Gallager et al. (6).

RESULTS

Experiment 1: 2-Deoxyglucose Autoradiography, Tolerance Study

The IV administration of acute diazepam (0.3 mg/kg caused a significant depression of glucose use in 44 of the 66 brain structures examined. This mean depression was of the order of 20%, and it was widespread, occurring in limbic, cortical, sensory, and motor structures [see (11)]. Following subacute diazepam pretreatment LCGU remained depressed in 30 of the 44 structures that had exhibited a reduction in LCGU after acute treatment ($p < 0.05$). This was particularly noticable in limbic structures such as the hippocampus (Fig. 1). However, tolerance was apparent in the dorsal tegmental nucleus and the locus coeruleus. A partial tolerance was evident in the lateral septal nucleus and in the infralimbic, entorhinal, and parietal cortices (Table 1).

Most structures associated with auditory processing (auditory cortex, inferior colliculus, cochlear nucleus) displayed tolerance to the depressant effects of diazepam upon LCGU after 3 days' pretreatment. Thus, following this treatment period, glucose use was no longer significantly depressed compared to the control group ($p > 0.05$) but was significantly increased compared to the acute group ($p < 0.05$) (Fig. 2). However, the medial geniculate only showed a partial recovery (Table 1). There was also tolerance in structures involved in reflexes associated with the visual system (anterior pretectal area and flocculus) (Table 1). However, tolerance was not apparent after 3 days of diazepam treatment in structures involved in visual processing (visual cortex and lateral geniculate). [For further details, see (11).]

Following chronic diazepam treatment, many limbic and functionally associated structures (hippocampus, raphe nuclei) still exhibited sensitivity to the depressant effects of diazepam ($p < 0.05$) (Fig. 1). However, one striking feature of the patterns of glucose utilization after 28 days of diazepam treatment was that tolerance to the depressant effects of diazepam occurred in the mammillary body and subiculum (Fig. 3). In the mammillary body, for example, glucose use was no longer significantly reduced compared to the control group after 28 days of treatment ($p > 0.05$), but glucose use was significantly greater than that in both the acute and subacute groups ($p < 0.05$). In the lateral habenula and interpeduncular nucleus there was a partial tolerance. Of those structures concerned with motor control, LCGU had returned to control values in the caudate nucleus. A partial recovery occurred in the sensory–motor cortex, substantia nigra, and subthalamic nucleus (Table 1).

It should also be noted that some structures that exhibited a partial recovery after 3 days' treatment, exhibited tolerance after 28 days treatment (e.g., medial geniculate), whereas other structures had still not developed full tolerance after 28 days of treatment (Table 1). These included the entorhinal and parietal cortices and the lateral septal nucleus.

TABLE 1

SUMMARY OF BRAIN STRUCTURES EXHIBITING TOLERANCE TO THE ACUTE DEPRESSANT EFFECTS OF DIAZEPAM UPON LCGU AFTER REPEATED TREATMENT

3-Day Pretreatment		28-Day Pretreatment	
Partial Tolerance	Tolerance	Tolerance	Partial Tolerance
Lateral septal nucleus	Dorsal tegmental nucleus	Mammillary body	Lateral habenula
Parietal cortex	Locus coeruleus	Subiculum	Interpeduncular nucleus
Infralimbic cortex	Auditory cortex	Caudate nucleus	Sensory-motor cortex
Entorhinal cortex	Inferior colliculus	Medial geniculate	Subthalamic nucleus
Medial geniculate	Cochlear nucleus		Substantia nigra
	Flocullus		Lateral septal nucleus
	Anterior pretectal area		Entorhinal cortex
			Parietal cortex

Rats were treated with diazepam (5 mg/kg IP daily) for 3 or 28 days prior to a challenge dose of diazepam (0.3 mg/kg IV) on the day of the LCGU measurements. In the above structures glucose use was significantly depressed after acute diazepam treatment compared to vehicle-treated controls. Tolerance was defined as those structures in which values for glucose use were significantly raised compared to the acute group. A less marked tolerance (partial tolerance) was judged to have occurred if the LCGU value was no longer depressed compared to the controls, yet was also not significantly raised compared to the acute treatment group. For further details of mean values see Laurie and Pratt 1989 and Figs. 1–3.

Experiment 2: 2-Deoxyglucose Autoradiography, Withdrawal Study

Flumazenil exerted markedly different patterns of change in LCGU in drug-naive rats compared to those chronically treated with diazepam. Acute flumazenil (5 mg/kg IV) had no effect on any of the 54 brain regions analyzed [Table 2; (12)] compared to the vehicle control group. These included limbic structures and those associated with sensory and motor function (Table 2). However, in rats chronically treated with diazepam, flumazenil precipitated withdrawal increases in glucose use in the mammillary body, anterior thalamic nuclei, and cingulate cortex (Fig. 4 and Table 2) in particular ($p < 0.01$). These increases in glucose use were substantial; approximately 40% compared to the chronic diazepam group and 13–20% compared to the control group. Less marked increases in glucose use were observed in the basolateral amygdala and the accumbens (approximately 25%) during flumazenil-precipitated diazepam withdrawal. Other structures that exhibited increases in LCGU during withdrawal were in those from the visual and extrapyramidal systems (see Table 2).

Experiment 3: Receptor Autoradiography Study

The level and regional variation in [^{3}H]-flunitrazepam binding was similar to that previously reported in control rats. For example, there were high levels of binding in cortex, hippocampus, mammillary body, and amygdala with more modest levels in other areas such as the nucleus accumbens. Chronic treatment with diazepam by either repeated daily injections or continuous subcutaneous release did not affect total flunitrazepam binding in any of the 47 area investigated [see Table 3; (3)].

The enhancement of flunitrazepam binding by 100 μM GABA varied from structure to structure (range 15–60%) in control rats. There was a significant effect of treatment in the nucleus accumbens, $F(2, 28) = 4.78$, $p < 0.025$, dorsal tegmental nucleus, $F(2, 26) = 4.37$, $p < 0.025$, and central gray, $F(2,$

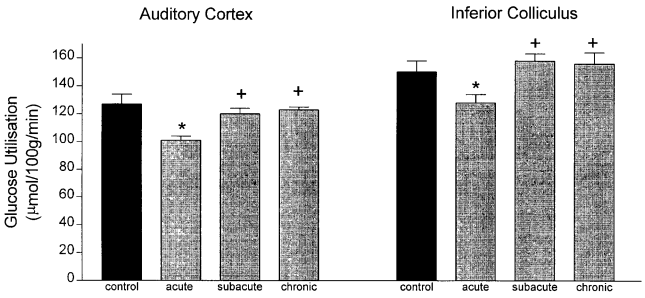

FIG. 2. Tolerance to the depressant effects of diazepam upon LCGU in auditory structures. Data are presented as mean glucose use ±SEM for $n = 7$–8 rats. Control rats received chronic vehicle treatment and an IV injection of vehicle on the 2-DG experimental day. The acute group received chronic vehicle followed by 0.3 mg/kg diazepam (IV). The subacute and chronic groups received daily injections of diazepam (5 mg/kg IP) for 3 and 28 days, respectively, followed by a challenge dose of diazepam (0.3 mg/kg IV) on the day of the LCGU measurements. *$p < 0.05$ compared to the control group; $^+p < 0.05$ compared to the acute diazepam group.

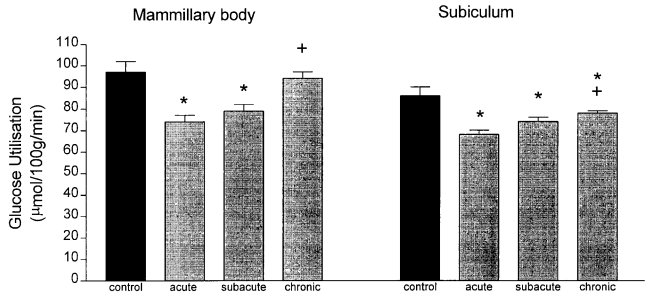

FIG. 3. Tolerance to the depressant effects of diazepam upon LCGU in the mammillary body and subiculum. Data are presented as mean glucose use ±SEM for $n = 7$–8 rats. Control rats received chronic vehicle treatment and an IV injection of vehicle on the 2-DG experimental day. The acute group received chronic vehicle followed by 0.3 mg/kg diazepam (IV). The subacute and chronic groups received daily injections of diazepam (5 mg/kg IP) for 3 and 28 days, respectively, followed by a challenge dose of diazepam (0.3 mg/kg IV) on the day of the LCGU measurements. *$p < 0.05$ compared to the control group; $^+p < 0.05$ compared to the acute diazepam group.

TABLE 2
EFFECT OF DIAZEPAM WITHDRAWAL UPON LCGU

	Control	Acute FZL	Chronic DZP	Precipitated Withdrawal
Limbic structures				
Cingulate cortex	97 ± 4	98 ± 2	89 ± 3	112 ± 4*§
Mammillary body	95 ± 4	97 ± 3	84 ± 3	117 ± 6†§
Anteroventral thalamic nucleus	103 ± 4	106 ± 3	88 ± 4	122 ± 5†§
Anteromedial thalamic nucleus	103 ± 4	103 ± 2	91 ± 5	117 ± 4*§
Subiculum	84 ± 4	83 ± 4	78 ± 4	88 ± 4
Basolateral amygdala	79 ± 4	77 ± 3	70 ± 3	86 ± 3§
Nucleus accumbens	80 ± 5	80 ± 3	74 ± 3	88 ± 3‡
Dorsal tegmental nucleus	94 ± 5	92 ± 6	85 ± 4	95 ± 4
Locus coeruleus	59 ± 2	60 ± 3	58 ± 3	59 ± 2
Dorsal raphe	80 ± 3	79 ± 4	71 ± 2	83 ± 3
Hippocampus CA2 molecular layer	79 ± 3	71 ± 4	71 ± 2	76 ± 2
Sensory/motor structures				
Sensory–motor cortex (layer IV)	100 ± 5	96 ± 2	88 ± 2	102 ± 4
Caudate nucleus	99 ± 4	97 ± 3	91 ± 3	105 ± 2‡
Visual cortex (layer IV)	105 ± 3	99 ± 5	89 ± 4	106 ± 6‡
Anterior pretectal area	85 ± 3	83 ± 2	76 ± 3	92 ± 5‡
Lateral geniculate body	89 ± 4	91 ± 3	73 ± 4	102 ± 6§
Auditory cortex	105 ± 3	99 ± 5	89 ± 4	109 ± 6‡
Inferior colliculus	166 ± 7	153 ± 8	157 ± 5	160 ± 6

Data are expressed as mean glucose use (μmol/100 g/min) with six rats per group.
*$p < 0.05$, †$p < 0.01$ vs. control; ‡$p < 0.05$, §$p < 0.01$ vs. chronic DZP. See experimental methods (section 2) for details of treatment regimes.

29) = 3.80, $p < 0.05$) (Table 3). While reductions in the GABA enhancement of benzodiazepine binding were apparent in the nucleus accumbens by both treatment routes after subacute and chronic diazepam treatment ($p < 0.05$), the reductions in binding in the central gray were only evident after subacute treatment ($p < 0.05$). GABA enhancement of benzodiazepine binding in the dorsal tegmental nucleus was significantly greater ($p < 0.05$) in the chronically treated animals than in the controls. No changes in GABA enhancement of benzodiazepine binding was apparent in structures of the Papez circuit such as the mammillary body and cingulate cortex (Table 3).

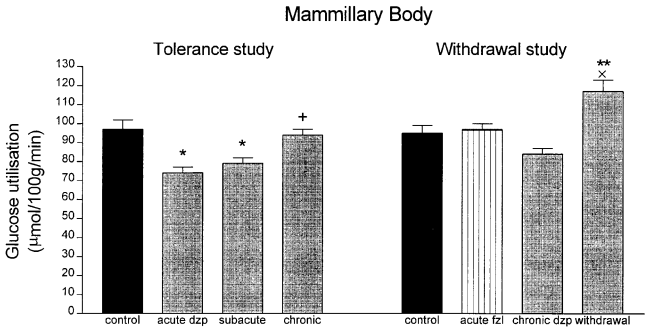

FIG. 4. Comparisons of tolerance- and withdrawal-induced changes in LCGU in the mammillary body after chronic diazepam treatment. See the Method section for treatment schedules. In the tolerance study, *$p < 0.05$ compared to the control group; $^{+}p < 0.05$ compared to the acute diazepam group. In the flumazenil (fzl)-precipitated diazepam withdrawal study, × $P < 0.01$ compared to chronic diazepam, **$p < 0.01$ compared to control.

In a number of anatomically related areas, there was a trend towards an effect of treatment. This included the lateral habenula and the dorsal raphe ($p < 0.1$) [see (3)]. There were no other effects of treatment or treatment route in any other brain area measured.

Experiment 4: In situ Hybridization Study

The effects of chronic exposure to diazepam upon mRNA levels for α_1 and γ_2 subunits are shown in Tables 4 and Figs. 5 and 6. Essentially, there were no changes in the mRNA levels for either subunit in the dentate gyrus or the pyramidal cell layers of the hippocampal fields after chronic diazepam treatment. In structures of the Papez circuit, there were moderate to high levels of hybridization to the α_1 subunit but relatively low levels of hybridization to the γ_2 subunit in control rats. Chronic diazepam treatment did not affect the expression of either subunit ($p > 0.05$). Similarly, we were unable to demonstrate any changes in γ_4 subunit expression after chronic diazepam (19).

Despite moderate amounts of binding to components of the $GABA_A$ receptor in the nucleus accumbens, low levels of mRNA were detected in the accumbens for both subunits in control rats. Interestingly, however, mRNA for both subunits was increased ($p < 0.05$) in the ventral pallidum (an output structure of the nucleus accumbens) after 1 day and chronic diazepam treatment.

There were no changes in subunit expression in cortical regions after chronic treatment, with the exception of the parietal cortex, which displayed an increase in α_1 receptor subunit mRNA after chronic diazepam ($p < 0.05$).

The general lack of effect of treatment on mRNA levels could not be attributable to the rats not receiving sufficient

TABLE 3

$GABA_A$ RECEPTOR AUTORADIOGRAPHY IN LIMBIC STRUCTURES AFTER CHRONIC DIAZEPAM TREATMENT

	[^{3}H]-Flunitrazepam Binding (p mol g^{-1} Tissue)			GABA Enhancement of [^{3}H] Flunitrazepam Binding (% Increase)		
	Control	Subacute	Chronic	Control	Subacute	Chronic
Mammillary body	105 ± 5	116 ± 10	111 ± 8	36 ± 3	47 ± 21	32 ± 13
Subiculum	85 ± 7	89 ± 6	86 ± 5	36 ± 12	38 ± 11	44 ± 10
Cingulate cortex	99 ± 8	103 ± 9	113 ± 10	42 ± 8	28 ± 9	35 ± 7
Nucleus accumbens	74 ± 5	85 ± 3	88 ± 6	53 ± 10	18 ± 5*	40 ± 6*
Hippocampus CA2 mol	142 ± 12	124 ± 8	140 ± 8	35 ± 12	42 ± 16	34 ± 6
Central gray	79 ± 6	83 ± 6	79 ± 4	32 ± 7	21 ± 7*	33 ± 5

Rats were pretreated with diazepam by IP injection (5 mg kg^{-1}) and by subcutanous release from silastic capsules. The above results depict those from the rats receiving IP injections daily for 3 (subacute) and 28 (chronic) days.

*$p < 0.05$ pooled IP and implant groups differ from pooled controls. Each value is the mean ± SE mean of six experiments. For further experimental results and details, see Brett and Pratt (1995).

quantities of drug. Brain diazepam levels were 11.8 ± 3.3, 295 ± 38, and 556 ± 37 ng/g brain tissue in the control, acute, and chronic groups, respectively.

DISCUSSION

2-DG studies: Tolerance and Withdrawal

The acute administration of diazepam produced widespread reductions in LCGU throughout the neuroaxis. As predicted, the results following acute administration do little to resolve the brain structures involved in mediating the various pharmacological effects of diazepam because these effects are superimposed upon one another. The widespread depression of glucose use is likely to reflect the functional consequences of benzodiazepine–receptor occupation, namely altered functional activity in both primary brain regions and those exhibiting neuronal connections with the target area. The pattern of glucose use following 3 days of diazepam pretreatment is consistent with the waning of a sedative effect and the restoration of normal sensory processing. The locus coeruleus is part of the reticular activating system, and is thought to be important in behavioral vigilance and in influencing activity in almost all cortical areas, possibly by enhancing signal processing capabilities (21). Indeed, LCGU of some cortical regions (e.g., parietal and entorhinal cortices) that are

TABLE 4

EFFECT OF ACUTE AND CHRONIC DIAZEPAM TREATMENT ON α_1 $GABA_A$ RECEPTOR SUBUNIT mRNA

	Control	Acute	Chronic
Dentate gyrus	147 ± 4	133 ± 6	150 ± 11
Hippocampus pyramidal cell layer			
CA_1	154 ± 27	153 ± 7	146 ± 5
CA_2	124 ± 11	132 ± 12	116 ± 9
CA_3	93 ± 12	96 ± 6	97 ± 5
Papez circuit			
Cingulate cortex	188 ± 16	169 ± 25	189 ± 15
Anteroventral thalamus	74 ± 6	85 ± 7	98 ± 7
Mammillary body	82 ± 9	67 ± 11	87 ± 4
Parietal cortex	112 ± 7	118 ± 8	140 ± 6*
Basolateral amygdala	37 ± 6	36 ± 3	47 ± 4
Ventral pallidum	73 ± 5	107 ± 16*	110 ± 10*

Data are expressed as mean ± SEM (nCi/g).

*$p < 0.05$ compared to control. Rats received diazepam either for 1 day (acute) or for 21 days (chronic) via subcutaneous capsules containing diazepam. Control rats were implanted with empty capsules.

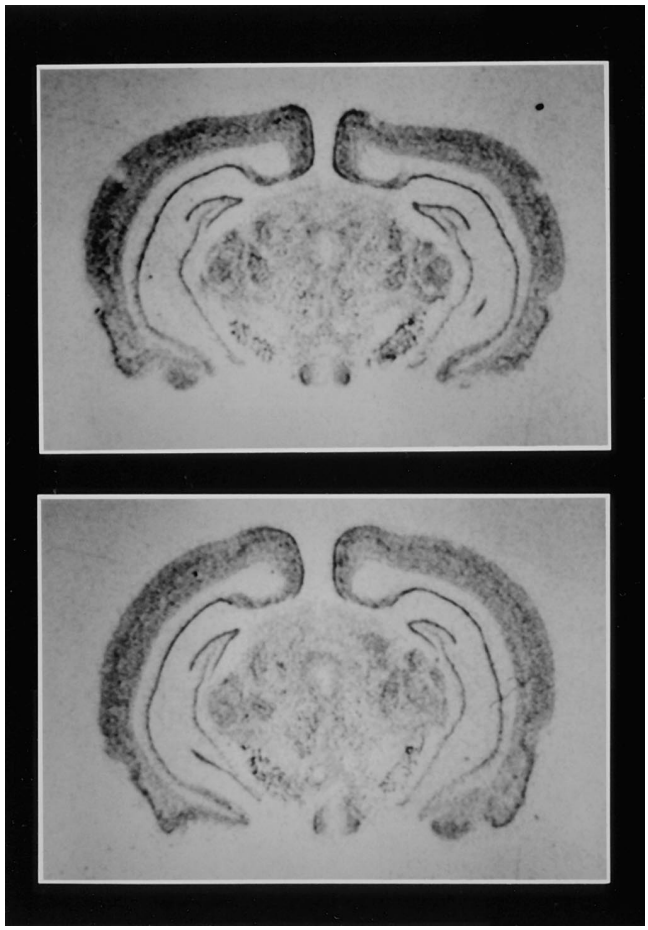

FIG. 5. Lack of effect of chronic diazepam upon mRNA levels for the α_1 subunit of the $GABA_A$ receptor at the level of the hippocampus and mammillary body. Top panel (control), bottom panel (chronic diazepam).

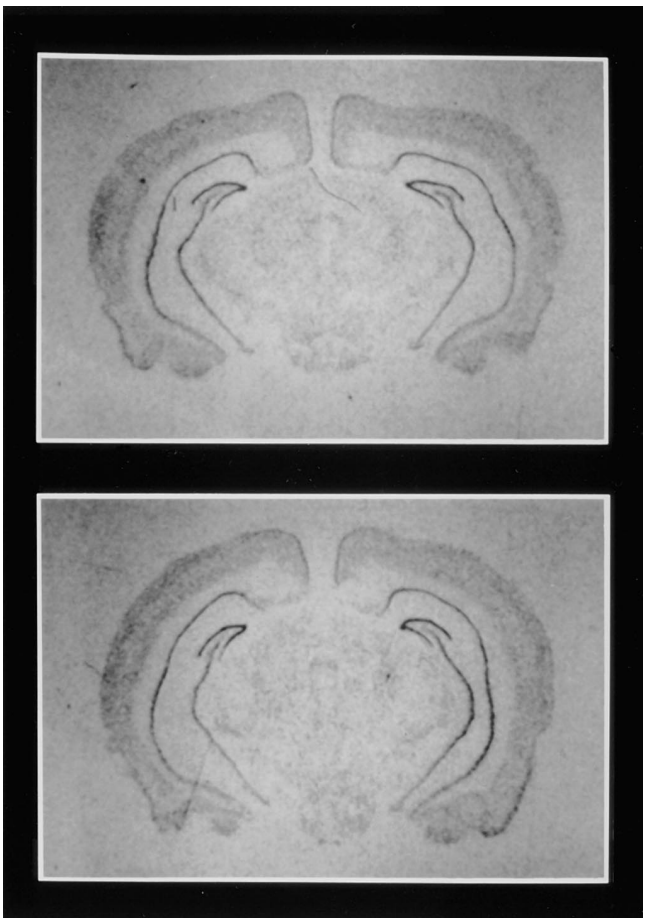

FIG. 6. Lack of effect of chronic diazepam upon mRNA levels for the γ_2 subunit of the $GABA_A$ receptor at the level of the hippocampus and mammillary body. Top panel (control), bottom panel (chronic diazepam).

considered to be involved in sensory association and integration displayed a partial recovery from the effects of diazepam following 3 days of pretreatment. The dorsal tegmental nucleus (DTN), besides being involved in the limbic system, is thought to be associated with the reticular activating system.

We have demonstrated that glucose use in most components of the polysynaptic pathway concerned with the receiving and processing of auditory signals (e.g., cochlear nucleus, inferior colliculus, and auditory cortex) is restored to control levels after 3 days of diazepam pretreatment. This is consistent with their involvement in the acute sedative effects of the drug where sedation may be interpreted as a reduced dedication to process sensory information. This effect was less apparent, however, in sensory areas associated with vision.

Glucose use remained depressed in most other limbic, thalamic, extrapyramidal, and cortical regions, which is in accord with the persistence of the anxiolytic, muscle-relaxant, and anticonvulsant actions of diazepam beyond 3 days of drug administration.

Following chronic diazepam treatment (28 days), the LCGU of a sizeable proportion of the brain regions examined, particularly limbic structures, still exhibited sensitivity to the depressant effects of diazepam. One striking feature of the present study was that glucose use remained depressed in most hippocampal layers but that a substantial functional recovery had occurred in the mammillary body and subiculum. The subiculum is one of the most important efferent relay structures of the hippocampal region, and one of the principal projections from this structure is to the mammillary body (10,25). Thus, it would appear that, after chronic benzodiazepine treatment, the outflow of neuronal activity from the hippocampal formation via the subiculum to the mammillary body had significantly returned towards control levels, despite an apparent lack of recovery of the inputs into, and interregional activity within, the hippocampus.

One interpretation of our results, assuming tolerance to the anxiolytic effect had occurred, is that the hippocampus–subiculum–mammillary body pathway (Papez circuit) is important in mediating the anxiolytic effect of benzodiazepines. Other limbic connections (e.g., the input into the hippocampus from the median raphe) would, therefore, be of lesser importance in the anxiolytic effect, because their functional activity remained depressed.

An interesting point to note is that none of the amygdaloid nuclei showed any change of LCGU in response to diazepam administration, although changes in glucose use in target areas (e.g., mediodorsal thalamic nuclei, frontal cortex, and anterior cingulate cortex) approached significance. It is perhaps surprising in view of the findings that focal injections of benzodiazepines into the basolateral and lateral amygdaloid nuclei produce an anticonflict action (26).

In structures associated with motor control, full tolerance to the depressant effect of diazepam on LCGU occurred in the caudate nucleus after chronic diazepam pretreatment and some degree of recovery, indicating partial tolerance, was apparent in the sensory–motor cortex and several extrapyramidal structures.

This lack of complete recovery is difficult to explain, particularly in view of the likelihood that tolerance to the muscle relaxant, anticonvulsant, and sedative effects had occurred following this period of treatment (8,14). Possibly animals learn to adapt to the motor deficit effects despite a reduction of brain metabolism in some extrapyramidal areas.

In summary, the findings that tolerance to the effects of diazepam upon LCGU does not occur at the same rate for each brain structure indicates that these neuroanatomical substrates are functionally important in tolerance to the various behavioral effects of the drug that occurs at different rates. This raises the question as to whether similar neurochemical adaptive mechanisms proceeding at different rates underlie the changes in LCGU or, whether distinct mechanisms are involved.

Whatever the mechanism(s), this study demonstrates that various brain regions differ in their responses to the continued presence of diazepam and this has important implications for the understanding of tolerance.

Withdrawal Studies

The acute administration of flumazenil (5 mg/kg IV) to drug-naive rats produced no change in LCGU in any of the brain regions examined and no overt changes in behavior. This is consistent with it being a benzodiazepine receptor antagonist. However, in rats undergoing "flumazenil-precipitated diazepam withdrawal," flumazenil induced behavior consistent with anxiety such as agitation and heightened awareness of surroundings such as movement and noise.

When compared to the "chronic diazepam" group, those brain regions responding to precipitated withdrawal were pri-

marily cortical and subcortical structures of the limbic system. In particular, there were increases in glucose use in structures that form part of the Papez circuit of emotion (mammillary body–anterior thalamus–cingulate cortex). In addition, increases in glucose use occurred in the septal nucleus, which provides an input to the Papez circuit, the basolateral amygdala, and the nucleus accumbens. Another neural system that was affected during flumazenil-precipitated withdrawal from diazepam was the visual system. Increases in glucose use occurred in structures involved in visual processing (visual cortex and lateral geniculate) and those involved in the coordination of reflex responses to visual stimuli (e.g., infralimbic cortex and flocculus). These data are consistent with the behavioral effects of apparent heightened anxiety and increased awareness of environmental surroundings noted following flumazenil administration.

In agreement with the findings of this study, others have reported increases in glucose use in the lateral geniculate nucleus, mammillary body, and lateral septum during flumazenil-precipitated withdrawal from diazepam (1). However, direct comparisons between the studies is difficult because they administered diazepam chronically to rats by subcutaneous implant (20 mg/kg/day) and examined fewer brain structures.

It is noteworthy that withdrawal from pentobarbitone and morphine also evokes marked increases in glucose use in the Papez circuit (18). Changes in this circuit could underlie some of the emotional responses experienced during drug withdrawal. Interestingly, only morphine withdrawal evoked substantial increases in LCGU in the mesolimbic reward pathway.

Common Neural Circuits in Tolerance and Withdrawal?

The results from these 2-DG studies provide some support for the hypothesis that diazepam tolerance and withdrawal affect common neuronal circuits in the CNS. In particular, the Papez circuit features strongly in both phenomena, indicating that a common neurochemical adaptive process may be involved. A similar situation would appear to exist for structures within the visual system. However, it is also clear from these studies that distinct neural systems are involved in diazepam tolerance and withdrawal. For example, structures of the auditory system exhibited tolerance to the acute depressant effects of diazepam upon LCGU but exhibited no rebound increase in glucose use during withdrawal. Moreover, some structures such as the nucleus accumbens and the basolateral amygdala appeared to be uniquely affected during withdrawal.

Taken together, these data highlight the importance of identifying the neural systems recruited in response to drug treatment as a means of providing a more rational basis for investigating the underlying neurochemical mechanisms in the identified regions.

Neurochemical Events Underlying the Changes in Brain Functional Activity

The results from the GABA receptor autoradiography studies and the in situ hybridization studies provide little support for changes in $GABA_A$ receptor characteristics or GABA receptor subunit gene expression as being responsible for the changes in neural activity, in particular brain structures, during diazepam tolerance and withdrawal. Thus, no changes in benzodiazepine binding per se or changes in the coupling between the GABA and benzodiazepine binding site (as determined by GABA enhancement of benzodiazepine binding) were apparent in the majority of brain regions measured. This included structures of the Papez circuit where marked changes in LCGU were observed in the 2-DG studies. Similarly, we observed minimal changes in low- and high-affinity GABA binding after chronic diazepam (3).

One brain pathway where some changes appeared to exist was the nucleus accumbens–lateral habenula pathway. Both these structures showed reductions in benzodiazepine/GABA coupling after subacute and chronic treatment. Further support for this finding stems from the results of the in situ hybridization studies where expression of α_1 subunit mRNA in the ventral pallidum (which receives a GABAergic projection from the accumbens) was reduced after both 1 day and chronic exposure to diazepam.

It is clear from numerous studies that the nucleus accumbens is critical to the reinforcing properties of many drugs that cause dependence (2). However, its exact role in benzodiazepine addiction remains unclear. Furthermore, the relationship between altered GABA receptor characteristics in this region after relatively short treatment periods yet no apparent change in glucose use until diazepam withdrawal requires further examination.

The results of our GABA autoradiography and gene expression studies are difficult to compare with the findings of some other groups who found downregulation of receptors and reduced mRNA levels after chronic benzodiazepine treatment. However, these groups used larger dosing regimes (27,28). More recently, Holt et al. (9) in an investigation of a wide range of α, β, and γ subunit isoforms mRNAs in rat cortex, provided evidence for $GABA_A$ receptor subunit isoform switching after chronic diazepam treatment.

In summary, there is clear evidence from our 2-DG studies that common and distinct neural circuits are recruited during diazepam tolerance and withdrawal. The underlying neurochemical mechanisms responsible for these changes remain to be resolved. It would appear that changes at the level of the $GABA_A$ receptor may only have a modest contribution to the phenomena of tolerance and withdrawal, and, that changes in the characteristics of other receptors, such as the glutamate receptor may have a larger role to play. Indeed, there is emerging evidence that supports a role for glutamate receptors in the various psychopharmacological effects of benzodiazepines (20,23).

ACKNOWLEDGEMENTS

We thank the Wellcome Trust and Mental Health Foundation for financial support and the Scottish Hospitals Endowments Research Trust for the provision of laboratory facilities. Diazepam was donated by Roche Products Ltd.

REFERENCES

1. Ableitner, A.; Wuster, M.; Herz, A.: Specific changes in local cerebral glucose utilization in the rat brain induced by acute and chronic diazepam. Brain Res. 359:49–56; 1985.
2. Altman, J.; Everitt, B. J.; Glautier, S.; Morkov, A.; Nutt, D.; Oretti, R.; Phillips, G. D.; Robbins, T. W.: The biological, social and clinical base of drug addition; Commentary and debate. Psychopharmacology (Berlin) 125:285–345; 1996.
3. Brett, R. R.; Pratt, J. A.: Changes in benzodiazepine–GABA receptor coupling in an accumbens–habenula circuit after chronic diazepam treatment. Br. J. Pharmacol. 116:2375–2384; 1995.

4. File, S. E.: Tolerance to the behavioural actions of benzodiazepines. Neurosci. Biobehav. Rev. 9:113–121; 1985.
5. Friedman, L. K.; Gibbs, T. T.; Farb, D. H.: γ-Aminobutyric acid (A) receptor regulation: Heterologous uncoupling of modulatory site interactions induced by chronic steroid, barbiturate, benzodiazepine, or GABA treatment in culture. Brain Res. 707:100–109; 1996.
6. Gallager, D. W.; Malcolm, A. B.; Anderson, S. A.; Gonsalves, S. F.: Continuous release of diazepam: Electrophysiological, biochemical and behavioural consequences. Brain Res. 342:26–36; 1985.
7. Gallager, D. W.; Marley, R. J.; Hernandez, T. D.: Biochemical and electrophysiological mehcanisms underlying benzodiazepine tolerance and dependence. In: Pratt, J. A., ed. Biological bases of drug tolerance and dependence. London: Academic Press; 1991:49–70.
8. Gonsalves, S. F.; Gallager, D. W.: Time course for development of anti-convulsant tolerance and GABA ergic subsensitivity after chronic diazepam. Brain. Res. 405:94–99; 1987.
9. Holt, R. A.; Bateson, A. N.; Martin, I. L.: Chronic treatment with diazepam or abecarnil differentially affects the expression of $GABA_A$ receptor subunit mRNAs in the rat cortex. Neuropharmacology 35:1457–1463; 1996.
10. Irle, E.; Markowitsch, H. J.: Connections of the hippocampal formation, mamillary bodies, anterior thalamus and cingulate cortex: A retrograde study using horseradish peroxidase in the cat. Exp. Brain Res. 47:79–94; 1982.
11. Laurie, D. J.; Pratt, J. A.: Local cerebral glucose utilization following subacute and chronic diazepam treatment; Differential tolerance. Brain Res. 504:101–111; 1989.
12. Laurie, D. J.; Pratt, J. A.: Flumazenil induces localised increases in glucose utilization during diazepam withdrawal in rats. Brain Res. 631:277–286; 1993.
13. Laurie, D. J.; Seeburg, P. H.; Wisden, W.: The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. J. Neurosci. 12:1063–1076; 1992.
14. Matsubara, K.; Matsushita. A.: Changes in ambulatory activities and muscle relaxation in rats after repeated doses of diazepam. Psychopharmacology (Berlin) 77:279–283; 1982.
15. McCabe, R. T.; Olsen, R. W.; Yezuita, J. P.; Wamsley, J. K.: Osmotic shock: A method to eliminate endogenous γ-aminobutyric acid and account for the influence on benzodiazepine binding affinity in autoradiographic studies. J. Pharmacol. Exp. Ther. 245:342–349; 1988.
16. Miller, L. G.; Greenblatt, D. J.; Roy, R. B.; Summer, W. R.; Shader, R. I.: Chronic benzodiazepine administration. II. Discontinuation syndrome is associated with upregulation of γ-aminobutyric $acid_A$ receptor complex binding and function. J. Pharmacol. Exp. Ther. 246:177–182; 1988.
17. Petursson, H.: The benzodiazepine withdrawal syndrome. Addiction 89:1455–1459; 1994.
18. Pratt, J. A.: Psychotropic drug tolerance and dependence: Common underlying mechanisms? In: Pratt, J. A., ed. The biological bases of drug tolerance and dependence. London: Academic Press; 1991:1–28.
19. Pratt, J. A.; Brett, R. R.; Laurie, D. J.: Expression of α_1, α_4, and γ_2 $GABA_A$ receptor subunit mRNAs in rat brain after chronic low dose diazepam treatment. Br. J. Pharmacol. 116:397P; 1995.
20. Pratt, J. A.; Gray, A.: Involvement of AMPA/kainate receptors in diazepam-induced conditioned place preference in rats. Soc. Neurosci. Abstr. 22:814.5; 1996.
21. Saper, C. P.: Function of the locus coeruleus. Trends Neurosci. 10:343–344; 1987.
22. Sokoloff, L.; Reivich, M.; Kennedy, C.; Des Rosiers, M. H.; Patlek, C. S.; Pettigrew, K. D.; Sakurada, O.; Shinohara, M.: The ^{14}C-deoxyglucose method for the measurement of local cerebral glucose utilisation: Theory, procedure and normal values in the conscious and anaesthetised albino rat. J. Neurochem. 28:897–916; 1977.
23. Stephens, D. N.: A glutamatergic hypothesis of drug dependence: Extrapolations from benzodiazepine receptor ligands. Behav. Pharmacol. 6:425–446; 1995.
24. Stephens, D. N.; Schneider, H. H.: Tolerance to the benzodiazepine diazepam in an animal model of anxiolytic activity. Psychopharmacology (Berlin) 87:322–327; 1986.
25. Swanson, L. W.; Cowan, W. M.: An autoradiographic study of the organisation of the efferent connections of the hippocampal formation in the rat. J. Comp. Neurol. 172:49–84; 1977.
26. Thomas, S. R.; Lewis, M. E.; Iversen, S. D.: Correlations of [^{3}H]diazepam binding density with anxiolytic locus in the amygdaloid complex of the rat. Brain Res. 342:85–90; 1985.
27. Tietz, E. I.; Huang, X.; Weng, X.; Rosenberg, H. C.; Chiu, T. H.: Expression of α_1, α_5, and γ_2, $GABA_A$ receptor subunit mRNAs measured in situ in rat hippocampus and cortex following chronic flurazepam administration. J. Mol. Neurosci. 4:277–292; 1993.
28. Tietz, E. I.; Rosenberg, H. C.; Chiu, T. H.: Autoradiographic localization of benzodiazepine receptor downregulation. J. Pharmacol. Exp. Ther. 236:284–292; 1986.
29. Young, W. S.; Kuhar, M. J.: Radiohistochemical localization of benzodiazepine receptors in rat brain. J. Pharmacol. Exp. Ther. 212:337–346; 1980.