Studies on $[^3H]$Diazepam and $[^3H]$Ethyl-$\beta$-Carboline Carboxylate Binding to Rat Brain In Vivo. I. Regional Variations in Displacement

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Abstract: The binding of $[^3H]$diazepam and $[^3H]$ethyl-$\beta$-carboline carboxylate ($\beta$-CCE) to rat brain membranes has been studied following injection of the ligand via a tail vein. "Ex vivo" binding was avoided by homogenising the tissue in an excess of unlabelled ligand. The dissociation rate constant for $[^3H]$diazepam and $[^3H]$$\beta$-CCE was approximately 0.46 min$^{-1}$ at 0°C. Displacement of $[^3H]$diazepam by $\beta$-CCE in vivo showed regional variation: the dose of $\beta$-CCE required to inhibit 50% of $[^3H]$diazepam binding in the cerebellum was one quarter of that required in the cortex, hippocampus, or striatum. However, when diazepam was used to displace $[^3H]$$\beta$-CCE in vivo the converse occurred: the dose needed for 50% inhibition in the cerebellum was more than four times that required in the other three regions. These findings support suggestions from in vitro experiments that two receptors exist with different affinities for benzodiazepines and $\beta$-carbolines. The benzodiazepine receptor antagonist Ro 15-1788 did not differentiate between the two receptor subtypes. Key Words: Benzodiazepines—$\beta$-Carbolines—In vivo binding. Minchin M. C. W. and Nutt D. J. Studies on $[^3H]$diazepam and $[^3H]$ethyl-$\beta$-carboline carboxylate binding to rat brain in vivo. I. Regional variations in displacement. J. Neurochem. 41, 1507–1512 (1983).

Many studies have described specific, high-affinity binding of benzodiazepines to brain membranes since the original observation of Squires and Braestrup (1977). Subsequently several $\beta$-carboline esters were found to bind to the same receptor, although their action at that receptor appeared to be opposite to that of the benzodiazepines (Nielsen and Braestrup, 1980; Cowen et al., 1981; Oakley and Jones, 1982). However, binding of $[^3H]$benzodiazepine ligands displays an unusual temperature dependence, being greater at 0°C than at 37°C (Braestrup and Squires, 1977), and this led to some doubts about the physiological relevance of the phenomenon. Subsequently these doubts were dispelled when several groups demonstrated that binding of benzodiazepines could occur in vivo (Williamson et al., 1978; Chang and Snyder, 1978; Tallman et al., 1979).

Recently it has become clear that the benzodiazepine receptor population is not homogeneous, as indicated by the interaction of triazolopyridazines and propyl-$\beta$-carboline carboxylate with the receptors (Squires et al., 1979; Nielsen et al., 1981), by the molecular heterogeneity exhibited on polyacrylamide gels (Seighart and Karobath, 1980)—although this has not been confirmed (Thomas and Tallman, 1981)—and by differential detergent solubility (Lo et al., 1982). However, the question arises as to whether this heterogeneity is present in vivo. Experiments by Hirsch and Lydigsen (1981), in which different regional sensitivity to ethyl-$\beta$-carboline carboxylate ($\beta$-CCE) displacement of $[^3H]$flunitrazepam binding in vivo was observed, suggest that this may be so. The experiments described here attempt to elucidate this question further and, in addition, provide data on some properties of the in vivo binding of both diazepam and $\beta$-CCE.

MATERIALS AND METHODS

Male Sprague-Dawley-derived rats weighing between 125 and 150 g were used throughout. They were injected...
via a tail vein with 0.2 ml saline containing either 40 μCi [3H]diazepam (97 Ci/mmol, Amersham) or 40 μCi [3H]β-CCE (24 Ci/mmol, Amersham). Two minutes later the animals were stunned and decapitated, and the brain was removed to ice. Previous studies have shown that at 1 to 2 min after i.v. injection the concentration of diazepam in the brain is at its highest and that little metabolism has occurred (Williamson et al., 1978). Similarly, behavioural studies suggest that peak response to β-CCE occurs 1–5 min following i.v. injection (Cowen et al., 1981; Oakley and Jones, 1982). The brain was quickly dissected and frozen on dry ice. The frozen portions were weighed and homogenised for 20 s in 25 volumes of ice-cold 50 mM Tris-HCl, pH 7.4, containing 10 mM β-CCE (for diazepam binding) or 10 μM β-CCE (for β-CCE binding) with an Ultra Turrax homogeniser. Immediately, duplicate 0.5-ml portions of this homogenate were filtered under vacuum on Whatman GF/B filters and the filters were rinsed twice with 5 ml ice-cold Tris buffer. The remaining homogenate was left on ice for 30 min to minimise dissociation of bound ligand. When frozen brain tissue was homogenised with [3H]diazepam (i.e. about 20 nCi/ml), there was a rapid association of label with the membranes, which was maximal after about 5 min of incubation at 0°C (Fig. 1). When an excess of unlabelled flurazepam was added to this mixture, [3H]diazepam dissociated from the membranes with a half-life of approximately 2.5 min (Fig. 1), which corresponds to a dissociation rate constant of 0.28 min⁻¹. If flurazepam was present in the buffer at the time of homogenisation, only nonspecific binding was seen; specific binding was stable at 0°C for at least 50 min (Fig. 1).

When in vivo bound [3H]diazepam was displaced by homogenising different portions of the cerebral cortex in increasing concentrations of flurazepam and measuring bound radioactivity immediately after homogenisation and after 30 min on ice, the IC₅₀ for flurazepam was 23 nM (Fig. 2). Accordingly, in subsequent experiments tissue was homogenised in an excess (10 μM) of unlabelled flurazepam to prevent ex vivo binding, and total binding was estimated immediately after homogenisation, which minimised dissociation of bound ligand. Similar considerations probably apply to β-CCE binding. For example, the half-time of in vitro bound [3H]β-CCE is 4 min (Marangos and Patel, 1981); thus, the same protocol was used in in vivo experiments with this ligand, homogenisation taking place in 10 μM unlabelled β-CCE.

The dissociation of in vivo bound [3H]diazepam and [3H]β-CCE was studied by measuring membrane-bound radioactivity at various times after homogenisation (Fig. 3). In these experiments with whole brain minus cerebellum and pons/medulla, the half-life of dissociation for both ligands was approximately 1.5 min, giving a dissociation rate constant of 0.46 min⁻¹.

**RESULTS**

The principal problem to be overcome in these experiments was to prevent ex vivo binding during homogenisation and to minimise dissociation of bound ligand at the same time. When frozen brain tissue was homogenised with an amount of [3H]diazepam corresponding roughly to that found in homogenates from animals previously injected with [3H]diazepam (i.e. about 20 nCi/ml), there was a rapid association of label with the membranes, which was maximal after about 5 min of incubation at 0°C (Fig. 1). When an excess of unlabelled flurazepam was added to this mixture, [3H]diazepam dissociated from the membranes with a half-life of approximately 2.5 min (Fig. 1), which corresponds to a dissociation rate constant of 0.28 min⁻¹. If flurazepam was present in the buffer at the time of homogenisation, only nonspecific binding was seen; specific binding was stable at 0°C for at least 50 min (Fig. 1).

![FIG. 1. Binding of [3H]diazepam to homogenate membranes in vitro. Frozen whole brain was homogenised with [3H]diazepam (20 nCi/ml) as described in Materials and Methods. Portions of the homogenates were filtered at various times and the membrane-bound radioactivity measured. Unlabelled flurazepam was added to the homogenate after 28 min, at a final concentration of 10 μM, and further samples were filtered at various times afterwards. The solid line indicates the association/dissociation curve of [3H]diazepam. The dashed line shows the binding in the absence of flurazepam and the broken line indicates binding occurring when 10 μM flurazepam was included during homogenisation—i.e. nonspecific binding. Each point is the mean of duplicate filtrations. The experiment was repeated four times with similar results.](image-url)
IN VIVO BINDING OF DIAZEPAM AND β-CCE

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Although the amount of radioactivity injected into each animal was identical and the weight of the rats was similar, the amount of [3H]diazepam or [3H]β-CCE appearing in the brain homogenates was rather variable. To correct for this, the specifically bound ligand was expressed as a percentage of the total radioactivity in the homogenate. However, for this to be a valid normalisation, the ratio of specifically bound ligand to free ligand in the homogenate should be constant over the range involved in the experiments. To establish whether this was true, plots were made of specifically bound radioactivity versus free radioactivity in the homogenate for both [3H]diazepam and [3H]β-CCE in the various brain regions (Fig. 4). It can be seen that in all cases the relationship was linear over the range of free concentrations observed in these experiments. The specific binding of [3H]diazepam and [3H]β-CCE in various brain regions is shown in Table 1.

β-CCE displaced [3H]diazepam from its in vivo binding sites in all the brain regions studied, although it was considerably more potent in the cerebellum than in the other areas (Table 2). By contrast, flurazepam displaced [3H]β-CCE with the least potency in the cerebellum. The benzodiazepine antagonist Ro 15-1788 displaced [3H]β-CCE with a fairly even potency over the four brain regions studied (Table 2).

DISCUSSION

Binding of [3H]diazepam to the brain during homogenisation was quite rapid, rather more rapid, in fact, than that seen in previous experiments with brain membrane preparations (Braestrup and Squires, 1977; Möhler and Okada, 1977). It was not possible to measure the association rate constant with any degree of accuracy, but the dissociation rate constant was measured and found to be greater than that reported for a washed membrane preparation (0.12 min⁻¹-Tallman et al., 1978). Inclusion of flurazepam in the homogenisation medium was found to abolish completely ex vivo association of unbound [3H]β-diazepam.

Dissociation of in vivo bound [3H]diazepam and [3H]β-CCE was rapid following homogenisation in excess flurazepam or unlabelled β-CCE, respectively. The dissociation rate constant was similar for both ligands and somewhat larger than that found for the dissociation of in vivo bound [3H]diazepam in the presence of an excess of chlordiazepoxide.
The explanation for this discrepancy may lie in the different homogenate strengths used; 25 volumes in the present study against 150 volumes in the study of Tallman et al. (1979). Accordingly, any endogenous effectors would be present at higher concentration in the present study, and it has been reported that one such endogenous effector can alter the dissociation rate of bound benzodiazepines. Thus Chiu et al. (1982) demonstrated that GABA slowed the dissociation of \[^{3}H\]flunitrazepam from brain membranes, and it is not inconceivable that other effectors may increase the dissociation of membrane-bound benzodiazepines. Similar considerations may apply to the different dissociation rates seen in the \textit{ex vivo} binding experiments and those using washed membranes. However, despite the presence of endogenous substances, the \textit{ex vivo} displacement of \textit{in vivo} bound \[^{3}H\]diazepam by flurazepam displayed a remarkably similar IC\(_{50}\) to that found in membrane preparations (Squires and Braestrup, 1977).

Following bolus injections of ligand it is not possible to determine ligand binding curves, since equilibrium conditions are not met. However, some indication of the relationship between bound \[^{3}H\]diazepam or \[^{3}H\]β-CCE and free ligand concentration may be obtained by comparing specifically bound radioactivity with the concentration of free ligand in the homogenates, as in Fig. 4. Since the amount of homogenate filtered in these experiments corresponds to 20 mg of brain tissue—and assuming that the benzodiazepine receptor ligands are distributed throughout the extracellular space, which corresponds to approximately 30% of the brain weight—the range of \[^{3}H\]diazepam concentrations found in these experiments is approximately 1.5–18 nM. The corresponding figures for

![Graphs](image_url)
for diazepam and P-CCE in membrane preparations of \[3H\]f3-CCE, for which the hippocampus and

versus-free plots for \[3H\]diazepam was similar in the four brain regions studied, it was not in the case of \[3H\]beta-CCE, for which the hippocampus and striatum showed lower slopes than the other two areas. In each area the slope of the \[3H\]P-CCE plot was less than that of \[3H\]diazepam; this was also
demonstrated in the normalised specific binding data in Table 1. Values for the normalised \[3H\]diazepam binding compare favourably with that found by Wil-

Specific binding of tritiated ligands was determined as described in Materials and Methods and is expressed as a percentage of the radioactivity in the homogenate. Flurazepam (0.3–10.0 mg/kg) and unlabelled beta-CCE (0.3–3.0 mg/kg) were injected i.v. 5 min before labelled ligand. Ro 15-1788 (1.0–5.0 mg/kg) was injected i.p. 15 min before the labelled ligand. Control animals were injected with the appropriate vehicle and the percentage displacement caused by the drugs was plotted on log-probit paper, from which ID$_{50}$ values were estimated. Each value is the mean of two or three experiments, except for the experiment involving Ro 15-1788, which is the result of a single experiment, determined in duplicate. The ID$_{50}$ values have not been corrected for the variability in regional

\[3H\]ligand concentration. 

[\[3H\]]beta-CCE are 3–43 nM. Considering that the K$_D$
for diazepam and beta-CCE in membrane preparations is 3.6 nM and 1.4 nM, respectively (Mohler and Okada, 1977; Patel et al., 1981), the linear relationship between bound and free ligand over these concentration ranges probably reflects the lack of equilibrium. However, it may also indicate that binding conditions in vivo are quite different from those found in vitro, particularly in regard to the presence of endogenous effectors or modulators of binding.

Interestingly, whereas the slope of the bound-versus-free plots for \[3H\]diazepam was similar in the four brain regions studied, it was not in the case of \[3H\]beta-CCE, for which the hippocampus and striatum showed lower slopes than the other two areas. In each area the slope of the \[3H\]beta-CCE plot was less than that of \[3H\]diazepam; this was also demonstrated in the normalised specific binding data in Table 1. Values for the normalised \[3H\]diazepam binding compare favourably with that found by William

Williamson et al. (1978) for whole brain; they also demonstrated that there was little difference in \[3H\]diazepam binding in vivo between several brain regions, which we have confirmed in the present study. No data are available for in vivo \[3H\]beta-CCE binding, but in vitro binding of the propyl ester of beta-carboline carboxylic acid showed marked variations between different brain regions (Nielsen et al., 1981). Thus, binding at a single concentration revealed the following preference: cortex > cerebellum >> hippocampus > striatum, which is remarkably similar to the order of preference shown for \[3H\]beta-CCE binding in the present study.

beta-CCE displays differential interaction with benzodiazepine receptors in vitro experiments, being about seven times more potent in the cerebellum than in the hippocampus (Nielsen and Braestrup, 1980), and exhibits mixed-type competitive inhibition of \[3H\]flunitrazepam binding in regions other than cerebellum (Braestrup et al., 1980). This suggests receptor heterogeneity or a single site with more than one conformation. Evidence for the former explanation comes from the identification on polyacrylamide gels of two benzodiazepine binding proteins in hippocampus, but only one in cere-

bellum (Seighart and Karobath, 1980), and for the latter from the kinetic experiments of Chiu et al. (1982). In any event, it is generally accepted that in vitro there is only a single type of benzodiazepine receptor in the cerebellum, whereas different forms or conformations exist in varying proportions in other regions of the brain. 

Braestrup and Nielsen (1981) have shown that propyl-beta-carbolino carboxylate preferentially labels the cerebellar or BZ1 type of receptor and have cal-

culated that whereas the cerebellum contains only BZ1 receptors, hippocampus and striatum have 60% of this type and cerebral cortex contains 80%. We have now demonstrated that these in vitro findings also apply in vivo. Thus beta-CCE was four times more potent in the cerebellum in \[3H\]diazepam displacement studies, whilst conversely flurazepam was several fold less potent in the cerebellum at displacing \[3H\]beta-CCE. These potency ratios are considerably greater than, but qualitatively in good agreement with, those recently reported by Jones and Oakley (1982), who compared the displacement of in vivo bound \[3H\]flunitrazepam by diazepam and propyl-beta-carbolino carboxylate in mice. Our results with the benzodiazepine antagonist Ro 15-1788 suggest that it is more or less equipotent in the four areas investigated when tested as a displacer of in vivo \[3H\]beta-CCE binding. Previous studies by Nutt et al. (1982) have shown that in both seizure threshold and superior cervical ganglion depolarisation tests Ro 15-1788 was a potent antagonist of both benzodiazepines and beta-CCE, and it has been shown to displace in vivo bound \[3H\]flunitrazepam

TABLE 2. In vivo displacement of \[3H\]diazepam and \[3H\]beta-CCE binding by flurazepam, beta-CCE, and Ro 15-1788

<table>
<thead>
<tr>
<th>Brain region</th>
<th>[3H]beta-CCE displacing</th>
<th>Flurazepam displacing</th>
<th>Ro 15-1788 displacing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum</td>
<td>0.5</td>
<td>0.5–0.6</td>
<td>0.9–2.6</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.2</td>
<td>1.5–2.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Striatum</td>
<td>2.1</td>
<td>1.4–2.8</td>
<td>0.4–0.8</td>
</tr>
<tr>
<td>Cortex</td>
<td>2.4</td>
<td>1.9–2.8</td>
<td>0.1</td>
</tr>
</tbody>
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<th>ID$_{50}$ (mg/kg)</th>
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J. Neurochem., Vol. 41, No. 6, 1985
(Möller et al., 1981). From the present results it appears that Ro 15-1788 does not discriminate between the two benzodiazepine receptor subtypes.

REFERENCES


