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Heterogeneous distribution of benzodiazepine receptors among rat neostriatal neurones

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1 The effects of benzodiazepine receptor (BZR) agonist were investigated in dissociated rat neostriatal neurones by a conventional whole-cell patch recording configuration at room temperature.

2 The dissociated neurones, with a longest somatic diameter of larger than 25 μm, were classified as 'large neurones', while those having soma measuring less than 15 μm were described as 'small neurones'. Large neurones were intensely positive for acetylcholinesterase staining, whereas the small ones were not.

3 CL218,872 enhanced the GABA response in both the large and small neurones with similar EC_{50}s. However, the potentiation efficacy of CL218,872 in large neurones was larger than that of small ones.

4 Zolpidem also potentiated the GABA response in both neuronal populations with similar EC_{50}s. This compound also enhanced the GABA response more strongly in large neurones than in small ones.

5 Zopiclone exerted a prominent potentiation in large neurones, although no difference was seen in the EC_{50}s in the large and small neurones.

6 It was concluded that the BZR in large neurones had a different pharmacological property from that in small ones and that the BZR agonists showed a prominent difference, not in EC_{50}, but in the potentiation efficacy between these neuronal populations.

Keywords: Rat neostriatum; GABA; benzodiazepine receptor

Introduction

γ-Aminobutyric acid (GABA) acts as a major inhibitory neurotransmitter within the neostriatum (Groves, 1983; Calabresi *et al.*, 1991). The $GABA_A$ receptor, a type of GABA receptor, is modulated by the benzodiazepine receptor (BZR) which is incorporated with the $GABA_A$ receptor-Cl^- channel complex (Sivilotti & Nistri, 1990). Radioligand studies have shown the presence of BZR in the neostriatum (Maloteaux *et al.*, 1988) and revealed that BZR in the neostriatum is altered in patients with Parkinson's disease (Nishino *et al.*, 1988; Maloteaux *et al.*, 1988). In the clinical field, diazepam, a BZR agonist, has been reported to decrease the severity of levodopa-induced diskinesia (Pourcher *et al.*, 1989). When zopiclone, a non-benzodiazepine BZR agonist, is used in combination with levodopa, it also ameliorates the motor symptoms of juvenile onset Parkinson's disease (Nagasato *et al.*, 1992). These facts suggest that BZR in the basal ganglia may thus have some functions and, as a result, could potentially be a therapeutic target.

Recently, immunohistochemistry has revealed the heterogeneous distribution of $GABA_A$ receptor subunits among neuronal populations in several brain areas (Gao *et al.*, 1993; 1995). These findings imply that the functional properties of BZR may be different among neuronal populations. In the neostriatum, two major kinds of neuronal populations have been recognized morphologically (Kawaguchi, 1992). These populations play distinct functional roles. Large aspiny neurones serve as cholinergic interneurones, while medium spiny neurones are GABAergic and project to the globus pallidus and the substantia nigra. The medium spiny neurones also send collateral fibres to both adjacent projecting neurones and cholinergic interneurones (Groves, 1983; Kawaguchi *et al.*, 1995). To understand the actions of BZR agonists in this brain area, it is therefore important to clarify the properties of BZR in each neuronal population.

For this purpose, we used neostriatal neurones freshly dissociated from the rat brain. In the dissociated neostriatal neurones, two major kinds of neuronal populations were also recognized (Munakata & Akaike, 1994; Surmeier *et al.*, 1995). Based on our findings, it is suggested that the BZR expressed in each population has different pharmacological properties.

Methods

Preparation

Single neurones of the neostriatum were acutely dissociated from 14–19 day-old Wistar rats of either sex, according to procedures described elsewhere (Ito *et al.*, 1991). Briefly, the rats were anaesthetized with ether and decapitated. The brain was removed and an area containing the neostriatum was cut into coronal slices (400 μm) with a microslicer (DTK-1000, D.S.K.). These slices were pre-incubated in solution saturated with 5% CO_2: 95% O_2 gas. Thereafter, the slices were treated with enzymes. The enzyme treatment consisted of incubation first in 0.016% pronase (Calbiochem) for 25 to 30 min at 31°C and subsequently in 0.01% thermolysin (Sigma) under the same conditions. Subsequently, the dorsal half of the neo striatum (the caudate-putamen) was micro-punched out and mechanically dissociated with fine Pasteur pipettes in a culture dish (Falcon) filled with normal external solution.

We have shown previously that suitable enzyme treatments do not alter the receptor properties for various neurotransmitters including glutamate, acetylcholine, 5-hydroxytryptamine and GABA (Nakagawa *et al.*, 1991; Shirasaki *et al.*, 1994; Nishikawa *et al.*,1994). The benzodiazepine receptors are also maintained in the enzymatically dissociated neurones with distinct pharmacological properties (Yakushiji *et al.*, 1993; Oh *et al.*, 1995). However, some receptors may be partially digested by the proteases, resulting in a decrease in the receptor density (Kaneda *et al.*, 1988). In the present study, the effects of the BZR agonists are described as the potentiation ratio to the control, and are discussed in comparison with that of diazepam, a non-selective BZR agonist.

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Acetylcholinesterase cytochemistry

In a separate series of experiments, the dissociated neurones were stained for acetylcholinesterase by the modified Karnovsky method (Karnovsky & Roots, 1964; Studer *et al.*, 1994). After the dissociated neurones adhered to the bottom of the dish, they were rinsed in Millonig phosphate-buffered saline (PBS; 13.6 mM NaH_2PO_4, 10.7 mM NaOH and 145.4 mM NaCl; pH 7.4). Next, the neurones were fixed with 4% formaldehyde and 0.5% glutaraldehyde in PBS at room temperature for 30 min. After rinsing in PBS, the neurones were incubated in a modified Karnovsky medium (Studer *et al.*, 1994) at 37°C for 60 min. After rinsing in PBS, the neurones were examined with a microscope.

Solutions and their application

The ionic composition of the incubation medium was (in mM): NaCl 125, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 24 and glucose 10, aerated with 95% O_2:5% CO_2 gas to a final pH of 7.4. The ionic composition of the standard external solution was (in mM): NaCl 150, KCl 5, $MgCl_4$ 1, $CaCl_2$ 2, N-2 - hydroxyethyl - piperazine - N' - 2 - ethanesulphonic acid (HEPES) 10 and glucose 10. The pH was adjusted to 7.4 with tris (hydroxy-methyl) aminomethane base (Tris-base). The ionic composition of the pipette (internal) solution was (in mM): NaCl 30, KCl 50, K-gluconate 70, $CsCl_2$ 0.25, $MgCl_2$ 6, Na_2 ATP 5, EGTA 5, HEPES 10. The pH was adjusted to 7.2 with Tris-base.

Electrical measurement

The current and voltage were measured by the conventional whole-cell patch-clamp technique (Hamill *et al.*, 1981). Patch pipettes were fabricated from glass capillaries (Narishige, 1.5 mm outer diameter) on a two-stage puller (Narishige, PB-7). The resistance between the patch-pipette filled with the internal solution and the reference electrode was 4 to 6 MΩ. Ionic currents were measured with a patch-clamp amplifier (List Electronic, EPC-7). Signals were filtered with a low-pass filter (NF Electronic Instruments, FV-665) at a cut off frequency of 1 kHz and were monitored simultaneously on a storage oscilloscope (Iwatsu, MS-5100A) and a pen-recorder (San-ei, RECTI HORIZ 8K). Records were stored on a video cassette recording system (Mitsubishi, HV-F93) through a pulse coded modulation processor (SONY, PCM 501) for subsequent analysis using the pCLAMP system (Axon Instruments). All the electrical measurements were performed at room temperature (25–27°C).

Drugs

The drugs were applied by a rapid application system termed the 'Y-tube' method, as described elsewhere (Murase *et al.*, 1989; 1990). With this technique, the solution surrounding a dissociated neurone could be completely exchanged within 30 ms.

The drugs used in the present experiments consisted of thermolysin (Sigma), tetrodotoxin (TTX) (Sankyo) and pronase (Calbiochem). Diazepam and 3-methyl-6-(3-trifluolomethyl-phenyl)-1,2,4-triazolo (4,3-β) pyridazine (CL218,872) were synthesized by the Yoshitomi pharmaceutical company, while zolpidem and zopiclone were gifts from the Fujisawa and Chugai pharmaceutical companies, respectively. Diazepam was dissolved in dimethyl sulphoxide (DMSO) at 10^{-3} M, and CL218,872, zolpidem and zopiclone were dissolved in DMSO at 10^{-2} M. These BZR agonists were diluted to the respective final concentration in the normal standard solution just before use. The maximal concentration of DMSO was less than 0.3%, at which concentration it did not affect the GABA-induced currents.

Statistical analysis

The data are presented as the mean ± standard error of the mean (s.e.mean) in the text, and the s.e.mean is indicated by a vertical bar in figures. Statistical significance was determined by Student's unpaired t test.

The continuous curves for concentration-response relationships were constructed according to a modified Michaelis-Menten equation (1), using a least-squares fitting routine:

$$I = I_{max} \frac{C^{n_H}}{C^{n_H} + EC_{50}{}^{n_H}} \quad (1)$$

where I is the drug-induced current amplitude and C is the corresponding drug concentration. EC_{50} and n_H denote the half-activation concentration and the Hill coefficient, respectively. The equation for the concentration-augmentation curves for BZR agonists is as follows (2):

$$I' = (I'_{max} - 1) \frac{C^{n_H}}{C^{n_H} + EC_{50}{}^{n_H}} + 1 \quad (2)$$

where I' is the current amplitude normalized by that of the control. When the current amplitudes were normalized to that of the control I'_{max} gives the maximal potentiation ratio for each BZR agonist.

Results

Acutely dissociated neurones of the rat neostriatum

Neostriatal neurones with longest somatic diameters of more than 25 μm were tentatively classified as 'large neurones' and those having soma less than 15 μm as 'small neurones'. In the present dissociated neurones, all large neurones ($n = 34$) were intensely positive for AChE staining. On the other hand, 88 (94.5%) out of 93 small neurones were AChE negative, while 5 neurones (5.5%) were slightly positive.

GABA-induced currents in the neostriatal neurones

When the neurones were perfused by external and internal (pipette) solutions containing 161 and 82.5 mM Cl^-, respectively, GABA induced an inward current(I_{GABA}) in both large and small neurones at a holding potential (V_H) of −50 mV (Figure 1a). Figure 1b summarizes the concentration-response relationships for GABA in large and small neurones. All current amplitudes were normalized to the current amplitude induced by 10^{-5} M GABA (shown by *). In large neurones, the EC_{50} and Hill coefficients for I_{GABA} were 2.2×10^{-5} M and 1.3, respectively. The estimated EC_{50} and Hill coefficients for I_{GABA} in small neurones were 2.0×10^{-5} M and 1.1, respectively.

The effects of BZR agonists on GABA response in large neurones

The effects of BZR agonists such as diazepam, CL218,872, zolpidem and zopiclone on I_{GABA} were examined in large neurones. Figure 2 shows typical cases of the effects of these agonists on the I_{GABA} induced by 3×10^{-6} M GABA. Neurones were pretreated with the agonists for 2 to 3 min, which gave the steady-state enhancement. All the agonists tested enhanced the GABA response in a concentration-dependent manner with different efficacies and potencies. None of the BZR agonists themselves evoked any current.

The concentration-potentiation relationships for BZR agonists in large and small neurones

Figure 3 shows the facilitatory effects of BZR agonist on I_{GABA} induced by 3×10^{-6} M GABA in large and small neurones. The

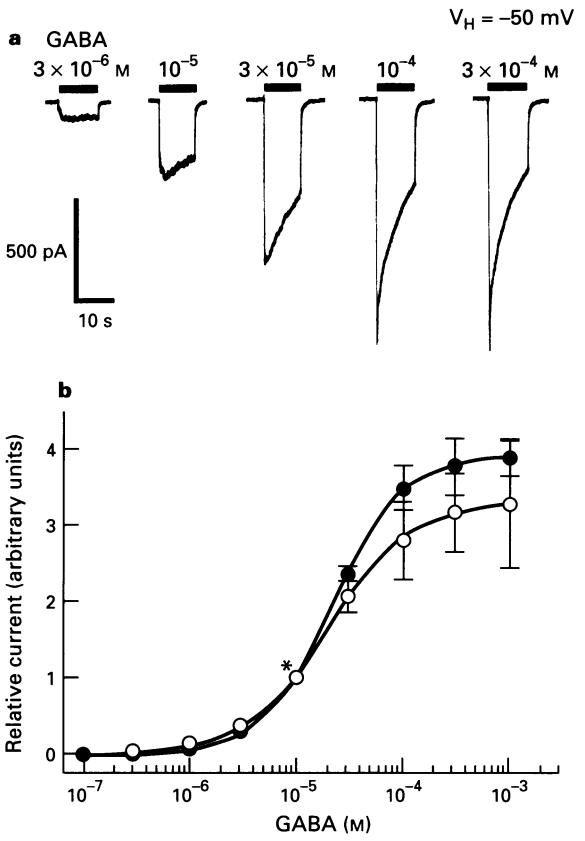

Figure 1 GABA-induced inward currents (I_{GABA}) in the neostriatal neurones. (a) Typical currents elicited by GABA in a large neurone. V_H was −50 mV. GABA was continuously applied by the Y-tube during the periods indicated by the bars above each trace. (b) Concentration-response curves for GABA in large (●) and small neurones (○). Each point is the average of 6 neurones with s.e.mean.

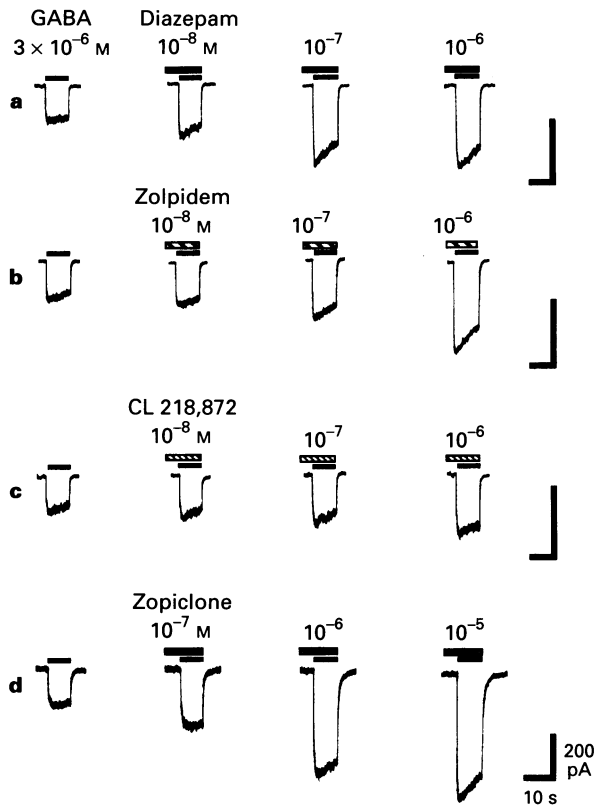

Figure 2 Augmentation of I_{GABA} induced by 3×10^{-6} M GABA in the presence of diazepam (a), zolpidem (b), CL218,872 (c) and zopiclone (d) in large neurones. All agonists potentiated the I_{GABA} in a concentration-dependent manner with different efficacies. V_H was −50 mV. The pretreatment time for each agonist at various concentrations was 2 min, and 3×10^{-6} M GABA and one of the agonists were applied simultaneously. The traces in (a), (b), (c) and (d) were obtained from different neurones.

data were fitted by equation (1). The estimated EC_{50}, Hill coefficient and maximal potentiation ratios are summarized in Table 1. All the BZR agonists tested were effective in both neuronal populations with distinct pharmacological properties. As shown in Table 1, BZR agonists showed prominent differences not in the EC_{50}, but in the maximal potentiation efficacy between large and small neurones. In Figure 3c, the maximal potentiation ratio for BZR agonists are compared in each neuronal population. Zolpidem (3×10^{-6} M) and zopiclone (10^{-5} M) were more efficacious than diazepam (10^{-6} M), while the efficacy of CL218,872 (3×10^{-6} M) was smaller than that of diazepam (Figure 3c(i)). On the other hand, there was no significant difference among efficacies for diazepam, zolpidem and zopiclone, whereas the efficacy of CL218,872 was much smaller than that of diazepam. In addition, the effect of CL218,872 in small neurons was smaller than that in large ones.

The concentration-response relationships for GABA in the presence of BZR agonists

Zopiclone binds to a different site from a recognition site for benzodiazepines (Trifiletti & Snyder, 1984). We thus investigated whether or not the prominent potentiation of I_{GABA} by zopiclone was based on a mechanism similar to that of diazepam. As a result, both compounds potentiated I_{GABA} by increasing the affinity of the GABA receptor for its ligand without changing either the maximal response amplitude or Hill coefficient. The concentrations of diazepam and zopiclone were 10^{-7} M and 3×10^{-6} M, respectively, which exerted the submaximal potentiation of the GABA response (Figure 3a). The EC_{50} for I_{GABA} in the control was 1.9×10^{-5} M and 5.2×10^{-6} M, respectively.

The effects of BZR agonists on the current-voltage (I-V) *relationship for the GABA response*

Figure 5 shows the *I-V* relationship for I_{GABA} with or without 10^{-7} M diazepam or 3×10^{-6} M zopiclone. The concentration of GABA was 3×10^{-6} M. Large neurones were voltage-clamped at a V_H of +10 mV. Subsequently, as shown in the inset, a voltage-ramp of 4 s duration from +10 to −45 mV was applied before and during the application of GABA with or without BZR agonists. In this figure, the *I-V* plots before and during the application of GABA were subtracted from each other. The reversal potential for I_{GABA} (E_{GABA}) without BZR agonists was -12.7 ± 0.6 mV ($n = 5$), which was close to the Cl^- equilibrium potential of −15.2 mV. None of the BZR agonists used changed the E_{GABA}, where the E_{GABA} in the presence of diazepam and zopiclone were -11.4 ± 0.9 mV ($n = 5$) or -11.9 ± 0.5 mV ($n = 5$), respectively. Therefore, the prominent potentiation of zopiclone in large neurones was due to the enhancement of the Cl^-current elicited by GABA without affecting any other ionic currents.

Discussion

We investigated the effects of BZR agonists in rat neostriatal neurones. The BZR in large neurones had a different pharmacological properties from that in small ones, and the BZR agonists showed a distinct difference, not in EC_{50}, but in the potentiation efficacy between these neuronal populations at room temperature.

In the mammalian neostriatum, there are two major morphological types of neurones. Large aspiny neurones (20–60 μm) are cholinergic interneurones (DiFiglia & Carey, 1986),

Table 1 The EC_{50}, maximal potentiation ratio and the Hill coefficient for BZR agonists in large and small neurones.

	Large neurones			*Small neurones*		
	EC_{50} (M)	*Potentiation ratio*	*Hill coefficient*	*EC_{50} (M)*	*Potentiation ratio*	*Hill coefficient*
Diazepam	2.1×10^{-8}	2.1	1.4	3.0×10^{-8}	1.9	0.8
CL218,872	2.1×10^{-7}	1.7	1.0	1.8×10^{-7}	1.3	1.1
Zolpidem	1.8×10^{-7}	2.8	0.9	9.2×10^{-8}	1.8	1.3
Zopiclone	2.2×10^{-7}	3.2	1.0	1.7×10^{-7}	1.9	1.0

All values were determined by fitting the data to an equation (2), using a least-squares fitting routine. EC_{50}, half-maximum concentration.

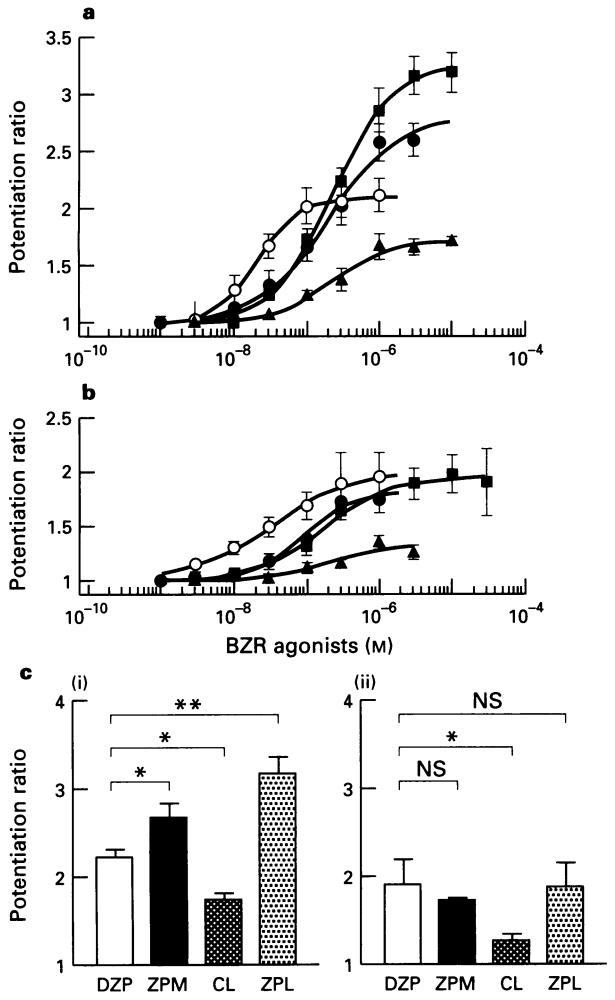

Figure 3 Concentration-potentiation relationships for BZR agonists in large (a) and small (b) neurones. The concentration of GABA was 3×10^{-6} M. V_H was -50 mV. BZR agonists tested were diazepam (○), zolpidem (●), CL218,872 (▲) and zopiclone (■). Each point is the average of 5 to 9 neurones. (c) Maximal potentiation ratios (efficacies) for BZR agonists in large (i) and small neurones (ii). The averaged effects of diazepam (DZP; 10^{-6} M), zolpidem (ZPM; 3×10^{-6} M), CL218,872 (CL; 3×10^{-6} M) and zopiclone (ZPL; 10^{-5} M) on GABA (3×10^{-6} M) are shown with s.e.mean ($n = 6-9$). $^*P < 0.05$; $^{**}P < 0.01$. NS; no significant difference.

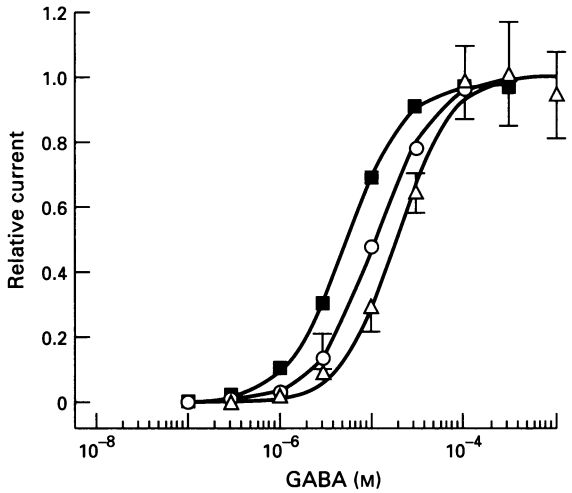

Figure 4 The effects of BZR agonists on the concentration-relationship for GABA. V_H was -50 mV. Both diazepam (10^{-7} M; ○) and zopiclone (3×10^{-6} M; ■) caused a parallel shift in the control GABA concentration-response curve (△) to the left without affecting the maximum current amplitude. Each point is the average of 4 to 6 neurones.

while medium spiny neurones (10–20 μm) are projecting neurones which comprise 90–95% of the neuronal populations (Graveland & DiFiglia, 1985). In the present study, the large neurones (>25 μm) were intensely stained for acetylcholinesterase (AChE). Although AChE is not a reliable marker for cholinergic neurones, the histochemical findings of combined AChE and choline acetyltransferase (ChAT) staining showed that the neurones which stained intensely for AChE are also ChAT-positive in the neostriatum (Eckenstein & Sofroniew, 1983; Levey *et al.*, 1983). Therefore, large neurones were considered to be cholinergic interneurones. On the other hand, the majority of small neurones was considered to be projecting neurones. However, other kinds of neurones such as parvalumin, calretinin on somatostatin positive interneurones may also be included in small neurones (Kawaguchi *et al.*,1995).

The $GABA_A$ receptor complex containing the α1 subunit displays the BZ_1 phenotype, while the receptor complex having α2, α3 or α5 subunits shows a BZ_2 property (Doble & Martin, 1992; Lüddens *et al.*, 1995). This heterogeneity of BZR was first recognized based as a result of studies on the pharmacology of CL218,872, a partial BZR agonist. CL218,872 has a 10 times higher affinity for BZ_1 than BZ_2 at 0 to 4°C in binding studies (Squires *et al.*, 1979). However, the difference in the affinity decreases to only 3 times at 37°C (Gee & Yamamura, 1982). In electrophysiological studies, the difference in the EC_{50} of the potentiation of I_{GABA} among BZ_1 and BZ_2 also decreases at room temperature (Yakushiji *et al.*, 1993; Wafford *et al.*, 1993). Instead, the efficacy of CL218,872 at BZ_1 is larger than that at BZ_2 (Yakushiji *et al.*, 1993; Wafford *et al.*, 1993). In the current study, CL218,872 caused greater enhancement in large neurones than in small ones, suggesting that large neurones may be of the BZ_1 subtype. In contrast, the small neurones are considered to be the BZ_2 subtype (Lo *et al.*, 1983). If so, this phenomenon is probably due to the differential expression of α1 subunit among neuronal populations. An *in situ* hybridization study reported that the major α subunits in the neostriatum are α2 and α4, but a small quantity of α1 mRNAs was also detected (Wisden *et al.*, 1992). This α1 subunit might thus be preferably expressed in large neurones.

Zolpidem, a BZ_1 selective agonist, also reduces the selectivity for BZ_1 at physiological temperatures (Byrnes *et al.*,

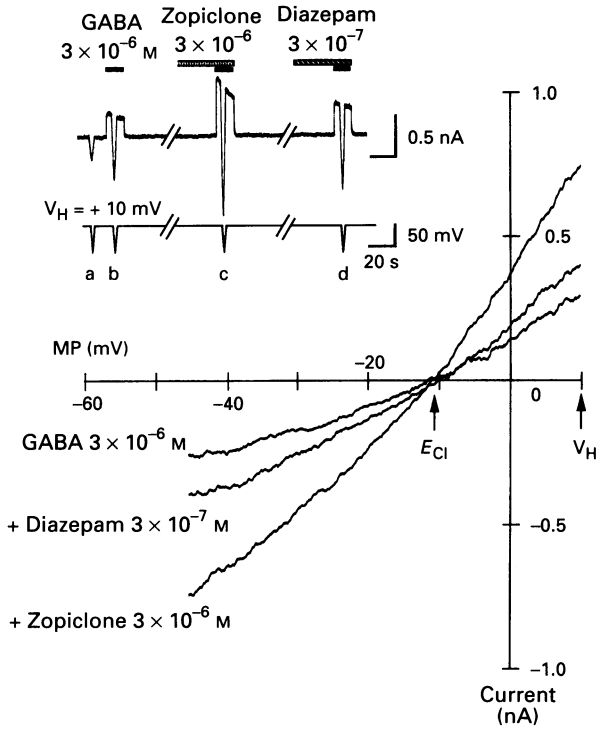

Figure 5 The effects of BZR agonists on the current-voltage (*I-V*) relationship for I_{GABA}. The *I-V* plots were obtained by the ramp-clamp method. As shown in the inset, neurones were voltage-clamped at a V_H of +10 mV and a voltage ramp command with 4 s-duration from +10 to −45 mV was applied before and during the application of GABA at points (a), (b), (c) and (d). Each curve was obtained by the subtraction of the *I-V* plots during the application of the drugs from the plot before application of the drugs. MP, membrane potential. E_{Cl}, the Cl^- equilibrium potential calculated from the Nernst equation.

1992; Wafford *et al.*, 1993). In the present study, there was no apparent difference between the EC_{50} for zolpidem in large neurones and in small ones, while the maximal potentiation ratio for zolpidem in large neurones was somewhat greater than that in small ones. Wafford *et al.* (1993) reported that zolpidem serves as full agonist in both $\alpha1\beta2\gamma2L$ (BZ_1 phenotype) and $\alpha3\beta2\gamma2L$ recombinant forms (BZ_2 phenotype) at room temperature. Thus, the differential efficacy of zolpidem in the present study could not simply be attributed to the difference in the BZ_1 and BZ_2 subtypes. Other subunit families (β, γ) might be involved in the difference in efficacy of zolpidem.

Interestingly, zopiclone produced prominent potentiation in large neurones. Binding studies showed that zopiclone did not recognize the difference between BZ_1 and BZ_2 phenotypes (Trifiletti & Snyder, 1984; Faure-Halley *et al.*, 1993). In an electrophysiological study, the potentiation efficacy of zopiclone was not altered by the presence of the $\alpha1$ subunit (Im *et al.*, 1993). These facts thus suggested that zopiclone does not interact differentially with BZ_1 and BZ_2 subtypes. Instead, the efficacy of zopiclone seems to be affected by the other subunits. This compound facilitated I_{GABA} more strongly in $\alpha1\gamma2$ recombinant form than $\alpha1\beta2\gamma2$ one (Im *et al.*, 1993). Therefore, the differential efficacy of zopiclone among neurones may be based on the differences in another subunit composition.

In the present study, 14–19 day-old Wistar rats were used. *In situ* hybridization studies show that the subunit composition of the $GABA_A$ receptors changes during development in various brain areas (Laurie *et al.*, 1992). In the neostriatum, the expression of $\alpha2$, $\alpha3$, $\alpha5$ and $\beta3$ mRNAs reach a peak at P6 and thereafter gradually decreases, while the $\alpha4$ increases until P12 and then declines slightly to adult levels (Laurie *et al.*, 1992). The low level of $\gamma1$, $\gamma2$, $\gamma3$, $\alpha1$ and $\beta2$ mRNAs is detected throughout the ontogenic stages. These findings suggest that the major changes in $GABA_A$ receptor take place up until P12 in this brain area, and thereafter the receptor may change only a little in the further developmental stages. Since the expression of $\alpha1$ is stable, the expression of the BZ1 phenotype may be maintained until the adult stage. In contrast, the pharmacological properties based on other subunits may change somewhat in the later developmental stages.

It was reported that low dosage of zopiclone improves juvenile onset Parkinson's disease without inducing sleepiness (Nagasato *et al.*, 1992). In the present study, zopiclone was remarkably efficacious in cholinergic interneurones, which are hyperactive in parkinsonism. Thus, the selective effect of zopiclone on the interneurones might partly explain its therapeutic effects. Further investigation on BZR regarding neuronal population in the related brain area might improve the selective therapeutic intervention targeting on the BZR receptors.

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