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BRIEF COMMUNICATION

Residual effect of a 7-amino metabolite of clonazepam on $GABA_A$ receptor function in the nucleus reticularis thalami of the rat

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Summary

A considerable amount of 7-aminoclonazepam (ACZP), a major metabolite of clonazepam (CZP), is present in the brain during CZP treatment, yet the pharmacological properties of ACZP remain unknown. We investigated the effects of ACZP on the $GABA_A$ receptor-mediated currents (I_{GABA}) in neurons from the nucleus reticularis thalami (NRT) of the rat, using a nystatin-perforated patch technique. Neurons in which CZP (10 nM) exerted prominent augmentation (>100% augmentation) of I_{GABA}, which comprised 32% of the neurons tested, were included for the analysis of ACZP. In these neurons, ACZP augmented I_{GABA}, which was blocked by 10 μM flumazenil, a benzodiazepine receptor (BZR) antagonist. The half-maximal effective concentration of ACZP was 124 nM, whereas that of CZP was 1.8 nM. The maximal enhancements induced by ACZP and CZP were 38% and 170%, respectively. In neurons from the ventrobasal complex of the thalamus, the effect of ACZP was negligible. Our results suggest that ACZP was a weak partial BZR agonist and that ACZP may competitively modify the effect of CZP, leading to clinical consequences for patients with high levels of ACZP.

KEY WORDS: 7-aminoclonazepam, Clonazepam, $GABA_A$ receptor, Benzodiazepine re-ceptor, Nucleus reticularis thalami, Withdrawal symptoms.

Clonazepam (CZP) is a benzodiazepine derivative widely used in the treatment of several types of epileptic symptoms, including myoclonic jerks and absence seizures (Patsalos, 2005). In humans, CZP is metabolized primarily to 7-aminoclonazepam (ACZP) by nitroreduction via hepatic cytochrome P450. ACZP is then N-acetylated to form 7-acetamidoclonazepam, which is excreted in the urine and feces following extensive biotransformation (Eschenhof, 1973; Seree et al., 1993). During CZP treatment, the serum concentration of ACZP can be as high as the CZP concentration or higher (Sjö et al., 1975; Edge et al., 1991). Administration of radiolabeled CZP revealed that ACZP radioactivity was 20% of the total radioactivity in the brain (Tateishi et al., 1976), indicating that a considerable amount of ACZP is distributed in the brain. Although no pharmacological activity of ACZP has been recognized in humans, ACZP exhibits a weak anticonvulsive effect in the rat (Fukushima et al., 1977). Conversely, patients with high serum concentrations of ACZP show withdrawal symptoms after discontinuing CZP treatment (Sjö et al., 1975). Thus, ACZP may have unknown pharmacological effects in the brain.

Our objective was to investigate the effects of ACZP on the $GABA_A$-mediated response, using a patch-clamp technique. We used freshly dissociated neurons from the nucleus reticularis thalami (NRT) of the rat. The NRT surrounds the thalamus and is involved in synchronized rhythm generation within the thalamocortical network, which may be a major target of CZP activity (Gibbs et al., 1996; Fuentealba & Steriade, 2005).

Materials and Methods

Single NRT neurons were isolated from 11- to 17-day-old Wistar rats of both sexes, as previously described (Sato et al., 2005). Briefly, pentobarbital-anesthetized rats were decapitated, the brains were removed, and coronal slices (400 μm thick) were made in a cutting solution

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using a vibratome (Microslicer DTK-1000, Dosaka EM, Kyoto, Japan). The brain slices were kept in an incubation solution saturated with 5% CO_2 and 95% O_2 at room temperature for 60 min. The slices were then treated with 0.017% pronase (Calbiochem, San Diego, CA, U.S.A.) for 25–35 min at 31 °C, followed by 0.017% thermolysin (Sigma, St. Louis, MO, U.S.A.) under the same conditions. The NRT and the ventrobasal (VB) complex of the thalamus were identified using a stereo zoom microscope and were micro-punched. Using small Pasteur pipettes, the NRT neurons were then mechanically dissociated in a small plastic culture dish (Falcon, Lincoln Park, NJ, U.S.A.) filled with normal external solution. All procedures involving animals were approved by the Animal Care and Use Committee, Tohoku University Graduate School of Medicine.

The ionic compositions of the solutions used were as follows: cutting solution (234 mM sucrose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM $MgSO_4$, 0.5 mM $CaCl_2$, 11 mM glucose), incubation solution (124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose), normal external solution (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM N´-2 ethanesulphonic acid (HEPES) adjusted to pH 7.4 with Tris-base), and pipette solution for the perforated patch (70 mM KCl, 80 mM potassium methanesulfonate, 10 mM HEPES adjusted to pH 7.2 with Tris-base).

Electrical measurements were performed in the whole-cell configuration, using the nystatin-perforated patch technique (Akaike & Harata, 1994). Nystatin pores are permeable to Cl^-, and the reversal potential for Cl^- depends on the Cl^- concentrations in the pipette and external solution (Horn & Marty, 1988). Thus, the driving force for Cl^- was stable throughout the experiments, which was suitable for the pharmacological characterization of the effect of ACZP on the $GABA_A$ receptor-mediated Cl^- currents. Nystatin (Sigma) was dissolved in acidified methanol (10 mg/ml), immediately adjusted to pH 7.0, and stored at −20 °C. The stock solution was added to the pipette solution at a final concentration of 240–300 μg/ml, and the pipette solution was filtered just before use. The resistance between the patch pipettes filled with internal solution and the reference electrode was 8–10 MΩ. Measurements were started after the stabilization of GABA-induced current amplitudes (about 15–25 min after cell attachment). Ion currents and voltages were measured using a patch-clamp amplifier (CEZ-2300; Nihon Kohden, Tokyo, Japan). A junction potential of 6.5 mV was measured to be subtracted from the recorded potentials. All experiments were carried out at 25 °C using a bath temperature controller (DTC-200; Daiya Medical System, Tokyo, Japan).

GABA (Wako, Osaka, Japan) was dissolved in external solution. ACZP (Lipomed, Arlesheim, Switzerland), CZP (Wako), and flumazenil (FMZ; Sigma) were dissolved in dimethylsulfoxide (DMSO; Sigma) and then diluted with standard external solution to the appropriate final concentrations just before use. The maximum concentration of DMSO was less than 0.1%, which did not affect the current response. The drugs were applied using a ''Y-tube'' rapid application system (Murase et al., 1989).

Continuous curve for the observed concentration–response relationship for GABA were constructed according to a modified Michaelis–Menten equation (1), using the Levenberg–Marquardt method:

$$I = I_{\max} \cdot \frac{C^n}{C^n + C_{50}^n}, \quad (1)$$

where I is the drug-induced current amplitude, $I_{\max}$ is the maximal current amplitude, C is the drug concentration, C_{50} is the half-maximal effective concentration, and n is the Hill coefficient.

The concentration–augmentation curves for ACZP and CZP were constructed according to the following equation:

$$\%\text{Augmentation} = P \cdot \frac{C^n}{C^n + C_{50}^n}, \quad (2)$$

where C is the corresponding drug concentration, C_{50} is the half-maximal effective concentration, and n is the Hill coefficient. P is the maximal augmentation induced by the drugs and is expressed as a percentage in the text and figures.

The theoretical concentration–enhancement relationships for CZP in the presence and absence of ACZP were calculated using LabVIEW software (National Instruments, Austin, TX, U.S.A.) and the following equation (Tallarida & Jacob, 1979):

$$\%\text{Augmentation} = P_{CZP} \cdot \frac{C_{CZP}}{C_{CZP} + C_{50CZP}\left(1 + \frac{C_{ACZP}}{C_{50ACZP}}\right)} + P_{ACZP} \cdot \frac{C_{ACZP}}{C_{ACZP} + C_{50ACZP}\left(1 + \frac{C_{CZP}}{C_{50CZP}}\right)}, \quad (3)$$

where C_{CZP} and C_{ACZP} are the concentrations of CZP and ACZP, respectively, and C_{50CZP} and C_{50ACZP} are the half-maximal effective concentrations for CZP and ACZP, respectively. P_{CZP} and P_{ACZP} are the maximal augmentation produced by CZP and ACZP, respectively. A value of 1 was assumed for the Hill coefficient in this equation.

Results

GABA treatment elicited an inward current (I_{GABA}) in a concentration-dependent manner at a holding potential (V_h) of −66.5 mV in NRT neurons (Fig. 1A). The application interval after the application of GABA at concentrations lower than 30 μM was 4 min, and it was 6 min at

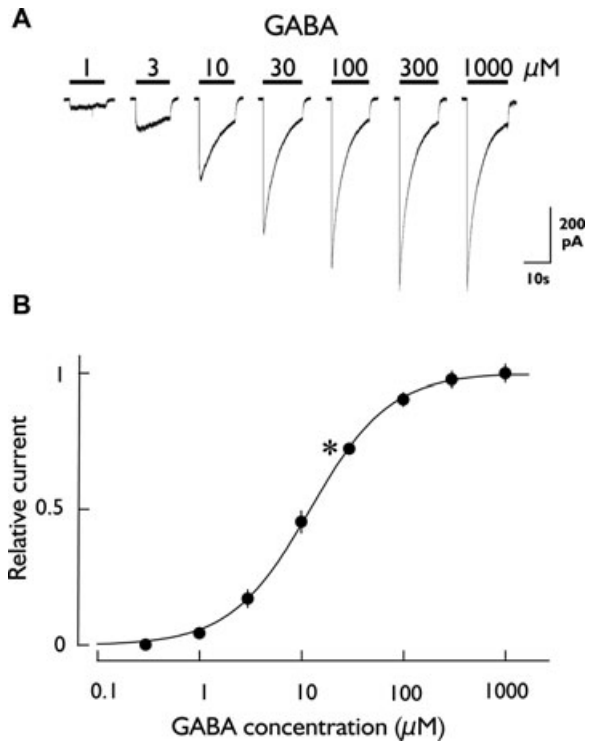

Figure 1.
GABA-induced inward currents (I_{GABA}) in neurons from the NRT. (**A**) Typical currents induced by GABA in a NRT neuron at a V_h of −66.5 mV. GABA was applied externally using a Y-tube method during the periods indicated by the solid bars above each trace. (**B**) Concentration–response relationship for GABA in NRT neurons. The peak current amplitudes for I_{GABA} were normalized to those observed when using 30 μM GABA (asterisk). Continuous curves are the result of fitting the data to equation (1). Units for relative current were determined so that the maximum value of the curve was 1.0. Each point represents the mean response of six neurons ± SEM.

30 μM or higher. The duration of GABA application was approximately 15 s. Fig. 1B summarizes the concentration–response relationship for GABA. The peak current amplitudes for I_{GABA} were normalized to those observed when using 30 μM GABA (Fig. 1B, asterisk). The continuous curve is the result of fitting the data to equation (1). The computer-estimated half-maximal effective concentration (C_{50}) and Hill coefficient for I_{GABA} were 13 μM and 1.0, respectively. Bicuculline (10 μM), a competitive antagonist of the $GABA_A$ receptor, reduced the I_{GABA} elicited by 10 μM GABA to 6.8 ± 3.6% of the control (mean ± SEM, n = 4), indicating that I_{GABA} was a $GABA_A$ receptor-mediated Cl^- current.

We then examined the effect of ACZP on the I_{GABA} elicited by 3 μM GABA in NRT neurons in comparison with the effect of CZP. Because the efficacy of CZP varied among neurons (Gibbs et al., 1996), neurons in which CZP (10 nM) exerted prominent augmentation (>100% augmentation) of I_{GABA}, which comprised 32% of the neurons tested, were included for the analysis of ACZP. In such neurons, ACZP (1 μM) enhanced I_{GABA} by 34.0 ± 3.0% (n = 6), and this enhancement was abolished in the presence of 10 μM FMZ, a benzodiazepine receptor (BZR) antagonist (Fig. 2A). ACZP enhanced the I_{GABA} in a concentration-dependent manner (Fig. 2B). CZP potentiated the I_{GABA} to a much greater degree (Fig. 2C). Fig. 2D summarizes the concentration–enhancement relationships for ACZP and CZP. The maximum augmentation produced by CZP (0.1 μM) was 170 ± 29% (n = 6), significantly larger than the 38 ± 6% (n = 6) produced by ACZP (3 μM) (Student's *t*-test, $p < 0.05$). The ratio for maximal efficacy of ACZP (1 μM) to that of CZP (0.1 μM) in each neuron was 0.24 ± 0.02 (n = 6). The computer-estimated C_{50} and maximum augmentation were 124 nM and 38% for ACZP and 1.8 nM and 170% for CZP, respectively.

The CZP-induced augmentation of the $GABA_A$ response was inhibited in the presence of 1 μM ACZP (Fig. 3A). As summarized in Fig. 3B, ACZP significantly reduced the net CZP-induced augmentation ($p = 0.02$). In Fig. 3C, the plots show the concentration–augmentation relationships for CZP in the presence of 1 μM ACZP. Continuous curves represent theoretical concentration–augmentation relationships in the absence (blue line) and presence of 1 μM ACZP (orange line), calculated using equation (3), with values of C_{50ACZP}, C_{50CZP}, P_{ACZP}, and P_{CZP} estimated in the present study. The plots were close to the corresponding theoretical curve.

Fig. 3D shows the effects of ACZP (1 μM) or CZP (10 nM) on I_{GABA} (3 μM) in a neuron from the VB complex of the thalamus. As summarized in Fig. 3E, the enhancement by ACZP was negligible and that by CZP was very weak (n = 6).

Discussion

In the present study, GABA elicited $GABA_A$ receptor-operated inward currents (I_{GABA}) in NRT neurons; the currents were almost identical to those in a previous report, although the estimated C_{50} of 14 μM was approximately two-fold lower than that previously reported (Gibbs et al., 1996). A possible explanation for this may be differences in recording conditions. The activity of $GABA_A$ receptors is regulated by multiple kinases via phosphorylation, which requires intracellular ATP (Kittler & Moss, 2003). Reduced ATP levels cause decreases in $GABA_A$ receptor activity, with a shift of C_{50} for GABA to higher concentrations (Shirasaki et al., 1992). The previous report used a

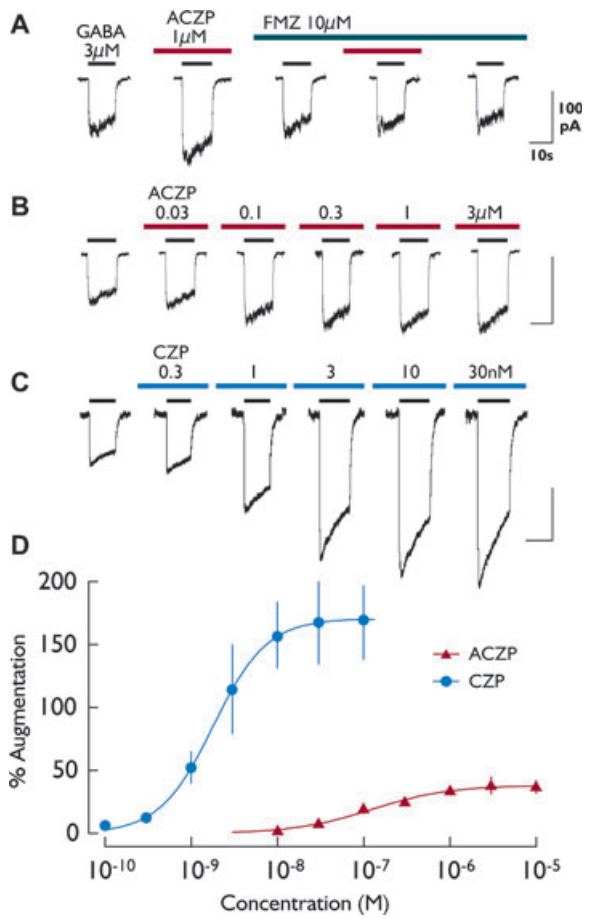

Figure 2.
Effects of ACZP or CZP on the I_{GABA} in NRT neurons. (**A**) Treatment with 1 μM ACZP (red line) enhanced the I_{GABA} elicited by 3 μM GABA (black line). This effect was blocked in the presence of 10 μM FMZ (green line; n = 4). (**B**) ACZP-induced enhancement of the I_{GABA}, at various ACZP concentrations. (**C**) CZP-induced enhancement of the I_{GABA}, at various CZP concentrations (blue line). Neurons were pretreated with ACZP, CZP, or FMZ for 3 min before application of GABA. (**D**) Concentration–enhancement relationships for ACZP and CZP. GABA was used at 3 μM. Each point for ACZP (red; ▲) or CZP (blue; ●) is the mean response of 7–9 neurons ± SEM. Continuous curves are the result of fitting the data to equation (2). All experiments were performed at a V_h of −66.5 mV.
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conventional whole-cell configuration, which supplied ATP by including an ATP regenerating system in the pipette solution, whereas the perforated-patch configuration has ATP from native metabolic production (Akaike & Harata, 1994). This may result in the difference in C_{50}.

CZP strongly enhanced I_{GABA} in subsets of NRT neurons but had little effect on thalamic VB neurons, owing to differences in the subunit composition of the $GABA_A$ receptors. The primary subunits for the $GABA_A$ receptor in the adult rat NRT are α3, β1, β3, and γ2 (Pirker et al., 2000). Among these subunits, the α3 subunit underlies the greater efficacy of CZP in NRT neurons, versus α1 in VB neurons (Browne et al., 2001). The $GABA_A$ receptor in the thalamus reaches a mature configuration by the end of the third postnatal week (Bentivoglio et al., 1990). Along with this, the efficacy of CZP in NRT neurons becomes gradually more prominent during postnatal development, which may parallel ontogenic changes in the dominant α subunit, from α5 to α3 (Pangratz-Fuehrer et al., 2007). The α3 subunit has been detected in subsets of NRT neurons at the age of the rats used in the present study (Browne et al., 2001; Studer et al., 2006). In the CZP-responding neurons, ACZP enhanced I_{GABA} with much lower efficacy and potency than CZP, and ACZP exerted negligible efficacy in VB neurons. The effect of ACZP was blocked by FMZ, confirming that ACZP recognized the BZR. Thus, ACZP was recognized as a weak partial agonist for the BZR.

Benzodiazepines are diversely metabolized to produce compounds with different efficacies. For example, diazepam is biotransformed to three major metabolites, N-desmethyldiazepam, N-methyloxazepam, and oxazepam, all of which are still potent BZR agonists (Marcucci et al., 1968). Midazolam is primarily transformed to α-hydroxymidazolam, which has less efficacy (Ziegler et al., 1983). Conversely, nitrazepam, an analog of CZP, loses its pharmacological activity via biotransformation to 7-aminonitrazepam (Schalleck et al., 1972). The 7-amino metabolite of medazepam (Ro 5-4964) also has very weak effects (Zbinden & Randall, 1967). Similarly, ACZP, the 7-amino metabolite of CZP, exhibits only very weak anticonvulsant activity in mice in vivo, with a half-maximal effective dose 80 times that of CZP (Fukushima et al., 1977). In the present study, the C_{50} of ACZP was 70 times that of CZP, consistent with the results of previous in vivo studies (Fukushima et al., 1977). This enhancement is considered to underlie the weak anticonvulsant activity of ACZP observed in mice in vivo.

The brain concentration of ACZP during chronic administration of CZP is unknown. Tateishi et al. (1976) reported that 4 h after administration of a single oral dose of ^{13}C-clonazepam to male rats, ACZP radioactivity within the brain reached 20% of total brain radioactivity, and CZP radioactivity reached 55%. These findings indicate that considerable amounts of ACZP are distributed in the brain during the chronic use of CZP. In humans, the serum concentration of ACZP can be as high as that of CZP during chronic CZP administration, although the concentration of ACZP varies widely, from 10 to 364 ng/ml (0.035–1.3 μM), presumably as a result of genetic variation in the rate of ACZP acetylation (Walson & Edge, 1996). If only ACZP were to exist in the CNS, it might exert weak effects as a partial agonist for the BZR. However, when ACZP

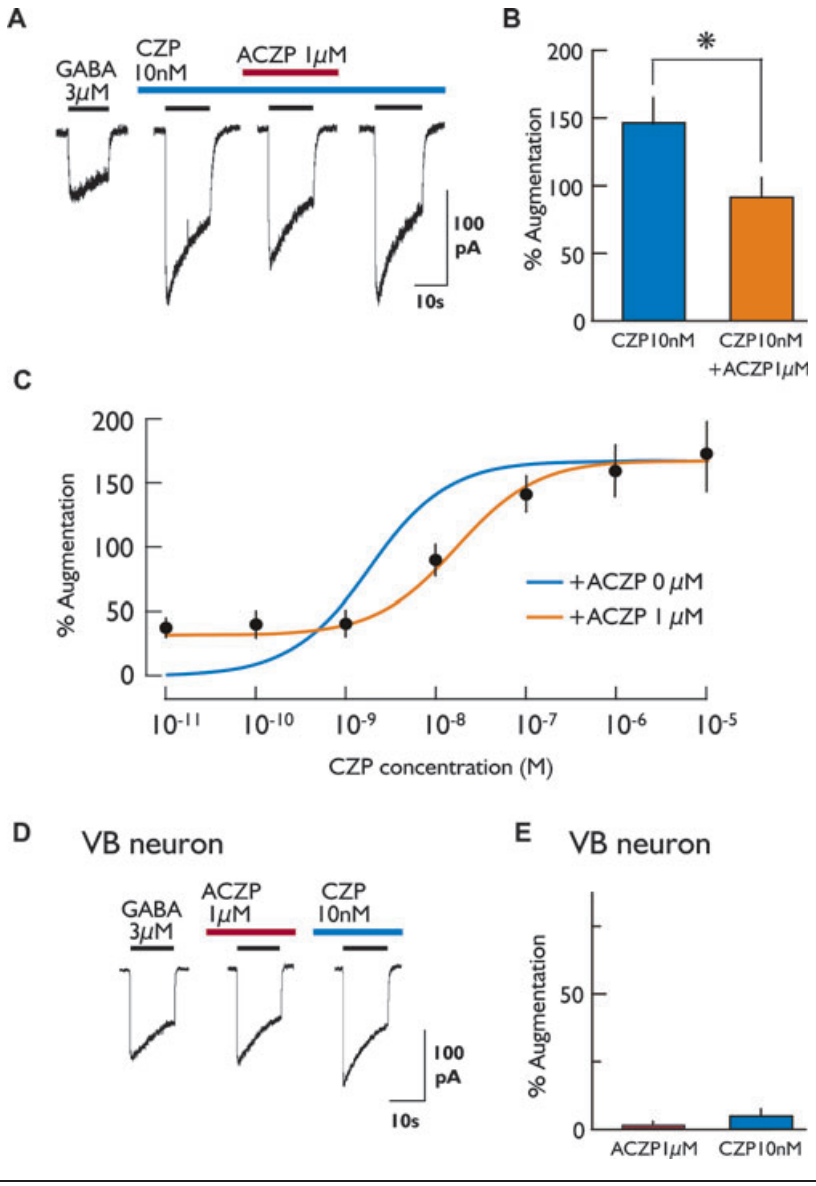

Figure 3.
Effects of ACZP on CZP-induced enhancement of the I_{GABA} in NRT neurons (**A**, **B**, **C**) and effects of ACZP or CZP on the I_{GABA} in neurons from the VB complex (**D**, **E**). (**A**) The I_{GABA} at 3 μM GABA was enhanced by treatment with 10 nM CZP (blue line); this effect was inhibited in the presence of 1 μM ACZP (red line; n = 4). Neurons were pretreated with CZP for 3 min; neurons were pretreated with ACZP and CZP for 9 min to replace previous agonists. $V_h = -66.5$ mV. (**B**) Summary of the effect of 1 μM ACZP on 10 nM CZP-induced enhancement of the $GABA_A$ response (n = 6). ACZP significantly reduced the net enhancement of the I_{GABA} ($p = 0.02$). (**C**) Effects of CZP at various concentrations on I_{GABA} at 3 μM GABA in the continuous presence of 1 μM ACZP (n = 6). Continuous curves are theoretical concentration–enhancement relationships for CZP in the absence (blue line) and presence of 1 μM ACZP (orange line), calculated using equation (3) with the half-maximal effective values and maximal efficacies for CZP and ACZP measured in the present study. (**D**) The effects of 1 μM ACZP or 10 nM CZP on I_{GABA} at 3 μM GABA in neurons from the VB complex. (**E**) Summary of the augmentation effects of 1 μM ACZP and 10 nM CZP on I_{GABA} at 3 μM GABA in VB neurons (n = 6).

and CZP coexist, which is the case in antiepileptic therapy, ACZP may competitively modify the action of CZP, as described in Fig. 3C. If the brain concentration of ACZP were to reach that in serum (~1 μM), its modifying effect may have clinical significance. If so, dual monitoring of the CZP and ACZP concentrations may be clinically useful.

Patients have been found to exhibit withdrawal symptoms in association with high ACZP levels, 3–4 times the levels in patients without symptoms; in contrast, the CZP levels did not differ (Sjö et al., 1975). Generally, benzodiazepine-induced withdrawal symptoms are believed to result from plastic changes in both the GABAergic and glutamatergic systems; chronic enhancement of the $GABA_A$ response causes functional uncoupling of the BZR from $GABA_A$ receptors and compensating up-regulation of glutamatergic signal transduction, which then results in excessive neuronal activity with rapid decreases of benzodiazepines (Allison & Pratt, 2003). Although it remains unclear how ACZP may be involved in this scheme, the clinical implications of ACZP in the withdrawal phase of CZP therapy may depend on the systemic efficacy of ACZP in humans. If the efficacy of ACZP were to be insufficient, the effect of ACZP might be rather antagonistic; ACZP might compete to occupy the BZR and cause a more rapid decrease of CZP binding when the CZP concentration decreases after discontinuing CZP treatment, thus transiently facilitating withdrawal symptoms. Conversely, if the efficacy of ACZP were to be still significant, ACZP might serve as a partial agonist to

mask symptoms when the CZP level is reduced. Further investigation into the pharmacokinetics and the pharmacodynamics of ACZP in the human brain is required.

In conclusion, ACZP had affinity for and efficacy at the BZR. The affinity of ACZP was much lower than that of CZP, and thus the potential effect of ACZP in patients receiving CZP would be small. However, in patients with high levels of ACZP, it may compete with CZP and thus modify the net augmentation of the $GABA_A$ response, which may have clinical consequences.

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Conflict of interest: We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. None of the authors has any conflict of interest to disclose.

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