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Changes in benzodiazepine-GABA receptor coupling in an accumbens-habenula circuit after chronic diazepam treatment

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1 The effects of subacute and of chronic diazepam treatment upon binding to the $GABA_A$ receptor have been examined by use of receptor autoradiography for determining flunitrazepam (FNZP) binding, GABA enhancement of FNZP binding, SR 95531 2-(3'-carboxy-2',propyl)-3-amino-6-*p*-methoxyphenylpyridazinium bromide) binding and GABA binding in parallel sections from rat brain. Prior to the autoradiographic procedures, a behavioural assessment of the rats was made in the elevated plus-maze test of anxiety.

2 Rats receiving diazepam either subacutely (3 days) or chronically (28 days) by both continuous release, from previously implanted subcutaneous silastic capsules, or by daily injection (5 mg kg^{-1}) did not display changes in FNZP or GABA binding in any of the 47 brain structures analysed. Similarly, there were no significant effects of treatment upon mean total entries or on the open:total ratio for entries in the elevated plus-maze.

3 There were reductions in the GABA enhancement of FNZP binding in the nucleus accumbens and central grey after subacute diazepam treatment. This effect persisted in the nucleus accumbens after chronic treatment. Less marked effects occurred in the lateral habenula, dorsal raphe and substantia nigra pars compacta. In the dorsal tegmental nucleus, GABA enhancement of FNZP binding was enhanced after chronic treatment and this was accompanied by reductions in SR 95531 binding. Treatment did not otherwise affect SR 95531 binding, with the exception of the dorsal raphe where binding was decreased after subacute treatment.

4 In general, the patterns of binding produced by the two different treatment routes were very similar. However, SR 95531 binding was lower in certain hippocampal fields in the i.p. treated animals compared to the rats implanted with silastic capsules.

5 It is concluded that repeated administration of diazepam evokes changes in benzodiazepine and GABA receptor coupling, and to a lesser extent changes in low affinity GABA binding, in certain interrelated brain structures of which an accumbens-habenula circuit is a central feature. These changes occur soon after the initiation of diazepam treatment, suggesting that they are unlikely to account for tolerance to the anxiolytic effects of diazepam but may trigger and/or accompany other critical neurochemical events.

Keywords: $GABA_A$ receptor; receptor autoradiography; chronic diazepam; nucleus accumbens; benzodiazepine-GABA coupling

Introduction

There is considerable concern about the dependence liability of benzodiazepines (BZs) since this may occur following treatment with therapeutic doses. It is well established that tolerance occurs very rapidly to the sedative effects of this group of drugs, and rapidly to the anticonvulsant effects; tolerance to the anxiolytic effect has been more difficult to establish in human subjects. In animal tests, however, tolerance to the anxiolytic effects of various benzodiazepines has been demonstrated (Gallager *et al.*, 1991). The mechanism underlying this tolerance remains uncertain. It is unlikely to be due to altered pharmacokinetics (Greenblatt & Shader, 1986). Downregulation of BZ receptor number has been shown with chronic high dosage regimes, by radioligand binding to brain homogenates (Chiu & Rosenberg, 1978; Rosenberg & Chiu, 1981). Some workers using low dose regimes have demonstrated downregulation of the BZ receptor (Miller *et al.*, 1988a, Allan *et al.*, 1992) and, interestingly, upregulation upon withdrawal (Miller *et al.*, 1988b). Many investigators, however, have shown no change, either in number or affinity of BZ receptors (Möhler *et al.*, 1978; Rauch & Gallager, 1983; Stephens & Schneider, 1985, Heninger & Gallager, 1988).

The pharmacological actions of BZs are believed to be mediated by facilitation of the actions of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). The $GABA_A$ receptor complex is an oligomeric transmembrane protein with binding sites for GABA and BZs, in association with a chloride ion channel (Sieghart, 1992). It is therefore possible that tolerance is associated with alterations in other components of the complex. Electrophysiological studies have shown subsensitivity to GABA following chronic diazepam treatment (Gallager *et al.*, 1991). However, while some studies have found no change in GABA receptor binding (Rauch & Gallager, 1983; Heninger & Gallager, 1988), other investigations have revealed a decrease (Möhler *et al.*, 1978) or an increase (Gallager *et al.*, 1984b; 1985; Marangos & Crawley, 1982; Abbracchio *et al.*, 1983) in binding to GABA receptors. This question is complicated by the existence of different affinity states of the GABA receptor; BZs are thought to modulate the low-affinity form rather than the high-affinity one. There is some evidence that chronic BZ treatment may convert the low-affinity GABA receptor to a high-affinity, desensitized, form (Gallager *et al.*, 1984b; 1985). Alteration in the phosphorylation state of the GABA receptor subunits is a possible mechanism for a change in sensitivity to GABA (Swope *et al.*, 1992). Another explanation for subsensitivity to exogenously applied GABA might be that the coupling between the BZ receptor and the GABA receptor is reduced. There is evidence for this in studies which have demonstrated a reduction in the ability of GABA to enhance BZ binding after chronic BZ treatment (Gallager *et al.*, 1984a; Mele *et al.*, 1984; Tietz *et al.*, 1989), although Stephens & Schneider (1985) found a change in the opposite direction.

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The studies which have attempted to determine the mechanism of tolerance to BZs vary greatly in the drugs used and the treatment regimes. Furthermore, most of the studies employ grossly dissected brain regions, which may mask changes in specific areas. Two studies have employed receptor autoradiography. Tietz *et al.* (1986) found reductions in BZ binding in some layers of the hippocampus in rats after chronic treatment. Isihara *et al.* (1993) found reductions in the binding of [^{3}H]-muscimol in a number of brain areas after chronic BZ treatment in mice. Both of these studies used high dose regimes.

We have adopted a different approach to investigate the processes involved in benzodiazepine tolerance and withdrawal. Before an understanding of the cellular and molecular mechanisms underlying tolerance can be explored in detail, it is important to have some knowledge of the brain structures and neural circuits functionally affected as a consequence of chronic drug treatment. We have therefore used the technique of 2-deoxyglucose autoradiography for measuring local rates of cerebral glucose use to identify the brain regions functionally important in BZ tolerance and withdrawal (Laurie & Pratt, 1989; 1993; Pratt, 1991). These studies have implicated the Papez circuit as well as structures associated with sensory processing in tolerance to low-dose diazepam treatment.

The aim of the present study was therefore to determine if the identified regional changes in brain functional activity are associated with regional changes in the binding characteristics of the $GABA_A$ receptor in an attempt to elucidate further the neuronal mechanisms underlying tolerance. We have investigated whether changes occur in BZ receptor binding, low- and high-affinity GABA receptor binding, or in the coupling between the BZ receptor and the low-affinity GABA receptor, using receptor autoradiography in parallel sections. No previous study has attempted to study simultaneously, and by receptor autoradiography, the effect of chronic BZ treatment on the BZ and GABA receptor, and on the coupling between them.

Our original intention was to investigate the biochemical changes in animals in which tolerance to the anxiolytic effects of DZP was demonstrable behaviourally. However, in the course of earlier behavioural studies, it was discovered that the chronic handling protocol implicit in this treatment mitigated the acute anxiolytic effect of DZP in a parallel group receiving chronic vehicle pretreatment (Brett & Pratt, 1990), thus preventing any demonstration of tolerance, and was to some extent anxiolytic in itself (Brett, 1992). Other preliminary work indicated some evidence for changes in BZ-GABA receptor coupling induced by chronic handling (Brett & Pratt, 1990; however, this phenomenon proved not to be robust (Brett, 1992). There is, however, considerable evidence from the work of Biggio's group that handling habituation modifies GABA receptors (Corda & Biggio, 1986). In order to investigate further both the behavioural and biochemical effects of handling, therefore, we compared two treatment regimes, administering DZP once-daily by i.p. injection as in the previous studies or by continuous release from subcutaneously implanted silastic capsules, a regime which reduced handling.

Methods

Animals

Male Long-Evans rats (bred at Strathclyde University) weighing 215–270 g were housed in groups of 3–4, maintained on a 12 h light/dark cycle (lights on at 06 h 00 min) and allowed free access to food and water.

Experimental design

Three groups of rats ($n=8-11$) received once daily i.p. injections, either 28 days vehicle (control i.p.), 25 days vehicle and 3 days DZP 5 mg kg^{-1} (subacute i.p.) or 28 days DZP 5 mg kg^{-1} (chronic i.p.). Four further groups ($n=8-11$) were each implanted with two silastic capsules, either empty (control implanted, and subacute implanted) or filled with DZP (chronic implanted, and chronic implanted plus i.p.). The chronic implanted plus i.p. group also received daily i.p. injections of vehicle for 28 days. On days 26–28, the other 3 implanted groups received i.p. vehicle (control implanted and chronic implanted) or DZP 5 mg kg^{-1} i.p. (subacute implanted). All capsules were removed on day 28. Animals were tested in the elevated plus-maze on day 29. All animals were killed on day 30 and the brains dissected and frozen for autoradiography.

Drug treatment

I.p. injection: DZP was suspended in 0.9% saline containing 1% Tween 20. Silastic capsules: Capsules were prepared and implanted according to the method of Gallager *et al.* (1985), with minor modifications. Briefly, 63 mm lengths of silastic tubing (0.095" o.d., 0.065" i.d.; Dow-Corning) were filled with DZP (previously recrystallized in ethanol and ground to a fine powder) and sealed with medical grade silastic adhesive (Dow-Corning). Control capsules were of the same size and materials, but empty. Before implantation, the capsules were immersed in absolute ethanol for 30 min and then floated in 1% bovine serum albumin for 30 min. The capsules were inserted subcutaneously under anaesthesia through an incision in the skin of the back. In order to maintain consistent release over the 28 day period, two capsules were implanted on day 1 and an additional capsule (through a separate incision) on day 15. These were withdrawn from the animals (again through a separate incision) on day 28. This treatment protocol is similar to that employed by Gallager *et al.* (1985), who demonstrated that rats implanted with silastic capsules containing DZP displayed relatively constant levels of DZP in the blood over the treatment period.

Behavioural testing

Animals were tested for 15 min in the elevated plus-maze 30 min after an i.p. injection of vehicle or DZP (2.5 mg kg^{-1}) following the procedure previously described by Brett & Pratt (1990). Briefly, the plus-maze consisted of two opposite open arms 9 × 44 cm with a 3 cm lip and two opposite enclosed arms 9 × 44 × 15 cm extending from a central area 9 × 9 cm, and was elevated to a height of 1 m. The number of entries into, and time spent in, each type of arm were recorded by an observer present in the room, and the total number of entries and the open:total ratios for number of entries and time were calculated. Injections on the test day were given in the test room. Behavioural testing was carried out between 09 h 30 min and 13 h 00 min.

Receptor Autoradiography

The animals were killed by cervical dislocation, decapitated, and the brains removed and frozen in isopentane prechilled to −42°C, coated in embedding medium and stored at −70°C until sectioning. 20 μm coronal sections were cut from selected brain areas in a cryostat maintained at −22°C, and mounted onto room temperature gelatine-chrome alum subbed slides. At each level, two sections were taken for total binding and one for non-specific binding for each of the ligands. The slides were dried in a stream of air for 1 h, packed into boxes containing silica gel 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 (McCabe *et al.*, 1988a) by a 3 min wash in room temperature distilled deionised 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 enhancement of flunitrazepam binding Sections were incubated for 90 min with 1 nM [^{3}H]-FNZP (87 Ci mmol^{-1}; New England Nuclear) in 50 mM Tris-citrate buffer pH 7.1 containing 200 mM NaCl in the absence (total binding) or the presence (non-specific binding) of 2 μM clonazepam (Roche). For GABA enhancement of FNZP binding, both the total and the non-specific binding solutions also contained 100 μM GABA (Sigma). 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 Young & Kuhar, 1980).

*SR 95531 (2-(3-carboxy-2-'-propyl)-3-amino-6-*p*-methyoxyphenylphyridazinium bromide) binding* For the measurement of low-affinity GABA binding, sections were incubated for 30 min with 6 nM [^{3}H]-SR 95531 (44 Ci mmol^{-1}; New England Nuclear) in 100 mM Tris-citrate buffer pH 7.1 in the absence (total binding) or the presence (non-specific binding) of 10 mM GABA. Following a brief dip in ice-cold buffer, slides were given a single 1 min rinse in ice-cold buffer, followed by a dip in ice-cold distilled deionised water to remove buffer salts (Bristow & Martin, 1988).

GABA binding For the measurement of high-affinity GABA binding, sections were incubated for 30 min with 50 nM [^{3}H]-GABA (35 Ci mmol^{-1}; New England Nuclear) in 50 mM Tris-HCl (pH 7.4) containing 190 mM sucrose, and including 100 μM (±)-baclofen to displace any binding to $GABA_B$ receptors. Non-specific binding was defined by 100 μM isoguvacine. Following a brief dip in ice-cold buffer, slides were given a single 20 s rinse in ice-cold buffer, followed by a dip in ice-cold distilled deionised water to remove buffer salts (Bristow & Martin, 1988).

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 (^{3}H-Hyperfilm; Amersham) in light-tight cassettes, together with a set of precalibrated ^{3}H standards (Amersham) for 8–9 days ([^{3}H]-FNZP and GABA enhancement of [^{3}H]-FNZP binding) or 23–25 days ([^{3}H]-SR 95531 binding and [^{3}H]-GABA binding). The films were developed according to the manufacturer's instructions (Kodak D19 developer, Kodak FX-24 fixer, Kodak Photoflo 600 rinse).

Optical density measurements were determined with a computer-based image analyser (MCID) and converted to pmol g^{-1} tissue by reference to ^{3}H standards (Amersham). Corrections to the values for the radiation standards were made to allow for the different white matter content of brain structures, according to the classification of Geary & Wooten (1985). For each of the 47 structures analysed, including cortical areas, primary auditory and visual areas and limbic and functionally associated areas, bilateral measurements were taken from as many sections as possible (usually 2–4) and averaged.

The non-specific binding of [^{3}H]-FNZP was very low (less than 2%) and difficult to measure accurately as it was indistinguishable from background; it did not appear to vary with region or with treatment. The analysis was therefore performed on total [^{3}H]-FNZP binding, since 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.

Non-specific [^{3}H]-SR 95531 binding varied between 20% and 50% according to the brain structure, and was subtracted from the total binding.

Non-specific [^{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 i.p. injection group were analysed by 2-way analysis of variance with drug treatment and treatment route as the factors. A separate one-way analysis of variance was used to compare the three chronic groups (chronic i.p., chronic capsules, and chronic capsules plus i.p. injection). Analysis of variance was followed by Neuman-Keuls multiple range test where appropriate.

Results

Elevated plus-maze behaviour

There were no significant effects of treatment or treatment route on the mean total entries, or on the open/total ratio for entries, and no interaction between the two factors (Table 1).

There was a significant effect of treatment route on the open/total ratio for time (F 1,40 = 4.92, $P < 0.05$). The ratios were lower in the capsule-implanted animals than the i.p. treated animals. There was no effect of treatment, and no interaction between treatment and treatment route.

Receptor autoradiography

[^{3}H]-FNZP binding The level and regional variation in [^{3}H]-FNZP binding was similar to that observed in other studies of BZ receptor binding (e.g. Young & Kuhar, 1980; Unnerstall *et al.*, 1981). There were high levels of binding in cortex, hippocampus and dentate gyrus (particularly the molecular layer), mammillary body, lateral amygdala and medial septum. Moderate levels of binding were found in other limbic and associated structures, such as the dorsal and ventral tegmental nuclei and nucleus accumbens. Rather lower levels were found in the thalamic nuclei, medial geniculate and lateral habenula. In agreement with other authors (Niehoff & Kuhar, 1983; Marcel *et al.*, 1986; Tietz *et al.*, 1986), there were clear postero-anterior gradients of BZ receptor density in the cingulate cortex, amygdala and substantia nigra pars reticulata.

Table 1 Effect of diazepam (2.5 mg kg^{-1}) on behaviour in the elevated plus-maze in rats receiving subacute and chronic diazepam by i.p. injection or subcutaneously via silastic capsules

	I.p. injection			*Implant*			
	Control	*Subacute*	*Chronic*	*Control*	*Subacute*	*Chronic*	*Chronic plus i.p.*
Total entries	30.6 ± 3.5	35.0 ± 4.0	32.1 ± 5.9	25.4 ± 3.4	31.5 ± 5.3	21.0 ± 3.9	23.4 ± 4.9
Open:total ratio - entries	0.34 ± 0.05	0.33 ± 0.03	0.32 ± 0.05	0.28 ± 0.02	0.34 ± 0.04	0.30 ± 0.04	0.26 ± 0.04
Open: total ratio - time	0.24 ± 0.08	0.24 ± 0.06	0.27 ± 0.09	0.11 ± 0.03*	0.20 ± 0.06*	0.12 ± 0.05*	0.18 ± 0.10

Values are mean total entries or mean open: total ratio ± s.e.mean. *Pooled implant groups differ from pooled i.p. groups, $P < 0.05$.

There were no significant effects of treatment or treatment route on [^{3}H]-FNZP binding, in any brain area, and no treatment × treatment route interaction (Table 2). There were also no significant differences between the three chronic groups.

GABA enhancement of [^{3}H]-FNZP binding In control rats, the mean enhancement of [^{3}H]-FNZP binding by 100 μM GABA over all structures measured was of the order of 35% (range 15%–60%). This is similar to that described by McCabe *et al.* (1988a), but less than that found by Unnerstall *et al.* (1981), who found increases of between 70% and 230%, with 10 μM GABA. There was considerable within-group variation in GABA enhancement of BZ binding.

There were significant effects of treatment on GABA enhancement of [^{3}H]-FNZP binding in the dorsal tegmental nucleus (F 2,26 = 4.37, $P<0.025$), nucleus accumbens (F 2,28 = 4.78, $P<0.025$), central grey (F 2,29 = 3.80, $P<0.05$) (Table 3).

Enhancement of binding in the dorsal tegmental nucleus was significantly greater ($P<0.05$) in the chronically treated animals than in controls, in the nucleus accumbens significantly lower in both subacutely and chronically treated animals compared with controls, and in the central grey significantly ($P<0.05$) lower in subacutely treated animals as compared with controls (Table 3).

In a number of anatomically releated areas, there was a trend towards an effect of treatment, significant only at the 10% level. These areas were: dorsal raphe, lateral habenula and substantia nigra pars compacta (Table 3).

There was an effect of treatment route on GABA enhancement of [^{3}H]-FNZP binding in the lateral part of the inferior colliculus (F 1,28 = 5.55, $P<0.05$). Enhancement of binding was lower in i.p. treated animals than in implanted animals.

There were no other significant effects of treatment or treatment route in any other brain area measured. There were also no significant differences between the three chronic groups.

[^{3}H]-SR 95531 binding Tenfold higher levels of [^{3}H]-SR 95531 binding were found than previously reported (Bristow &

Table 2 [^{3}H]-flunitrazepam binding (total binding, pmol g^{-1} tissue) in rats after subacute and chronic diazepam treatment

	I.p. injection			*Implant*			
	Control	*Subacute*	*Chronic*	*Control*	*Subacute*	*Chronic*	*Chronic plus i.p.*
dorsal tegmental nucleus	84±5	76±5	68±5	75±3	78±3	70±5	78±2
dorsal raphe	79±7	82±5	82±7	78±6	80±5	77±6	87±5
nucleus accumbens	74±5	85±3	88±6	72±6	82±4	79±5	77±5
central grey	79±6	83±6	79±4	80±6	81±4	79±8	84±3
lateral habenula	52±4	51±4	52±6	51±4	52±4	52±3	58±3
substantia nigra pars compacta	57±3	55±3	59±3	56±5	52±3	57±3	58±4
substantia nigra pars reticulata	89±10	88±8	85±7	90±10	89±12	87±8	95±8
medial mammillary body	105±5	116±10	111±8	112±10	101±7	95±7	101±4
dentate gyrus	138±12	130±9	132±9	136±10	127±10	132±10	135±4
CA2-oriens	94±9	93±4	98±5	98±7	94±6	92±10	100±3
CA2-pyramidal	93±6	86±4	92±6	88±6	88±6	85±7	91±2
CA2-radiatum	97±7	94±5	96±7	99±6	95±6	89±7	98±2
CA2-molecular	142±12	124±8	140±8	136±11	131±10	126±14	137±3
subiculum	85±7	89±6	86±5	91±8	91±4	92±7	95±4
lateral septum	53±7	57±4	58±6	54±8	55±5	59±6	61±2
caudate	53±3	56±3	57±3	52±3	51±3	53±3	54±2
visual cortex	170±9	103±9	98±12	96±8	107±4	98±8	110±5
anterior cingulate cortex	99±8	103±9	113±10	96±10	105±9	94±11	99±3

Each value is the mean ± s.e.mean of 6 experiments

Table 3 GABA enhancement of [^{3}H]-flunitrazepam binding (% increase) in rats after subacute and chronic diazepam treatment

	I.p. injection			*Implant*			
	Control	*Subacute*	*Chronic*	*Control*	*Subacute*	*Chronic*	*Chronic plus i.p.*
dorsal tegmental nucleus	15±13	18±9	51±16	35±6	23±10	44±3*	30±5
dorsal raphe	39±4	25±4	43±17	44±6	19±11^0	40±11	39±11
nucleus accumbens	53±10	18±5*	40±6*	61±14	39±10*	35±7*	48±12
central grey	32±7	21±7*	33±5	34±11	12±5*	41±10	26±6
lateral habenula	35±13	27±7^0	31±9^0	58±12	22±11^0	22±7^0	22±4
substantia nigra pars compacta	22±6	27±7	9±5†	22±6	34±8	18±10†	24±7
substantia nigra pars reticulata	29±8	33±10	24±7	32±5	18±5	19±9	26±6
medial mammillary body	36±3	47±21	32±13	29±14	54±17	72±8	59±10
dentate gyrus	38±11	37±7	40±10	44±6	40±11	38±5	42±5
CA2-oriens	54±9	41±16	43±4	31±6	34±8	39±9	36±5
CA2-pyramidal	38±8	38±12	35±5	28±6	26±6	36±8	35±6
CA2-radiatum	35±6	29±10	42±11	28±6	25±7	40±11	32±5
CA2-molecular	35±12	42±16	34±6	33±6	32±9	36±9	32±8
subiculum	36±12	38±11	44±10	54±20	27±8	28±7	36±5
lateral septum	23±11	20±6	39±13	34±13	42±16	22±4	25±7
caudate	24±3	18±4	25±8	33±5	31±17	25±3	31±5
visual cortex	23±10	34±11	62±29	48±16	26±12	40±10	46±4
anterior cingulate cortex	42±8	28±9	35±7	49±10	35±9	54±21	49±4

Each value is the mean ± s.e.mean of 6 experiments. * Pooled i.p. and implant groups differ from pooled controls, $P<0.05$. ^{0} Pooled i.p. and implant groups differ from pooled controls, $P<0.1$. † Pooled chronic i.p. and chronic implant groups differ from pooled subacute groups, $P<0.1$.

Table 4 [^{3}H]-SR 95531 binding (specific binding, pmol g^{-1} tissue) in rats after subacute and chronic diazepam treatment

	I.p. injection			*Implant*			
	Control	*Subacute*	*Chronic*	*Control*	*Subacute*	*Chronic*	*Chronic plus i.p.*
dorsal tegmental nucleus	98±5	75±6*	66±6*	93±9	81±8*	87±6*	76±7
dorsal raphe	107±8	90±5*	93±6	134±16	86±14*	112±9	102±10
nucleus accumbens	111±10	94±8	123±19	123±11	107±8	103±7	114±15
central grey	85±4	82±4	89±5	94±8	83±9	98±6	83±8
lateral habenula	38±4	38±5	46±4	47±7	39±7	31±6	41±10
substantia nigra pars compacta	42±2	52±7	43±4	44±5	39±4	46±3	44±7
substantia nigra pars reticulata	83±9	80±4	77±10	91±5	78±12	95±5	83±11
medial mammillary body	103±18	128±9	93±9	117±11	112±10	107±12	115±12
dentate gyrus	223±11	218±3	213±12	226±15	214±12	227±14	204±8
CA2-oriens	175±13	160±3	170±16	194±7	160±8	171±15	167±21
CA2-pyramidal	160±12	167±4	146±10	191±11	159±5	173±10	165±11
CA2-radiatum	158±11	160±7	149±9	185±9	164±14	166±11	143±9
CA2-molecular	232±14	225±4	211±8	260±13	229±15	232±23	212±17
subiculum	125±8	121±7	122±7	141±17	111±9	131±9	138±9
lateral septum	133±5	125±8	127±7	137±12	122±17	123±15	125±16
caudate	61±4	59±3	59±3	67±6	58±5	56±2	64±4
visual cortex	130±10	114±15	120±12	127±13	125±11	121±23	118±10
anterior cingulate cortex	116±9	116±9	118±13	122±13	109±17	129±18	126±10

Each value is the mean±s.e.mean of 6 experiments. * Pooled i.p. and implant groups differ from pooled controls, $P<0.05$.

Table 5 Effect of treatment route on [^{3}H]-SR 95531 binding (specific binding, pmol g^{-1} tissue)

	All i.p.	*All implant*
CA1-radiatum	154±5	173±5*
CA1-molecular	206±7	229±9^0
CA2-pyramidal	158±5	174±6*
CA2-radiatum	156±5	171±7^0

Values are the mean±s.e.mean of 18 experiments. * $P<0.05$; $^0P<0.1$.

Martin, 1988; McCabe *et al.*, 1988b; Olsen *et al.*, 1990). The distribution of binding sites was similar, but not identical, to BZ binding, with high levels of binding in the hippocampus and dentate gyrus, mammillary body, cortex, amygdala and lateral septum. Binding of [^{3}H]-SR 95531 was relatively higher in the hippocampal formation and relatively lower in cortex than [^{3}H]-FNZP binding. Moderate levels of [^{3}H]-SR 95531 binding were seen in various other limbic and associated structures such as the dorsal and ventral tegmental nuclei and the nucleus accumbens. Binding of [^{3}H]-SR 95531 appeared relatively more dense than [^{3}H]-FNZP binding in the nucleus accumbens. As with [^{3}H]-FNZP binding, there were low levels of binding in the thalamic nuclei, medial geniculate and lateral habenula. The lateral habenula appeared relatively less dense in [^{3}H]-SR 95531 binding than [^{3}H]-FNZP binding. Some of these discrepancies between [^{3}H]-FNZP and [^{3}H]-SR 95531 binding are also reported by Olsen *et al.* (1990). These authors also reported lower [^{3}H]-SR 95531 binding than [^{3}H]-FNZP binding in the substantia nigra, superior colliculus and periaqueductal (central) grey; however, these differences were not found in the present study.

There were significant effects of treatment on [^{3}H]-SR 95531 binding in the dorsal tegmental nucleus (F 2,23=4.52, $P<0.025$) and the dorsal raphe (F 2,23=5.10, $P<0.025$). In the dorsal tegmental nucleus, there was significantly less binding in both subacutely and chronically treated animals as compared with controls. In the dorsal raphe, binding was significantly ($P<0.05$) lower in the subacutely treated animals only (Table 4).

There were significant effects of treatment route on [^{3}H]-SR 95531 binding in the CA1 radiatum (F 1,23=5.45, $P<0.05$) and CA2 pyramidal layer (F 1,22=4.89, $P<0.05$) of the hippocampus. In both cases, binding was lower in the i.p. treated than in the implanted animals. There was an effect of treatment route significant only at the 10% level in two other layers of the hippocampus, the CA1 molecular layer and the CA2 radiatum; in these cases the trend was to lower binding in the i.p. treated animals (Table 5).

There were no significant differences between the three chronic groups.

[^{3}H]-GABA binding The pattern of [^{3}H]-GABA binding in control brains was similar to that described by Bristow & Martin (1988), with high levels of binding in most cortical areas, the thalamus and dentate gyrus, moderate levels in the mammillary body, amygdala, nucleus accumbens and caudate nucleus. Rather low levels of binding were seen in the lateral habenula, dorsal tegmental nucleus, ventral tegmental nucleus, raphe nuclei and central grey. In contrast to Bristow & Martin (1988), who found low to moderate levels of [^{3}H]-GABA in the hippocampus, our study showed relatively high levels of binding in the molecular layer of the CA fields of the hippocampus, with more moderate levels in other layers.

There were no significant effects of treatment or of treatment route on [^{3}H]-GABA binding in any brain area measured, and no interaction between treatment and treatment route (Table 6). There were also no significant differences between the three chronic groups.

Discussion

Receptor autoradiography

The patterns of distribution of benzodiazepine, low-affinity GABA and high-affinity GABA binding sites demonstrated in this study are broadly consistent with those previously reported in the literature. However, we found 10 fold higher levels of [^{3}H]-SR 95531 binding than previously reported by Bristow & Martin (1988) McCabe *et al.* (1988b) and Olsen *et al.* (1990). The method employed was that of Bristow & Martin (1988), except that the 30 min pre-incubation in buffer which they used was replaced by a short pre-incubation in distilled water to shock the tissue osmotically. Heaulme *et al.* (1987) noted that freezing and thawing the membrane pellets at −70°C overnight increased [^{3}H]-SR 95531 binding threefold; it is possible that disruption of the tissue sections occasioned by osmotic shock in addition to freezing is responsible for re-

Table 6 [^{3}H]-GABA binding (specific binding, pmol g^{-1} tissue) in rats after subacute and chronic diazepam treatment

	I.p. injection			*Implant*			
	Control	*Subacute*	*Chronic*	*Control*	*Subacute*	*Chronic*	*Chronic plus i.p.*
dorsal tegmental nucleus	27±3	45±8	34±4	35±9	37±7	39±7	47±5
dorsal raphe	34±8	55±3	47±6	47±6	44±6	37±10	43±5
nucleus accumbens	56±6	73±12	44±5	56±11	59±18	47±7	54±6
central grey	35±3	35±5	42±4	44±9	43±7	43±1	41±3
lateral habenula	23±4	30±6	33±8	17±4	20±5	23±6	19±4
substantia nigra pars compacta	26±4	33±4	32±4	22±4	32±8	33±7	30±6
substantia nigra pars reticulata	50±8	64±9	57±10	53±7	59±10	65±7	59±15
medial mammillary body	31±5	34±8	59±15	37±8	44±8	34±6	38±7
dentate gyrus	81±6	81±7	92±11	80±9	75±8	75±5	82±10
CA2-oriens	63±12	70±6	65±9	65±5	58±5	56±5	64±8
CA2-pyramidal	57±12	72±6	62±13	62±8	50±6	48±4	58±6
CA2-radiatum	59±9	65±3	60±11	60±6	48±7	49±3	57±6
CA2-molecular	79±12	87±8	83±11	80±7	68±7	77±4	94±12
subiculum	67±4	83±10	81±6	93±8	88±6	70±12	89±5
lateral septum	51±5	57±15	49±7	50±3	49±11	43±6	41±6
caudate	41±5	47±9	40±3	44±5	45±12	34±6	33±5
visual cortex	95±2	100±7	79±5	95±10	93±16	91±10	106±7
anterior cingulate cortex	87±6	93±7	95±5	102±7	97±8	91±5	90±5

Each value is the mean±s.e.mean of 6 experiments.

vealing receptors not otherwise accessible, or for altering the affinity of the receptors. This does not seem to be the case for BZ binding, which, as noted above, is similar to that reported by other authors. The binding of [^{3}H]-SR 95531 is increased 2.5–6 fold and non-specific binding reduced after osmotic shock (Brett, unpublished observations). Since the regional variation in [^{3}H]-SR 95531 binding density in control animals is very similar to that reported in the previous studies, [^{3}H]-SR 95531 does seem in the present study to be binding to the same population of low-affinity GABA receptors as in previous work.

In this study, the enhancement of [^{3}H]-FNZP binding by GABA is used, rather than the more physiologically relevant, but technically rather more difficult, enhancement of GABA binding by benzodiazepines, as a measure of the coupling between these two receptors. There appears to be a good correlation between the ability of various ligands for the benzodiazepine receptor to alter the responses to GABA in spinal cord neurones and the GABA shift values for these ligands (Chan & Farb, 1985), and this measure is used by many groups.

Mean levels of GABA enhancement of BZ binding varied between brain structures, an observation consistent with that of other groups. Mennini & Gobbi (1990) suggest that variation between regions in the level of GABA enhancement of [^{3}H]-FNZP binding can be related to the levels of type I BZ receptors, with areas rich in this BZ receptor subtype showing maximum enhancement. However, the variations found in our study do not entirely match those found by Mennini & Gobbi (1990). Ruano *et al.* (1993) also demonstrate high, but not uniform, enhancement of binding to type 1 (or ω_1) sites by GABA. It may be that variations in enhancement are due to differences in the exact subunit composition of the GABA receptor complex (Puia *et al.*, 1991).

Effect of subacute and chronic DZP treatment on the $GABA_A$ receptor

In contrast to the study of Tietz *et al.* (1986), there was no significant effect of subacute or chronic treatment on [^{3}H]-FNZP binding. These authors found significant BZ receptor downregulation after 1 week of chronic flurazepam treatment in the dentate gyrus, hippocampus, temporal cortex, superior colliculus, lateral amygdala and lateral hypothalamus. Most regions were downregulated to the same degree after 4 weeks treatment. This accords with their finding of reduced BZ binding in the hippocampus by radioligand binding methods (Tietz *et al.*, 1989). The present study demonstrated no such changes. However, it is difficult to make direct comparisons between these results and those of Tietz *et al.* (1986) since these investigators employed a different BZ at very large doses for the chronic treatment.

Subacute and chronic treatment effected alterations in GABA enhancement of BZ binding in the nucleus accumbens and a number of limbic system structures interconnected with the nucleus accumbens via the lateral habenula. However, it is necessary to take into account any changes in low-affinity GABA receptors, since a reduction in GABA enhancement of BZ binding may result if low-affinity GABA receptors are downregulated. Figure 1 illustrates the effect of BZ treatment on GABA enhancement of BZ binding and low-affinity GABA binding in 6 brain structures affected. It can be seen that the pattern of changes is different in different structures.

In the dorsal raphe, there is a trend towards a reduction in GABA enhancement in the subacute, but not the chronic groups. This is accompanied by a reduction in low-affinity GABA binding; therefore the reduction in GABA enhancement of BZ binding does not necessarily reflect reduced BZ/GABA receptor coupling. In the other structures, however, alterations in GABA enhancement of BZ binding appear to be independent of low-affinity GABA receptor changes. In the dorsal tegmental nucleus, there is a reduction in [^{3}H]-SR 95531 binding in both subacute and chronic groups; however, there is an increase of GABA enhancement in chronically, but not subacutely, treated animals. There is therefore a change in GABA enhancement independent of GABA binding with chronic treatment; interestingly, it is in the opposite direction to that found by most investigators. In the remaining structures, however, BZ/GABA coupling appears to be reduced. In the lateral habenula, nucleus accumbens, central grey and substantia nigra pars compacta, there is a reduction, or a trend towards a reduction, in GABA enhancement of BZ binding unaccompanied by any change in low-affinity GABA binding. In the lateral habenula and nucleus accumbens, this occurs in both subacute and chronic groups, whereas in the central grey it occurs only in subacutely treated animals and in the substantia nigra pars compacta only in chronically treated animals. Taken together, these findings show that some adaptive neurochemical processes have taken place by three days of diazepam treatment and as such do not directly support a hypothesis that tolerance to the anxiolytic actions of BZs is directly associated with a reduced coupling between the BZ and GABA receptors. However, these adaptive changes may trigger additional neurochemical processes critical to tolerance development.

Figure 1 shows the interconnections between the 6 structures in which changes in BZ/GABA coupling were observed. Central to their interrelationship is the lateral habenula, which

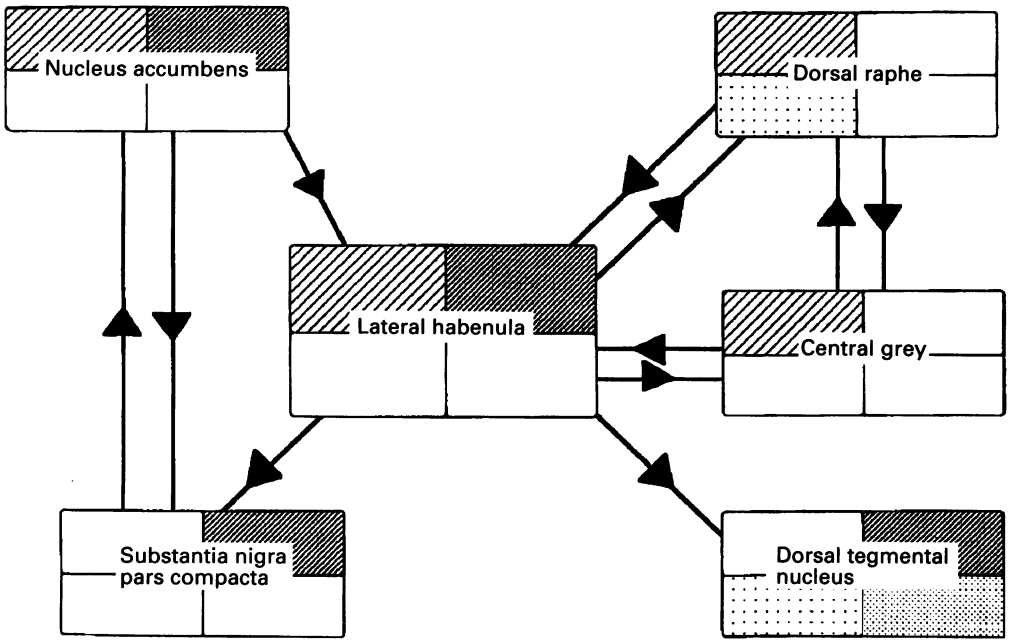

Figure 1 Changes in $GABA_A$ receptor binding in the lateral habenula and interconnected structures after subacute (3 days) and chronic (28 days) treatment with diazepam: Changes in GABA enhancement of [^{3}H]-flunitrazepam binding after subacute (▨) and chronic treatment (▨). Changes in [^{3}H]-SR 95531 after subacute (▨) and chronic (▨) diazepam treatment.

is a structure considered to act as an interface between limbic and motor neuronal circuitry. The limbic system is important in emotion and is involved in the actions of anxiolytic drugs. Clearly, subacute and chronic BZ treatment alters the $GABA_A$ complex in this system. However, the picture of changes in GABA enhancement and in low-affinity GABA binding in these interconnected structures does not provide a clear mechanism for BZ tolerance. It appears that even within this neuronal circuit the response to chronic BZ treatment is differently regulated in different structures, both in terms of the component of the $GABA_A$ complex which is altered and in terms of the time course of the response.

Changes in the nucleus accumbens are interesting, not only because there is an input from this structure to the lateral habenula, but also because the accumbens is clearly involved in the reinforcing properties of psychomotor stimulants and other drugs of abuse (Pratt, 1991; Koob, 1992). If changes in $GABA_A$ complex binding in this structure contribute towards dependence on BZs, then these results suggest that the process begins at an early stage of treatment, since these changes were observed in both subacutely and chronically treated animals.

There is general agreement that BZ receptors are coupled to low-affinity $GABA_A$ receptors. BZs enhance binding to the low-affinity, but not the high-affinity GABA site (Skerritt *et al.*, 1982; Skerritt & Johnston, 1983). Regional distributions of binding to the various components of the GABA receptor complex also suggest that it is the low-affinity GABA receptor to which BZ receptors are coupled (Olsen *et al.*, 1990). It is therefore perhaps to be expected that chronic BZ treatment has no effect on high-affinity GABA receptor binding.

Chronic diazepam: relationship between regional changes in the $GABA_A$ receptor and regional changes in glucose use

It is intriguing that the brain structures which exhibited clear tolerance to diazepam in our previous 2-deoxyglucose studies (Laurie & Pratt, 1989; 1993; Pratt 1991) do not, for the most part, show changes in $GABA_A$ receptor binding properties in the present autoradiography study.

In structures of the Papez circuit, which includes the mammillary body and anterior thalamus, there were marked tolerance and withdrawal effects upon cerebral glucose use after chronic DZP (Laurie & Pratt, 1989; 1993; Pratt 1991). However, no changes in $GABA_A$ receptor characteristics were observed in this circuit in the present study. There are a number of possible explanations for this mismatch. Firstly, changes in glucose use are generally attributed to net alterations in nerve terminal activity. Hence, changes in GABA receptor activity in one brain region could lead to downstream effects in interconnected brain structures that release other neurotransmitters. If this were the case, however, then we might have been expected to observe alterations in $GABA_A$ receptor properties in structures that have neural connections with the Papez circuit. Indeed the mammillary body has a reciprocal connection with the dorsal tegmental nucleus in which BZ/GABA receptor coupling was increased in this study. It is possible that this change in the dorsal tegmental nucleus is a compensatory response to the depression of GABAergic function in the accumbens-habenula-dorsal tegmental nucleus circuity (Figure 1). The resulting functional tolerance observed in the mammillary body could conceivably be a net result of these changes, although it is unlikely to be the sole explanation.

Alternatively, it is possible that there are changes in the $GABA_A$ receptor in structures that exhibited functional tolerance after repeated DZP treatment, but that the changes are in subunit composition. The autoradiographic procedure used in the present study does not permit an analysis of changes in the expression of the genes encoding the various subunits of the $GABA_A$ receptor, as the ligands used do not clearly discriminate between the subunits. In recent years investigators have determined if chronic BZ treatment can alter $GABA_A$ receptor gene expression. There are inconsistent effects of chronic BZs upon the expression of the α_1 and γ_2 subunits in the cortex, hippocampus and cerebellum, with some groups reporting a reduced expression and others finding no change (Heninger *et al.*, 1990; Kang & Miller, 1991; O'Donovan *et al.*, 1992b; Zhao *et al.*, 1994). One study in whole brain has found decreases in α_5 and increases in α_3 and α_6 expression, but no change in α_1 or α_2 (O'Donovan *et al.*, 1992b). The expression of the varous β subunits is reported not to be changed (Heninger *et al.*, 1990; O'Donovan *et al.*, 1992a). The variations between the findings in these studies may be due to the differences in BZ

ligand used, the dose, duration and route of treatment. Only one study has used *in situ* hybridisation, which is capable of revealing more subtle alterations of the subunit composition. Tietz *et al.* (1993) found a reduction in α_1, but not α_5 or γ_2, subunit mRNA in the CA1 region of the hippocampus and in layers II–III and IV of the cortex after chronic flurazepam treatment. The distribution of the various subunits and their likely coassembly is potentially quite complex (Wisden *et al.*, 1992; Laurie *et al.*, 1992). It is somewhat difficult at present to relate these changes in subunit mRNA levels to altered binding and function. It is not yet clear on which subunits the various binding sites are located. It is also uncertain to what extent the mRNA changes are accompanied by alterations in the subunit proteins and their coassembly. The study of Tietz *et al.* (1993) used a high dose regime. We are currently using *in situ* hybridization to determine if there are regional differences in the expression of subunit composition after chronic DZP treatment using a low-dose procotol.

The changes in BZ/GABA coupling observed in the accumbens-habenula-dorsal tegmental nucleus circuit (Figure 1) could also be due to alterations in the expression of $GABA_A$ receptor subunits. The γ_2 subunit is a good candidate as there is evidence that this subunit is required for a robust enhancement of GABA function. Indeed, there is some evidence of changes in the mRNA for γ_2 in the hippocampus and cortex after chronic high dose flurazepam treatment (Zhao *et al.*, 1994). However, our preliminary findings, using *in situ* hybridization, do not support changes in γ_2 or α_1 and α_4 subunit expression as a mechanism for the altered BZ/GABA coupling observed after chronic diazepam treatment in the present study (Pratt *et al.*, 1995).

Finally, the mismatch between structures revealed in the 2-deoxyglucose studies and the present receptor autoradiographic study may be because there are indeed two distinct adaptive changes occuring during DZP tolerance. The underlying molecular bases of these require further exploration. Possibilities include changes in the phosphorylation state of the $GABA_A$ receptor, alterations in the expression of immediate early genes or changes in the regulation of protein kinases.

Effect of different treatment regimes on GABA receptor complex binding

While treatment route did not affect binding to the BZ receptor or the high-affinity GABA receptor, there was an effect of treatment route on low-affinity GABA binding in several regions of the hippocampus, such that the density of [^{3}H]-SR 95531 binding was lower in animals treated by i.p. injection. Presumably this is attributable to the increased handling in the i.p.-treated animals; however, if so, the direction of the change is not what would be expected from the work of Biggio's group, who have shown that low-affinity GABA binding is increased in handling-habituated rats compared with naive rats, and that foot-shock stress reduces GABA binding to 'naive' levels in handling-habituated rats, but does not further reduce it in naive rats (Corda & Biggio, 1986). The decreased low-affinity GABA binding which we find would not seem consistent with an anxiolytic effect of handling. The reason for our finding is not clear, although there may be a confounding effect of the surgical treatment of the implanted animals.

The two treatment regimes differ not only in the amount of handling received by the animals, but also in the nature of the exposure of the animal to the drug. Once-daily i.p. injection will result in short-term exposure of brain receptors to a high level of DZP, which is rapidly eliminated, as is (in the rat) its major metabolite, desmethyldiazepam (Friedman *et al.*, 1986), whereas the implanted animals will receive continuous exposure to a lower level of the drug. However, the physiological consequences, as measured by sensitivity to iontophoretically applied 5-hydroxytryptamine (5-HT) and GABA, of chronic treatment in these two ways seem similar (Gallager *et al.*, 1985). Furthermore, had there been differences due to the different drug exposure, the statistical analysis of our results would have revealed interactions between the treatment and the treatment route.

Behaviour

Unlike most groups, we have attempted to demonstrate behavioural tolerance to the anxiolytic effects of DZP in chronically-treated animals and investigate receptor changes in the same animals. We have previously been unable to draw firm conclusions about the time course of tolerance to the anxiolytic effects of DZP since the chronic injection protocol prevents the demonstration of an acute anxiolytic effect of DZP in vehicle-pretreated animals (Brett & Pratt, 1990). This suggests an interaction between the handling and the effects of the drug in the behavioural test. In an attempt to overcome this, we have compared the effects of DZP treatment on behaviour in animals receiving the drug by daily injections and those receiving it continuously by silastic capsule.

The present behavioural results indicate that an experimental protocol involving chronic treatment by implanted silastic capsules rather than daily injection does not improve the detection of the anxiolytic effect in subacutely treated rats. The reduction in open/total ratios for time in the plus-maze in the implanted animals as a whole might indicate that they were more anxious than the i.p.-treated animals. However, this did not permit an anxiolytic effect of the subacute diazepam to be measured. Therefore the inability to observe an anxiolytic effect of acute and subacute DZP in chronic treatment protocols is not simply due to an anxiolytic effect of handling habituation. Our findings are in contrast to those of Luscombe *et al.* (1994) who were able to demonstrate tolerance to the anxiolytic effect of chlordiazepoxide, buspirone and dothiepin after 28 days treatment by osmotic minipump. Alternatively, discomfort from the previous day's surgery to remove the pumps may be reducing activity in the maze, although the total entries are not significantly reduced in the implanted animals, as might be expected if this was the explanation. In the study of Luscombe *et al.* (1994), the pumps remained in place. Support for the explanation that in the present study the reduction in the ratio of time spent on the open arms is due to previous-day surgery is provided by our finding, in a study of the behavioural effects of chronic FG 7142 (Jedrusik *et al.*, 1995), that a group implanted for 14 days with osmotic minipumps containing vehicle, which were not removed prior to testing, showed significantly higher open/total ratios for time than an unoperated and unhandled control group.

Our data suggest that the elevated plus-maze test of anxiety is not a robust test for the assessment of tolerance to anxiolytic actions of BZs because of the confounding effect of procedural variables. It is clear that such variables affect measurements in the elevated plus-maze (Handley & McBlane, 1993) and there is now some controversy about the use of this test for measuring anxiolytic effects (Dawson & Tricklebank, 1995). Nevertheless we can reliably demonstrate acute and subacute effects of DZP in the plus-maze using comparable doses in rats that are not repeatedly handled (Brett & Pratt, 1989; 1990). We also have evidence from previous studies that rats are tolerant to the sedative effects of the drug after 3 days treatment (Laurie & Pratt, 1989) and are tolerant to the anticonvulsant effects of DZP within 7 days of treatment using similar treatment regimes as the present study. Continuous DZP treatment (via silastic capsules) for 1 day significantly raised bicuculline seizure threshold from 0.34 ± 0.02 (in vehicle controls) to 0.45 ± 0.01 mg/kg whereas there was no significant effect after 7 days treatment: 0.38 ± 0.02 and 0.40 ± 0.05 respectively (unpublished data). It remains to be clarified with which components of behavioural tolerance the changes at the receptor level which we have demonstrated are associated.

Conclusion

In summary, diazepam treatment of 4 days or more does appear to have some effects on BZ/GABA receptor coupling and to a lesser extent on low affinity GABA binding in an accumbens-habenula circuit; the picture, however, is complex. This study, which in contrast to electrophysiological experiments on individual brain areas or biochemical assays on grossly dissected brain structures has examined effects simultaneously in multiple discrete neuronal structures, suggests that tolerance cannot simply be explained by global changes in $GABA_A$/BZ receptor coupling. It is unlikely that one single mechanism can explain tolerance to BZs. It is probable that several different processes with different time courses are occurring during the course of DZP treatment. From the present data, changes in the coupling between the BZ and GABA receptor in the habenula-accumbens circuitry occur soon after the initiation of DZP treatment, suggesting that they are unlikely to be directly responsible for tolerance to the anxiolytic effects. However, they may represent one of several neurochemical changes that trigger adaptive processes in other neural circuits that ultimately lead to the behavioural expression of tolerance. Superimposed on these alterations are changes induced by the experimental manipulations of the animals, which complicate the interpretation and have implications both for the conduct of animal experiments and for the understanding of the neurobiology of anxiety.

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