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Differential Effect of γ-Aminobutyric Acid on Benzodiazepine Receptor Subtypes Labeled by [^{3}H]Propyl β-Carboline-3-Carboxylate in Rat Brain[1]

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ABSTRACT

The regulation of benzodiazepine receptor subtypes (BZ1 and BZ2) by γ-aminobutyric acid (GABA) was studied by assessing the effect of GABA on flunitrazepam (FLU) binding to [^{3}H]FLU and [^{3}H]propyl β-carboline-3-carboxylate ([^{3}H]-PCC) labeled receptors in various brain regions. In the hippocampus, the BZ1 receptor (high affinity) labeled by a low concentration (0.04 nM) of [^{3}H]PCC at 0°C did not appear to be GABA regulated. This was based upon the observation that GABA (10^{-4} M) has no significant effect on the FLU/[^{3}H]PCC competition curves in the hippocampus. In contrast, when [^{3}H]FLU (0.05 nM) or a high concentration of [^{3}H]PCC (0.5 nM) was used to label both BZ1 and BZ2 (low affinity) receptors under similar conditions, GABA (10^{-4} M) caused a significant increase (1.7-fold) in the affinity of FLU as measured by FLU/[^{3}H]FLU and FLU/[^{3}H]PCC competition experiments. In cerebral cortex, GABA enhancement of FLU inhibition of [^{3}H]PCC (0.04 nM) binding was present but significantly less than the enhancement observed with [^{3}H]FLU (0.05 nM) or a high concentration of [^{3}H]PCC (0.5 nM). An effect of GABA on FLU inhibition was also observed in the cerebellum when either [^{3}H]PCC (0.04 nM) or [^{3}H]FLU (0.05 nM) was used as the ligand. Dissociation kinetics of high (0.5 nM) and low (0.04 nM) concentrations of [^{3}H]PCC in the hippocampus support the notion that 0.04 nM [^{3}H]PCC labels a single binding site, whereas the high concentration of [^{3}H]PCC labels both the high (BZ1) and low affinity (BZ2) sites. In cerebral cortex, a low concentration of [^{3}H]PCC (0.04 nM) predominantly labels the high affinity site with a small proportion of the low affinity site also being labeled. [^{3}H]PCC dissociation curves and PCC/[^{3}H]FLU competition curves in the cerebellum indicate PCC binds predominantly to a single benzodiazepine receptor site in this brain region. Overall, these findings suggest that GABA regulation may be predominantly associated with the BZ2 site found in the cerebral cortex and hippocampus. In contrast, BZ1 receptors show a regional difference in the way they are associated with GABA receptors. In terms of GABA regulation, BZ1 receptors in the cerebellum appear to be different from the BZ1 receptor in hippocampus and cerebral cortex. Consequently, functional coupling to a GABA site may be a determinant of benzodiazepine receptor heterogeneity.

Certain pharmacological effects of the BZDs appear to be mediated primarily through GABA neuronal systems (Costa *et al.*, 1975; Haefely *et al.*, 1975; Guidotti, 1978). Consistent with this observation is the intimate association between BZD receptors and GABA receptors in the central nervous system (Tallman *et al.*, 1980). It has been suggested that the BZD receptor is part of a macromolecular complex which consists of a GABA receptor and a chloride ionophore (Tallman *et al.*, 1978; Ehlert *et al.*, 1981; Olsen, 1981). GABA may be an allosteric modulator of BZD receptor affinity by enhancing the affinity of the BZD receptor for its ligand (Martin and Candy, 1978; Tallman *et al.*, 1978; Wastek *et al.*, 1978).

Compelling evidence for BZD receptor heterogeneity has been recently derived from the use of non-BZD compounds that interact specifically with BZD receptors (Squires *et al.*, 1979; Braestrup *et al.*, 1980; Ehlert *et al.*, 1981; Yamamura *et al.*, 1982). These novel compounds include the triazolopyridazines, such as CL 218872, and the β-carboline carboxylates, ethyl and propyl β-carboline-3-carboxylate. Based upon these data, BZD receptor subtypes discriminated by CL 218872 have been classified as type I and type II receptors (Klepner *et al.*, 1979). The BZD receptor subtypes recognized by PCC were designated BZ1 (high affinity) and BZ2 (low affinity) receptors (Nielsen *et al.*, 1981; Braestrup and Nielsen, 1981). BZDs are believed to have equal affinity for these subtypes. There is also evidence indicating a regional distribution of these receptor

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ABBREVIATIONS: BZD, benzodiazepine; GABA, γ-aminobutyric acid; PCC, propyl-β-carboline-3-carboxylate; FLU, flunitrazepam.

subtypes in the rat brain (Young *et al.*, 1981). Recently, the most compelling evidence for the existence of type I and type II receptors has been provided by the actual physical separation of type I and type II receptors (Lo *et al.*, 1982). In addition, type I receptors showed high affinity for PCC and were found to be highly concentrated in cerebellum and corpus striatum (Lo *et al.*, 1982). These data suggest that BZ1 and BZ2 receptors, defined by [^{3}H]PCC binding, may correspond to type I and type II receptors, respectively. However, the equivalence of BZ1 and BZ2 sites with type I and type II sites has not been fully established.

Based upon the unique spectrum of pharmacological effects of the triazolopyridazines, a hypothesis has been proposed to explain the action of these compounds. The selective anxiolytic effects of CL 218872 are thought to be mediated through GABA-independent type I receptors, whereas the anticonvulsant and sedative-hypnotic effects are mediated through GABA-dependent type II receptors (Klepner *et al.*, 1979; Lippa *et al.*, 1982). BZDs which have equal affinity for type I and type II receptors are consequently effective anxiolytics, anticonvulsants and sedative-hypnotics.

Because GABA enhances the affinity of BZD receptors for BZDs, it was of interest to determine whether [^{3}H]PCC labeled BZD receptor subtypes (*i.e.*, BZ1 and BZ2) are differentially regulated by GABA as hypothesized earlier for type I and type II sites (Klepner *et al.*, 1979). [^{3}H]PCC has been reported to recognize BZD receptor subtypes and have high affinity for BZ1 receptors (Braestrup and Nielsen, 1981; Ehlert *et al.*, 1981; Lo *et al.*, 1982). The selectivity of [^{3}H]PCC for the BZ1 receptor would allow a direct determination of the ability of GABA to regulate the binding of BZDs to the BZ1 receptor. Consequently, [^{3}H]PCC at low concentrations was used to selectively label the high affinity receptor and [^{3}H]FLU or high concentrations of [^{3}H]PCC was used to label both high and low affinity receptors. The effect of GABA on the affinity of FLU for these differentially labeled receptors was then assessed. We found that BZ1 receptors in the cerebral cortex and hippocampus were not GABA regulated. In contrast, functional coupling to a GABA site was observed with BZ1 receptors in the cerebellum. On the other hand, BZ2 receptors in both the cerebral cortex and hippocampus were found to be subject to GABA regulation.

Methods

The hippocampus, cerebral cortex and cerebellum from male Sprague-Dawley rats (200–250 g) were removed over ice immediately after sacrifice. The tissues were homogenized in 100 volumes of ice-cold 50 mM Na/K phosphate buffer, pH 7.4. Tissue homogenates were washed five times by centrifugation at 48,000 × *g* for 10 min. The final pellets were resuspended in the same buffer and used immediately in the binding assays.

BZD receptors were labeled by [^{3}H]FLU (87.3 Ci/mmol, New England Nuclear, Boston, MA) or [^{3}H]PCC (48.3 Ci/mmol, New England Nuclear). One hundred microliter aliquots of tissue homogenate were incubated with the radioligand, various concentrations of FLU (Hoffmann-LaRoche Inc., Nutley, NJ) or PCC (Hoffmann-La Roche Inc.) and in the presence or absence of 10^{-4} M GABA. Binding in the presence of 10^{-6} M clonazepam (Hoffmann-LaRoche Inc.) was defined as nonspecific. Incubations at 0°C lasted 90 min, while those at 37°C lasted 30 min. Incubations were terminated by rapid filtration through Whatman GF/B glass fiber filters. Filters were immediately washed three times with 5-ml aliquots of ice-cold buffer. Filter-bound radioactivity was quantitated by liquid scintillation spectrophotometry with an efficiency of 46%. Competition curves were constructed by plotting ^{3}H-ligand specifically bound as a percentage of that bound in the absence of inhibitor *vs.* log [I], where I = the inhibitor concentration in moles/liter. IC_{50} values and Hill slopes were determined by a weighted nonlinear least squares regression using a computerized iterative procedure.

Dissociation kinetics of [^{3}H]PCC were determined by incubating cerebellar, hippocampal or cortical homogenates (50 mg of original wet weight/ml) with 0.04 or 0.5 nM [^{3}H]PCC at 0°C for 90 min (equilibrium conditions). Binding in the presence of 10^{-6} M PCC was defined as nonspecific. At time 0, dissociation was initiated by the addition of 10^{-6} M PCC. The percentage of specifically bound [^{3}H]PCC was determined at various time intervals and plotted according to the equation in $(B_t/B_t) = -k_{-1}t$, where B_t is the amount of specifically bound [^{3}H]PCC at time 0, B_t is the specific binding at time t and $-k_{-1}$ is the dissociation rate constant.

[^{3}H]FLU (0.05–2 nM) and [^{3}H]PCC (0.0125–2.5 nM) saturation binding experiments were performed in cerebellar homogenates which had been washed five times. One hundred microliter aliquots of tissue homogenate were incubated with various concentrations of either ^{3}H-ligand in 50 mM Na/K phosphate buffer (pH 7.4) for 90 min at 0°C. Incubations were terminated and radioactivity quantitated as described earlier. Binding data were evaluated by Scatchard analysis using a nonlinear computerized iterative procedure to obtain the apparent K_D and maximum binding values.

Results

Initially it was necessary to determine the conditions under which PCC is able to discriminate between BZD receptor subtypes and selectively label only BZ1 sites. Figure 1 depicts a typical PCC/[^{3}H]FLU competition curve in hippocampus at two different incubation temperatures. The presence of multiple receptor subtypes is suggested by the shallow Hill slope (0.68 ± 0.012, n = 3) observed at 0°C. In contrast, Hill slopes at 37°C were approximately unity (0.93 ± 0.005, n = 3) and significantly greater ($P < .001$, Student's t-test) than that observed at 0°C. Apparently, PCC loses its ability to discriminate between receptor subtypes at physiological temperatures.

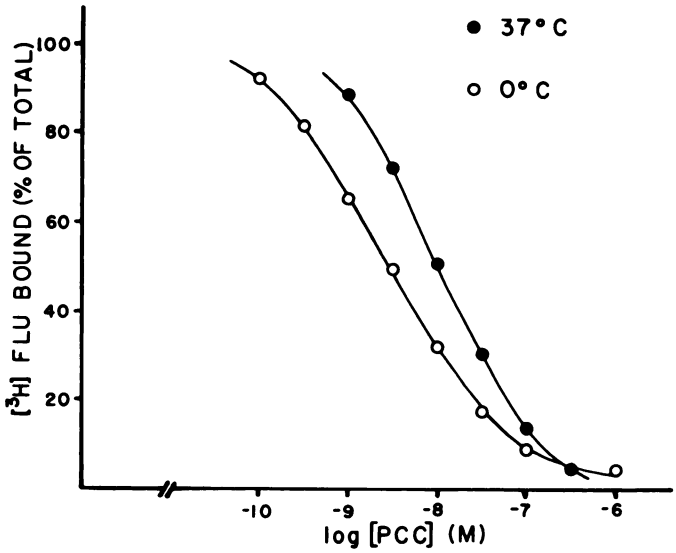

Fig. 1. Effect of incubation temperature on the competitive inhibition of [^{3}H]FLU by PCC in rat hippocampus. Curves are representative of three separate determinations. The concentration of [^{3}H]FLU used was 0.05 nM. Incubation times were 90 and 30 min at 0 and 37°C, respectively. The IC_{50} and Hill slope for PCC at 0°C were 5.0 ± 1.8 and 0.68 ± 0.01 nM, respectively. Under 37°C conditions, the IC_{50} and Hill slope were 13.7 ± 1.5 and 0.93 ± 0.005 nM, respectively.

TABLE 1

Inhibition of [^{3}H]FLU binding in rat cerebellum by PCC and FLU

All values represent the mean ± S.E.M. of three to seven determinations. [^{3}H]FLU (0.05 nM) was used in these competition experiments. Cerebellar homogenates (60 mg of original wet weight/ml) washed five times were used. Incubations were carried out in 50 mM Na/K phosphate buffer, pH 7.4, at 0°C for 90 min. The IC_{50} value represents the concentration of inhibitor that caused half-maximal inhibition of specific [^{3}H]FLU binding.

Inhibitor	IC_{50}	Hill Slope
	nM	
FLU	2.24 ± 0.18	1.01 ± 0.05
PCC	0.86 ± 0.09	1.06 ± 0.07

Similar effects of temperature were observed in PCC/[^{3}H]FLU competition curves derived from the cerebral cortex (data not shown). Consistent with previous reports, a single homogeneous class of BZD receptors was observed in the cerebellum at 0°C (Klepner *et al.*, 1979). PCC/[^{3}H]FLU competition curves displayed mass action behavior similar to that of FLU/[^{3}H]FLU competition curves (table 1). Based upon these observations, it was necessary to perform the experiments investigating the influence of GABA on BZD receptor subtypes under conditions that allow PCC to discriminate between BZ1 and BZ2 receptors (0°C).

Dissociation kinetics in the hippocampus support the notion that very low concentrations of [^{3}H]PCC will label a single high affinity site, whereas higher concentrations of [^{3}H]PCC will interact with both high and low affinity sites. Consistent with a simple bimolecular interaction, the dissociation of 0.04 nM [^{3}H]PCC binding in hippocampus was a linear first order decay, with a half-time of approximately 3.3 min (fig. 2). In addition, similar half-times of dissociation were observed under pre-equilibrium (3 min) and equilibrium (90 min) incubation conditions with 0.04 nM [^{3}H]PCC (data not shown). The dissociation kinetics of 0.5 nM [^{3}H]PCC was markedly different from that observed with 0.04 nM [^{3}H]PCC. The half-time for the decay of binding was approximately 1.0 min and the decay curve appeared curvilinear (fig. 2). The semilog plot of dissociation suggested the presence of fast and slow components of dissociation. Extrapolation (by linear regression) of the slowly dissociating component of the curve back to the ordinate indicated that at steady state, approximately 45% of [^{3}H]PCC receptor complex was of the slowly dissociating type.

In the cerebral cortex, semilog plots of the dissociation of 0.04 and 0.5 mM [^{3}H]PCC at 0°C yielded half-lives for the decay of binding of approximately 1 and 2 min, respectively (fig. 2). Both decay curves showed some degree of curvature suggesting the presence of rapidly (presumably BZ2 receptors) and slowly (presumably BZ1 receptors) dissociating complexes. Extrapolation of the slowly dissociating components of the decay curves back to the ordinate suggested that approximately 32% of the [^{3}H]PCC receptor complex dissociates slowly when 0.5 nM [^{3}H]PCC was used. In contrast, approximately 70% of the 0.04 nM [^{3}H]PCC labeled receptors were slowly dissociating complexes. Collectively, the dissociation kinetics indicate that high concentrations of [^{3}H]PCC (0.5 nM) label two types of BZD receptors, whereas low concentrations of [^{3}H]PCC (0.04 nM) predominantly label one site in the cerebral cortex.

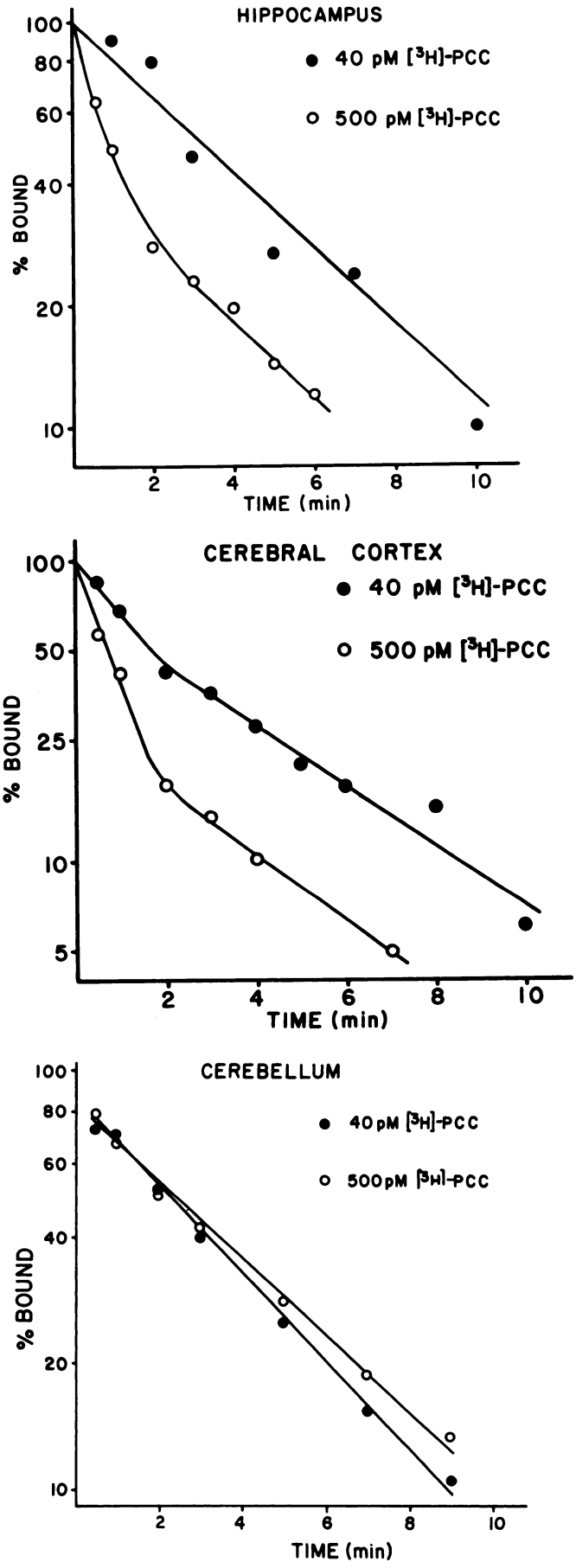

Fig. 2. Dissociation kinetics of 0.04 and 0.5 nM [^{3}H]PCC in hippocampus and cerebral cortex and cerebellum. Homogenates were incubated with [^{3}H]PCC for 90 min at 0°C followed by the addition of excess nonlabeled PCC (10^{-6} M) to initiate dissociation. Curves are representative of two independent determinations. Triplicate samples were taken at each time point.

Half-lives for decay of 0.04 and 0.5 nM [^{3}H]PCC in cerebellum were 2.3 and 2.5 min, respectively (fig. 2). Extrapolation of both decay curves to the ordinate indicate that ~88% of these sites dissociate slowly from [^{3}H]PCC, whereas the remaining sites dissociate rapidly. These data suggest that both high and low concentrations of [^{3}H]PCC label a nearly homogeneous population of sites in the cerebellum.

Table 2 shows the results of the FLU/[^{3}H]FLU and FLU/[^{3}H]PCC competition experiments (±10^{-4} M GABA) in the hippocampus. FLU inhibition of [^{3}H]FLU was significantly enhanced in the presence of 10^{-4} M GABA. IC_{50} values went from 1.5 nM in controls to 0.91 nM in the presence of GABA, approximately a 1.7-fold increase in affinity. Interestingly, when a low concentration (0.04 nM) of [^{3}H]PCC was used to selectively label high affinity BZD sites, the IC_{50} of FLU was unaltered by the presence of GABA (table 2). To ascertain that the lack of effect of GABA was not a peculiarity of the complex interaction between [^{3}H]PCC and FLU, additional competition experiments were performed using a concentration of [^{3}H]PCC (0.5 nM) that would label both high and low affinity sites. The results of these experiments are also shown in table 2. When both BZ1 and BZ2 receptors were labeled by 0.5 nM [^{3}H]PCC, GABA (10^{-4} M) produced a significant 1.8-fold increase in the affinity of FLU.

The results of FLU/[^{3}H]FLU and FLU/[^{3}H]PCC competition experiments in cerebral cortex performed in the presence and absence of GABA (10^{-4} M) are presented in table 3. Similar to the observations in hippocampus, GABA (10^{-4} M) caused a significant increase (≃ 1.8-fold) in the affinity of FLU for [^{3}H]

TABLE 2

Effect of GABA on the affinity of FLU for [^{3}H]FLU and [^{3}H]PCC labeled BZD receptors in rat hippocampus

[^{3}H]-Ligand concentrations and IC_{50} values are in nanomolar. All values represent the mean ± S.E.M. of four determinations. The IC_{50} value represents the concentration of FLU that caused half-maximal inhibition of specific [^{3}H]-ligand binding. Hippocampal homogenates (40 mg of original wet weight/ml) washed five times were used. Incubations were carried out in 50 mM Na/K phosphate buffer, pH 7.4 ± GABA (10^{-4} M) at 0°C and lasted 90 min.

^{3}H-Ligand	^{3}H-Ligand Conc.	FLU		IC_{50} (control)[b]
		IC_{50} (control)	IC_{50} (+ GABA)[a]	IC_{50} (+ GABA)
[^{3}H]FLU	0.05	1.50 ± 0.09	0.91 ± 0.04***	1.67 ± 0.11**
[^{3}H]PCC	0.04	3.10 ± 0.20	2.75 ± 0.30	1.14 ± 0.08
[^{3}H]PCC	0.50	7.47 ± 0.70	4.23 ± 0.27**	1.80 ± 0.20*

[a] Significantly different from control at $P < .01$ and $P < .005$ by Student's t-test.
[b] Significantly different from one at $P < .05$ and $P < .01$ by Student's *t*-test.
* $P < .05$; ** $P < .01$; *** $P < .005$.

TABLE 3

Effect of GABA on the affinity of FLU for [^{3}H]FLU and [^{3}H]PCC labeled BZD receptors in rat cerebral cortex

[^{3}H]-Ligand concentrations and IC_{50} values are in nanomolar. All values represent the mean ± S.E.M. of four determinations. The IC_{50} value represents the concentration of FLU that caused half-maximal inhibition of specific [^{3}H]-ligand binding. Cortical homogenates (50 mg of original wet weight/ml) washed five times were used. Incubations were carried out in 50 mM Na/K phosphate buffer, pH 7.4 ± GABA (10^{-4} M) at 0°C and lasted 90 min.

[^{3}H]-Ligand	[^{3}H]-Ligand Conc.	FLU		IC_{50} (control)[b]
		IC_{50} (control)	IC_{50} (+ GABA)[a]	IC_{50} (+ GABA)
[^{3}H]FLU	0.05	1.90 ± 0.20	1.04 ± 0.09**	1.84 ± 0.09***
[^{3}H]PCC	0.04	3.90 ± 0.60	2.65 ± 44	1.47 ± 0.07**
[^{3}H]PCC	0.50	8.4 ± 0.60	4.90 ± 0.40***	1.80 ± 0.12*

[a] Significantly different from control at $P < .01$ and $P < .005$ by Student's *t*-test.
[b] Significantly different from one at $P < .05$, $P < .01$ and $P < .005$ by Student's *t*-test.
* $P < .05$; ** $P < .01$; *** $P < .005$.

TABLE 4

Effect of GABA on the affinity of FLU for [^{3}H]FLU and [^{3}H]PCC labeled BD receptors in rat cerebellum

[^{3}H]-Ligand concentrations and IC_{50} values are in nanomolar. All values represent the mean ± S.E.M. of four to seven determinations. The IC_{50} value represents the concentration of FLU that caused half-maximal inhibition of specific [^{3}H]-ligand binding. Cerebellar homogenates (60 mg of original wet weight/ml) washed five times were used. Incubations were carried out in 50 mM Na/K phosphate buffer, pH 7.4 ± GABA (10^{-4} M) at 0°C and lasted 90 min.

[^{3}H]-Ligand	[^{3}H]-Ligand Conc.	FLU		IC_{50} (control)[b]
		IC_{50} (control)	IC_{50} (+ GABA)[a]	IC_{50} (+ GABA)
[^{3}H]FLU	0.05	2.24 ± 0.18	1.53 ± 0.06**	1.50 ± 0.15*
[^{3}H]PCC	0.04	4.10 ± 0.10	2.85 ± 0.20***	1.46 ± 0.14*

[a] Significantly different from control at $P < .005$ and $P < .001$ by Student's *t*-test.
[b] Significantly different from one at $P < .05$ by Student's *t*-test.
* $P < .05$; ** $P < .005$; *** $P < .001$.

TABLE 5

Inhibition of [^{3}H]FLU binding in various regions of the rat brain by PCC and CL 218872

Numbers in parentheses represent the number of independent determinations. All values represent the mean ± S.E.M. [^{3}H]FLU (0.05 or 0.5 nM) was used in the PCC and CL 218872 competition experiments, respectively. All incubations were performed at 0°C for 90 min. K_i values were determined from the equation $K_i = IC_{50}(1 + [L]/K_D)$, where L is the free tritiated ligand concentration and the K_D is the independently determined apparent dissociation constant. The K_D used in these experiments was 1.5 nM.

Brain Area	K_i	Hill Slope
	nM	
Cerebral cortex		
PCC (3)	2.8 ± 0.05	0.78 ± 0.01
CL 218872 (5)	136.8 ± 22.8	0.67 ± 0.01
Hippocampus		
PCC (3)	4.8 ± 1.7	0.68 ± 0.01
CL 218872 (5)	243.0 ± 26.0	0.62 ± 0.04
Cerebellum[a]		
PCC (3)	0.83 ± 0.08	1.06 ± 0.07
CL 218872 (5)	41.0 ± 7.3	0.85 ± 0.03

[a] Both K_i and Hill slope values for PCC and CL 218872 found in the cerebellum were significantly different from those values observed in the hippocampus and cerebral cortex with $P < .02$ being the minimum level of significance by Student's *t* test.

FLU (0.05 nM) labeled receptors. In contrast to the hippocampus, GABA induced a small but significant increase (1.47-fold) in the affinity of FLU for cerebral cortex BZD receptors labeled by a low concentration of [^{3}H]PCC (0.04 nM). However, this increase was significantly less ($P < .02$, Student's *t*-test) than the 1.8-fold increase observed in the cortex when [^{3}H]FLU (0.05 nM) was used to label the receptors. When a greater proportion of the BZ2 sites were labeled with a high concentration of [^{3}H]PCC (0.5 nM), the effect of GABA was significantly increased (1.8-fold, $P < .05$, Student's *t*-test) over the effect observed with 0.04 nM [^{3}H]PCC (table 3).

The effects of GABA on FLU/[^{3}H]FLU and FLU/[^{3}H]PCC competition experiments in the cerebellum were similar (table 4). GABA (10^{-4} M) enhanced the affinity of FLU for both [^{3}H]FLU (0.05 nM) and [^{3}H]PCC (0.04 nM) labeled receptors (approximately 1.5-fold). The similarity in the magnitude of the GABA effect is consistent with a previous report that almost all BZD binding sites in the cerebellum belong to a homogeneous population of receptors (Klepner *et al.*, 1979). The steep Hill slopes of the PCC/[^{3}H]FLU and CL 218872/[^{3}H]FLU competition curves and the greater affinity (smaller K_i values) of PCC and CL 218872 for the BZD receptors labeled by [^{3}H]FLU in the cerebellum, when compared with that found in the cerebral cortex and hippocampus, provide additional

evidence that the cerebellum is populated by a nearly homogeneous population of BZ1 receptors (table 5). Furthermore, dissociation kinetics of [^{3}H]PCC in cerebellum are consistent with the contention that the cerebellum contains a nearly homogeneous population of BZD binding sites (fig. 2). Linear Scatchard plots for [^{3}H]PCC and the similarity in maximum binding values for [^{3}H]FLU and [^{3}H]PCC binding (715 *vs.* 682 fmol/mg of protein, respectively) in cerebellum suggest that both [^{3}H]FLU and [^{3}H]PCC label the same binding site (fig. 3).

Discussion

The findings from the hippocampus and cerebral cortex coincide with those of Klepner *et al.* (1979) and suggest that like type II receptors, BZ2 sites are regulated by GABA, whereas similar to type I sites, BZ1 receptors in these two brain regions may not be subject to GABA regulation. This hypothesis is based upon the inability of GABA to enhance the affinity of FLU for the high affinity BZD receptor labeled by 0.04 nM [^{3}H]PCC in hippocampus at 0°C. It was assumed that only the high affinity, BZ1 receptor is labeled under such conditions. This assumption was supported by the data obtained from the dissociation kinetics of 0.04 nM [^{3}H]PCC. The apparent effect of GABA on the affinity of FLU for BZD receptors in cortex labeled by 0.04 nM [^{3}H]PCC is consistent with the dissociation kinetics (fig. 2). In the cerebral cortex, 0.04 nM [^{3}H]PCC appears to label a small proportion (≃ 30%) of BZD sites that form rapidly dissociating complexes with [^{3}H]PCC (BZ2 sites) as suggested by the curvilinear dissociation curve. These sites may be responsible for the small GABA effect on FLU potency (1.47-fold increase) observed in the cerebral cortex when low concentrations of [^{3}H]PCC were used.

The apparent ability of GABA to enhance the potency of FLU in the cerebellum, a brain area enriched with BZ1 receptors (Nielsen *et al.*, 1981) suggests the possibility that these BZ1 receptors are different from those found in the hippocampus and cerebral cortex. Evidence from PCC/[^{3}H]FLU and CL 218872/[^{3}H]FLU competition experiments, [^{3}H]PCC dissociation kinetics and linear Scatchard plots in cerebellum suggest that this brain region is populated by a nearly homogeneous population of BZD binding sites with high affinity for PCC and CL 218872, in agreement with the findings of Klepner *et al.* (1979), Lippa *et al.* (1980) and Nielsen et al. (1981) (table 5). In contrast to the BZ1 receptors in the hippocampus and cerebral cortex, those in the cerebellum may be functionally associated with a GABA receptor. These results are in opposition to the earlier suggestion that BZD receptors in cerebellum may be independent of GABA regulation (Klepner *et al.*, 1979). The magnitude of the GABA effect in the cerebellum was less than that observed in the hippocampus and cerebral cortex when BZ2 receptors in these two brain regions were also labeled. However, this difference in magnitude was not statistically significant. Nevertheless, this observation raises the possibility that the coupling between GABA receptors and BZ2 receptors in the hippocampus and cerebral cortex may be qualitatively different from GABA receptor-BZ1 receptor coupling in the cerebellum. It is conceivable that the nature of the coupling between GABA receptors and BZ1 and BZ2 receptors may be a determinant of the differential binding properties of [^{3}H]PCC at 0°C.

Several alternative explanations are possible, for the differential effect of GABA in the cerebral cortex and hippocampus. For example, BZD receptors labeled by low concentrations of [^{3}H]PCC at 0°C may be uncoupled from a GABA receptor, whereas those labeled by a high concentration are functionally coupled. Under 37°C conditions, all receptors may appear coupled for thermodynamic reasons (Gee *et al.*, 1982). Alternatively, low concentrations of [^{3}H]PCC at 0°C may be labeling a BZD receptor subtype that is associated with a GABA receptor that is unresponsive to GABA or muscimol. This GABA receptor may have greater sensitivity for piperidine derivatives

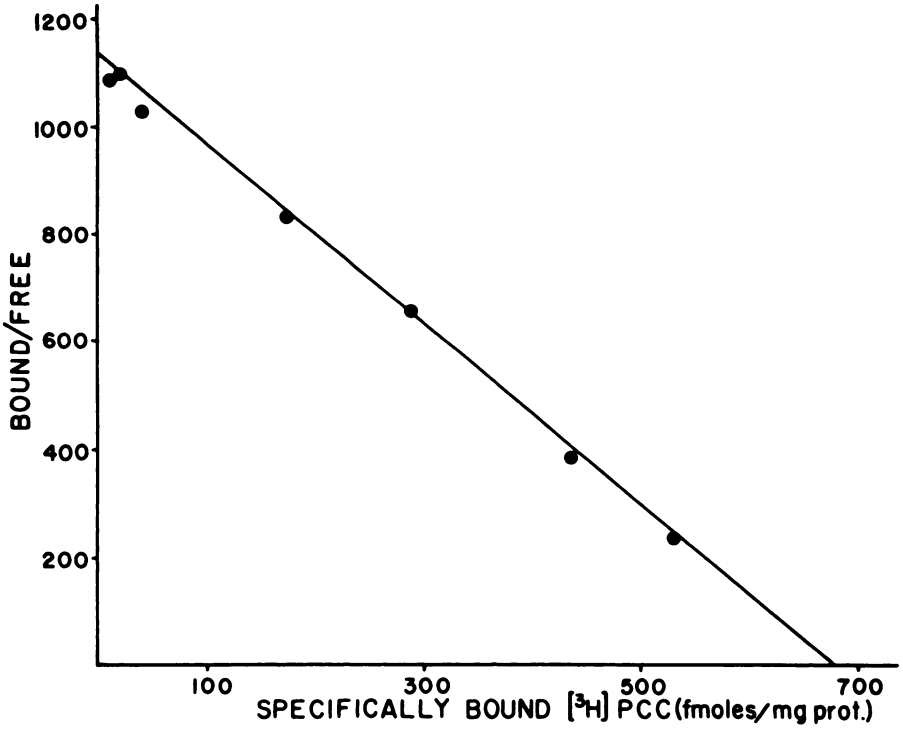

Fig. 3. Scatchard plot of [^{3}H]PCC (0.0125–2.5 nM) binding in rat cerebellum. Each point represents the result of a triplicate determination. Nonspecific binding was defined by 1 μM clonazepam. Plot is representative of three independent determinations. prot, protein.

such as 4,5,6,7-tetrahydroisoxazolo[5,4c]pyridn-3-ol and isoquvacine (Squires *et al.*, 1980). The apparent heterogeneity of GABA receptors makes it plausible that BZD receptors may be associated with different subtypes of GABA receptors (Braestrup *et al.*, 1979; Karobath and Sperck, 1979). Another model to explain the reduced sensitivity of BZ1 receptors in the hippocampus and cerebral cortex for GABA is based upon the possibility that these BZ1 receptors are already in a conformation with high affinity for FLU. If this conformation is similar to that induced by GABA, then the presence of GABA would have no further effect on the affinity of BZ1 receptors for FLU.

The effect of temperature on [^{3}H]PCC binding has important ramifications regarding the nature of BZD receptor heterogeneity. The conversion of the binding characteristics of PCC into a state of homogeneity at 37°C has also been observed when the triazolopyridazine, CL 218872, is used as the ligand (Gee *et al.*, 1982; Gee and Yamamura, 1982). This effect of temperature is reversible because receptor heterogeneity was observed in washed and unwashed tissue that had been preincubated at 37°C before use in PCC/[^{3}H]FLU and CL 218872/[^{3}H]FLU competition experiments performed at 0°C. The inability of PCC and CL 218872 to discriminate between BZD receptor subtypes at physiological temperature suggests that BZD receptor heterogeneity may be related to different conformational states of a single receptor. It may be reasonable to assume that the status of the coupling between GABA and BZD receptors influences the conformational state of the BZD receptor. Alternatively, the possibility that 37°C temperatures selectively denature one of the receptor subtypes cannot be absolutely ruled out.

In summary, low affinity BZ2 receptors in the rat cerebral cortex and hippocampus are more sensitive to the influence of GABA than the high affinity, BZ1 receptors. BZ1 receptors in the cerebellum are different from those found in the cerebral cortex and hippocampus. The difference is based upon whether the BZ1 receptor is coupled to a GABA receptor. This indicates that interregional heterogeneity of BZ1 receptors may occur (Gee and Yamamura, 1982). The functional significance of the differential effect of GABA on BZD receptor subtypes remains to be established. It is conceivable that coupling to a GABA receptor may influence the conformational state of the BZD receptor which may be reflected in the heterogeneous binding properties of PCC at 0°C.

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