



Determination of effects of antiepileptic drugs on SNAREs-mediated hippocampal monoamine release using *in vivo* microdialysis

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1 To elucidate possible mechanisms underlying the effects of carbamazepine (CBZ), valproate (VPA) and zonisamide (ZNS) on neurotransmitter exocytosis, the interaction between these three antiepileptic drugs (AEDs) and botulinum toxins (BoNTs) on basal, Ca²⁺- and K⁺-evoked release of dopamine (DA) and serotonin (5-HT) were determined by microdialysis in the hippocampus of freely moving rats.

2 Basal release of monoamine was decreased by pre-microinjection of the syntaxin inhibitor, BoNT/C, but only weakly affected by the synaptobrevin inhibitor, BoNT/B. Ca²⁺-evoked release was inhibited by BoNT/C selectively. K⁺-evoked release was reduced by BoNT/B predominantly and BoNT/C weakly.

3 Perfusion with low and high concentrations of CBZ and ZNS increased and decreased basal monoamine release, respectively. Perfusion with VPA increased basal 5-HT release concentration-dependently, whereas basal DA release was affected by VPA biphasic concentration-dependently, similar to CBZ and ZNS. This stimulatory action of AEDs on basal release was inhibited by BoNT/C predominantly.

4 Ca²⁺-evoked monoamine release was increased by low concentrations of CBZ, ZNS and VPA, but decreased by high concentrations. These effects of the AEDs on Ca²⁺-evoked release were inhibited by BoNT/C, but not by BoNT/B.

5 K⁺-evoked monoamine release was reduced by AEDs concentration-dependently. The inhibitory effect of these three AEDs on K⁺-evoked release was inhibited by BoNT/B, but not by BoNT/C.

6 These findings suggest that the therapeutic-relevant concentration of CBZ, VPA and ZNS affects exocytosis of DA and 5-HT, the enhancement of syntaxin-mediated monoamine release during resting stage, and the inhibition of synaptobrevin-mediated release during depolarizing stage.

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Abbreviations: AEDs, antiepileptic drugs; ANOVA, analysis of variance; BoNTs, botulinum toxins; BoNT/B, botulinum toxin type B; BoNT/C, botulinum toxin type C; CBZ, carbamazepine; DA, dopamine; HCMRS, high Ca²⁺ (3.4 mM) containing modified Ringer's solution; HKMRS, high K⁺ (50 mM) containing modified Ringer's solution; HPLC, high performance liquid chromatograph; 5-HT, serotonin; MRS, modified Ringer's solution; NSF, N-ethylmaleimide-sensitive factor; PKA, cyclic AMP-dependent protein kinase; PKC, calcium-phospholipid-dependent protein kinase; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SRF, sustained high-frequency repetitive firing; TTX, tetrodotoxin; VGSC, voltage-gated Na⁺ channel; VPA, valproate; VSCC, voltage-sensitive Ca²⁺ channel; ZNS, zonisamide

Introduction

The antiepileptic drugs (AEDs), carbamazepine (CBZ), zonisamide (ZNS) and valproate (VPA) have a wide clinical spectrum of use in epileptic (Scheffer *et al.*, 1994; Bourgeois, 1995; Loiseau & Duche, 1995; Seino & Ito, 1997) and mood (Okuma *et al.*, 1990; Kanba *et al.*, 1994; Norton & Quarles, 2000) disorders. Especially, CBZ has been established as a highly effective first line AED against both simple and complex partial seizures (Scheffer *et al.*, 1994; Loiseau & Duche, 1995), whereas VPA has been recognized as a highly-

effective first line AED against idiopathic and primary generalized seizures (Bourgeois, 1995). Several clinical trials have demonstrated ZNS to be a promising AED for treating a wide variety of seizures, including partial and generalised seizures (Seino & Ito, 1997).

The major mechanisms of antiepileptic actions of CBZ and ZNS were considered to be its inhibitory effects on voltage-gated Na⁺ channel (VGSC) activity (McLean & Macdonald, 1986; Rock *et al.*, 1989; Mattson, 1997). The effect of VPA on VGSC has been less extensively studied, and it is uncertain whether VPA has the same mechanism of action as CBZ and ZNS. Although VPA blocked sustained high-frequency repetitive firing (SRF) (Despland, 1994), detailed

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voltage-clamp investigations regarding the effects of VPA on Na^+ current have not been reported. It cannot be concluded that VPA has a mechanism of action similar to those of ZNS and CBZ until these studies have been performed (Bourgeois, 1995). Recently, ZNS was considered as one of the most effective AEDs against absence and myoclonic epilepsies similar to VPA (Seino & Ito, 1997). It has been considered that the putative mechanisms of action of VPA and ZNS may be modulated by their inhibition of T-type voltage-sensitive Ca^{2+} channel (VSCC) activity (Suzuki *et al.*, 1992; Kito *et al.*, 1994; 1996); however, their inhibitory effects were attained by doses higher than the plasma-concentration associated with their antiepileptic action (higher than therapeutic-relevant concentration) (Kito *et al.*, 1994; 1996; Bourgeois, 1995).

Numerous investigators have already studied the effects of these three AEDs on monoaminergic transmission (Pratt *et al.*, 1985; Elphick *et al.*, 1990; Okada *et al.*, 1992; 1995; 1997a, b, d; 1998a, b; 1999a; Takebayashi *et al.*, 1995; Ichikawa & Meltzer, 1999; Kawata *et al.*, 1999; 2001; Mizuno *et al.*, 1994; 2000), because monoaminergic function has been related to the aetiology of several psychiatric and neurological disorders. It has been established that inhibition of VGSC activity reduces basal neurotransmitter release (Westerink *et al.*, 1988; 1989). However, we have already demonstrated concentration-dependent biphasic effects of CBZ and ZNS on various neurotransmitter releases (Okada *et al.*, 1992; 1995; 1997a, d; 1998a, b; 1999a; Mizuno *et al.*, 1994; Kawata *et al.*, 1999; 2001). The plasma-concentration of CBZ and ZNS associated with their antiepileptic action (therapeutic-relevant concentration), enhanced basal release of monoamines and acetylcholine without affecting basal glutamate release, and inhibited the depolarization-induced release of monoamine and glutamate (Okada *et al.*, 1992; 1995; 1997a, d; 1998a, b; 1999a; Mizuno *et al.*, 1994; Kawata *et al.*, 1999; 2001), whereas higher than therapeutic-relevant concentration of CBZ and ZNS reduced them (Okada *et al.*, 1992; 1995; 1997a, d; 1998a, b; 1999a; Kawata *et al.*, 1999; 2001; Mizuno *et al.*, 2000). Contrary to CBZ or ZNS, several initial studies could not demonstrate the concentration-dependent biphasic action of VPA monoaminergic system (Takebayashi *et al.*, 1995).

It has been considered that a mechanistic model for neurotransmitter release, known as the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptor (SNARE) hypothesis, proposes that vesicle membrane SNAREs, synaptotagmin and synaptobrevin, binds to cognate proteins on the target membrane SNAREs, syntaxin and SNAP-25, in order to form complexes that are then recognized and dissociated by the general membrane trafficking factors α -SNAP and NSF (Sollner *et al.*, 1993; Sudhof, 1995). Recently, we have demonstrated that hippocampal serotonin (5-HT) release was regulated by two major functional complexes, 'N-type VSCC/calcium-phospholipid-dependent protein kinase (PKC)/syntaxin' and 'P-type VSCC/cyclic AMP-dependent protein kinase (PKA)/synaptobrevin', using *in vivo* microdialysis (Okada *et al.*, 2001).

Hence, using the *in vivo* microdialysis technique, the present study was designed to determine the interaction between AEDs (CBZ, VPA and ZNS) and SNAREs inhibitors, botulinum toxins (BoNTs) on hippocampal release of dopamine (DA) and 5-HT using *in vivo* microdialysis, in

order to: (1) clarify the mechanisms of concentration-dependent biphasic action of CBZ and ZNS on hippocampal neurotransmitter release, including basal, Ca^{2+} - and K^+ -evoked, (2) explore whether there is a concentration-dependent biphasic action of VPA on neurotransmitter release, and (3) determine the effects of these three AEDs on exocytosis.

Methods

All of the experiments described in this report were performed in accordance with the specifications of the Ethical Committee of Hirosaki University and met the guidelines of the responsible governmental agency. Male Wistar rats (Clea, Tokyo, Japan), weighing 250–300 g, were housed under conditions of constant temperature $22 \pm 2^\circ\text{C}$ with a 12 h light-dark cycle.

Microdialysis system preparation

Each rat was placed in a stereotaxic frame and kept under halothane anaesthesia (1.5% mixture of halothane and O_2 with N_2O). Before inserting the microdialysis probe, all rats used in this study were pre-treated with a microinjection of 0.3 μl modified Ringer's solution (MRS) with or without 0.3 ng botulinum toxins (BoNTs) (Capogna *et al.*, 1997; Pierce and Kalivas, 1997; Okada *et al.*, 2001). A concentric I-type dialysis probe (0.22 mm diameter; 3 mm exposed membrane; Eicom, Kyoto, Japan) was implanted in the hippocampus at anterior = -5.8 mm, lateral = 4.8 mm and vertical = -4.0 mm (relative to bregma) (Paxinos & Watson, 1986) and the perfusion experiments were started 18 h after the rats had recovered from anaesthesia (Okada *et al.*, 1997c; 1999b). The perfusion rate was always $1 \mu\text{l min}^{-1}$, using MRS composed of (in mM): Na^+ 145, K^+ 2.7, Ca^{2+} 1.2, Mg^{2+} 1.0, Cl^- 154.4, and buffered with 2 mM phosphate buffer and 1.1 mM Tris buffer adjusted to pH 7.40 (Okada *et al.*, 1998b, c). To study the effects of an increase in extracellular levels of Ca^{2+} (Ca^{2+} -evoked stimulation) and K^+ (K^+ -evoked stimulation) on the hippocampal extracellular monoamine level, MRS containing 3.4 mM Ca^{2+} (HCMRS) and 50 mM K^+ (HKMRS) was perfused for 20 min (Okada *et al.*, 1998c; 2001). The ionic composition was modified and isotonicity was maintained by an equimolar decrease of Na^+ (Okada *et al.*, 1998b). Each hippocampal dialysate was injected every 20 min into a high performance liquid-chromatograph (HPLC).

HPLC system preparation

The HPLC system used for determination of the extracellular levels of DA and 5-HT was equipped with an electrochemical detector (ECD-300, Eicom) with a EP-300 Eicom pump and a graphite carbon electrode set at +450 mV (versus an Ag/AgCl reference electrode). The analytical column (100 mm \times 1.5 mm internal diameter) was packed with mightysil RP-18 (3 μm particle size; a gift from Kanto Chemicals, Tokyo, Japan), by Masis INC. (Hirosaki, Japan). The mobile phase comprised 0.1 M phosphate buffer, containing 20% (v v $^{-1}$) methanol, 900 mg l $^{-1}$ octansulfonic sodium and 50 mg l $^{-1}$ EDTANa $_2$. The final pH was 5.9 and

the column temperature was maintained at 25°C with the flow rate set at 200 $\mu\text{l min}^{-1}$ (Okada *et al.*, 2001).

Chemical agents

The chemical agents used in this study were carbamazepine (Tokyo Chemical Industry, Tokyo, Japan), sodium valproate (Wako Chemicals, Osaka, Japan), zonisamide (Dainippon Pharmaceutical Co., Osaka, Japan), the synaptobrevin inhibitor, BoNT/B (Wako Chemicals) and the syntaxin inhibitor, BoNT/C (Wako Chemicals).

Drug administration

The perfusions were initiated with MRS, and hippocampal extracellular levels of DA and 5-HT were measured only at 6 h after starting the perfusion. When the coefficients of variation of hippocampal extracellular levels of DA and 5-HT reached less than 5% over 60 min (stabilization) (Okada *et al.*, 1998c), control data were obtained over a further 60 min, then the perfusion medium (MRS) was switched to MRS containing the target AED (pre-treatment period), in order to study the effects of CBZ, VPA and ZNS on basal monoamine release.

To study the effects of target agents on hippocampal Ca^{2+} - and K^{+} -evoked monoamine release, after confirming stabilization, the pre-treatment perfusion medium was switched to HCMRS or HKMRs containing the same agents for 20 min. After Ca^{2+} - or K^{+} -evoked stimulation, the perfusion medium was switched to the pre-treatment perfusion medium for another 100 min.

Diffusion rates of AEDs and recovery rate of monoamine

In order to accurately measure the concentration of CBZ, ZNS and VPA in the extracellular fluid perfused from hippocampus, *in vivo* probe diffusion was determined according to the 'reverse dialysis' procedure (Le Quellec *et al.*, 1995). Because solute diffusion occurs in both directions across the dialysis membrane, loss of solute from the perfusate occurs at the same rate as recovery of solute into the perfusate. During analysis the temperature was maintained at 37°C with a perfusion warmer. The concentration of CBZ and ZNS was measured by HPLC according to the methods of Juergens (1987). The concentration of VPA was measured by HPLC according to the methods of Liu *et al.* (1992). The rate at which CBZ, VPA and ZNS diffused from the dialysis probe (internal to external probes) was 19 ± 2 , 23 ± 4 and $21 \pm 4\%$ (mean \pm s.d., $n = 12$), respectively.

In *in vitro* experiments, the recovery rates of probes for DA and 5-HT (external to internal probes) were of 16.7 ± 2.4 and $13.6 \pm 1.9\%$ (mean \pm s.d., $n = 36$), respectively (Okada *et al.*, 1997c; 1998c; 2001).

Statistics

The concentration-dependent effects of CBZ, VPA and ZNS on basal, Ca^{2+} and K^{+} -evoked releases of hippocampal DA and 5-HT were compared using two-way analysis of variance (ANOVA) and Tukey's multiple comparison. The interaction between AEDs and BoNTs on basal, Ca^{2+} and K^{+} -evoked releases of DA and 5-HT were compared using one-way ANOVA and Tukey's multiple comparison.

Results

The basal hippocampal extracellular levels of DA and 5-HT were 3.1 ± 0.3 and 7.5 ± 0.6 fmol sample $^{-1}$ (20 μl) (mean \pm s.d., $n = 36$) in 20 min, respectively (Figures 1–3). In the pilot study, MRS containing tetrodotoxin (TTX) and Ca^{2+} -free decreased ($P < 0.01$) extracellular levels of DA and 5-HT to lower than 0.2 and 0.5 fmol sample $^{-1}$, respectively (data not shown). An increase in the extracellular K^{+} from 2.7 to 50 mM (K^{+} -evoked stimulation for 20 min) increased ($P < 0.01$) from 3.1 to 21.3 and from 7.5 to 42.5 fmol sample $^{-1}$ the extracellular DA and 5-HT levels, respectively (K^{+} -evoked release; DA 18.2 ± 2.6 fmol sample $^{-1}$, 5-HT 35.0 ± 5.6 fmol sample $^{-1}$, mean \pm s.d., $n = 36$) (Figures 1, 6 and 7). Therefore, these experiments show that under the microdialysis conditions employed, DA and 5-HT levels (basal release) were of neuronal origin, since there were TTX-sensitive, Ca^{2+} -dependent and K^{+} -sensitive (Westerink *et al.*, 1988; 1989). In addition, an increase in the extracellular Ca^{2+} level from 1.2 to 3.4 mM (Ca^{2+} -evoked stimulation) for 20 min increased ($P < 0.01$) from 3.1 to 6.0 and from 7.5 to 12.4 fmol sample $^{-1}$ the extracellular DA and 5-HT levels,

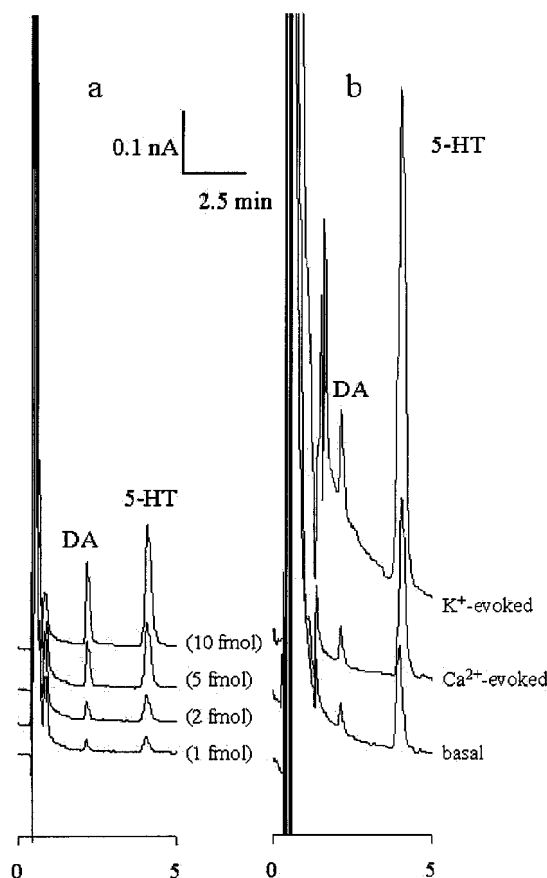


Figure 1 Typical chromatograms of ECD-HPLC. (a,b) Typical chromatograms obtained from 20 μl of a standard solution containing DA and 5-HT, and hippocampal perfusate, respectively. (a) The four lines express chromatograms of different concentrations of DA and 5-HT (1, 2, 5 and 10 fmol). The quantification limits for DA and 5-HT were both 100 amol 20 μl^{-1} . (b) The three lines express chromatograms of basal, Ca^{2+} - and K^{+} -evoked releases.

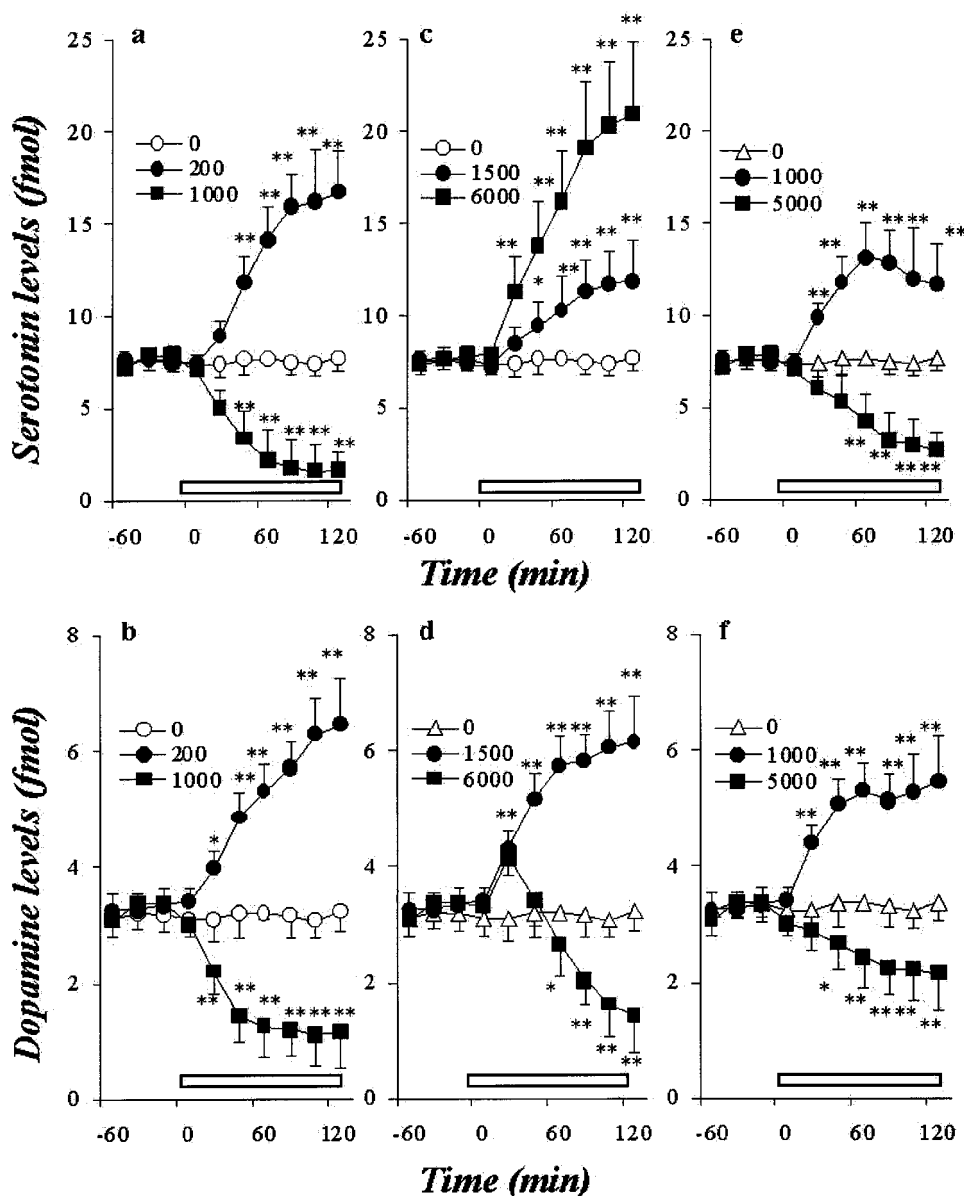


Figure 2 Effects of AEDs on hippocampal basal releases of DA and 5-HT. The effects of CBZ, VPA and ZNS on the hippocampal basal 5-HT release are shown in a, c and e, respectively. The effects of CBZ, VPA and ZNS on the hippocampal basal DA release are shown in b, d and f, respectively. The ordinates indicate the mean \pm s.d. ($n=6$) of extracellular levels of 5-HT or DA (fmol sample⁻¹), and abscissas show the time in minutes (min). The open bars indicate perfusion with AEDs. The effects of CBZ, VPA and ZNS on hippocampal basal releases of DA and 5-HT were compared using one-way ANOVA and Tukey's multiple comparison (* $P < 0.05$; ** $P < 0.01$).

respectively (Ca²⁺-evoked release; DA 2.9 ± 0.3 fmol sample⁻¹, 5-HT 4.9 ± 0.6 fmol sample⁻¹, mean \pm s.d., $n=36$) (Figures 1, 4 and 5).

Concentration-dependent effects of AEDs on basal hippocampal monoamine release

Figures 2 and 3 show the concentration-dependent effects of CBZ, VPA and ZNS on basal hippocampal releases of 5-HT and DA, respectively. The perfusion with CBZ (100 and 200 μ M, estimated concentrations in hippocampal tissue were 19 and 38 μ M, respectively) and ZNS (250 and 1000 μ M,

estimated concentrations in hippocampal tissue were 53 and 210 μ M, respectively) increased hippocampal basal DA and 5-HT releases, in a concentration-dependent manner ($P < 0.01$), whereas higher doses of CBZ (500 and 1000 μ M, estimated concentrations in hippocampal tissue were 95 and 190 μ M, respectively) and ZNS (2500 and 5000 μ M, estimated concentrations in hippocampal tissue were 530 and 1050 μ M, respectively) decreased them ($P < 0.01$) (Figures 2 and 3). The perfusion with VPA (250–6000 μ M, tissue concentrations 58–1380 μ M, respectively) increased basal 5-HT release concentration-dependently ($P < 0.01$) (Figures 2 and 3), whereas the hippocampal basal DA release was

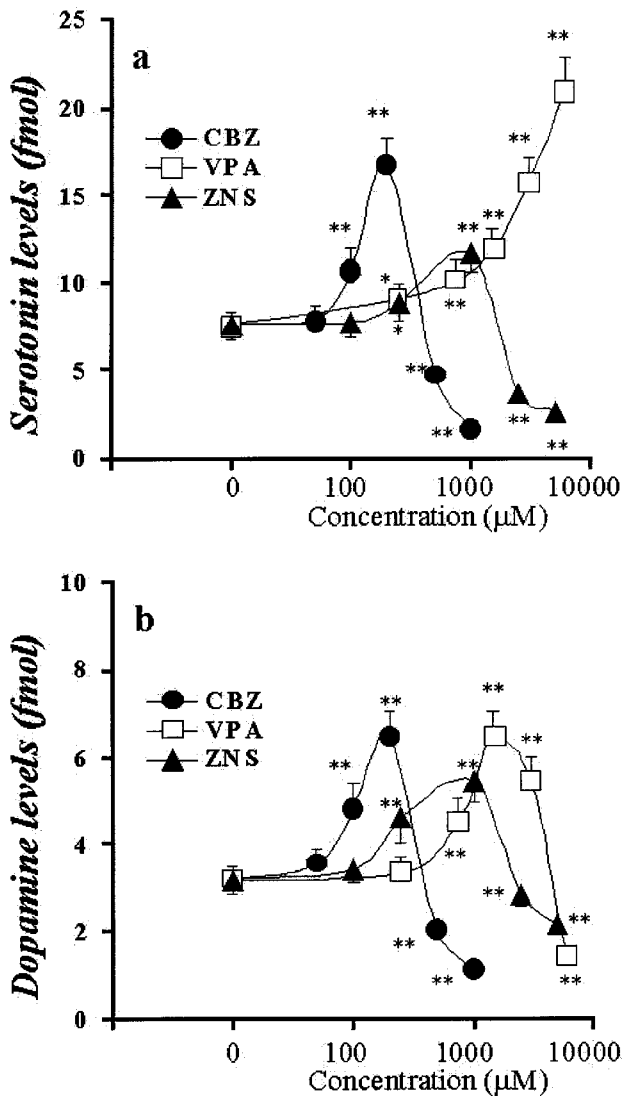


Figure 3 Concentration-dependent effects of AEDs on basal releases of hippocampal DA and 5-HT. The concentration-dependent effects of CBZ, VPA and ZNS on basal releases of hippocampal 5-HT and DA are shown in a and b, respectively. The ordinates indicate the mean \pm s.d. ($n=6$) of levels of basal releases of DA and 5-HT (fmol sample⁻¹), and abscissas indicate the concentration of AEDs (μM). The concentration-dependent effects of CBZ, VPA and ZNS on basal releases of hippocampal DA and 5-HT were compared using one-way ANOVA and Tukey's multiple comparison (* $P<0.05$; ** $P<0.01$).

increased by VPA concentration-dependently from 250 to 1500 μM (tissue concentrations 58–345 μM) ($P<0.01$); however, a dose of VPA higher than 3000 μM decreased DA release (Figures 2 and 3).

Concentration-dependent effects of AEDs on Ca^{2+} -evoked hippocampal monoamine release

Figures 4 and 5 show the concentration-dependent effects of CBZ, VPA and ZNS on Ca^{2+} -evoked hippocampal release of 5-HT and DA. The perfusion with CBZ (100 and 200 μM) and ZNS (250 and 1000 μM) increased hippocampal Ca^{2+} -evoked DA and 5-HT release ($P<0.01$), whereas perfusion

with CBZ (500 and 1000 μM) and ZNS (2500 and 5000 μM) decreased them ($P<0.01$) (Figures 4 and 5). The perfusion with VPA (250 to 1500 μM) increased Ca^{2+} -evoked DA and 5-HT release concentration-dependently ($P<0.01$), whereas VPA at doses greater than 3000 μM decreased them ($P<0.01$) (Figures 4 and 5).

Concentration-dependent effects of AEDs on K^{+} -evoked hippocampal monoamine release

Figures 6 and 7 show the concentration-dependent effects of CBZ, VPA and ZNS on K^{+} -evoked hippocampal releases of 5-HT and DA. The perfusion with these drugs reduced hippocampal K^{+} -evoked releases of DA and 5-HT in a concentration-dependent manner ($P<0.01$) (Figures 6 and 7).

Interaction between AEDs and BoNTs on basal monoamine release

The microinjection of both 0.3 ng BoNT/B and BoNT/C decreased the basal releases of 5-HT ($P<0.01$) and DA (BoNT/B: $P<0.05$; BoNT/C: $P<0.01$) (Figure 8). The inhibitory effect of BoNT/C on basal releases of 5-HT and DA was more predominant than that of BoNT/B ($P<0.05$) (Figure 8).

Inhibition of synaptobrevin by BoNT/B did not affect low concentrations of CBZ (200 μM) and ZNS (1000 μM) mediated stimulatory response of basal DA and 5-HT release, and high concentrations of CBZ (1000 μM) and ZNS (5000 μM) mediated inhibitory response of them (Figure 8). The concentration-dependent stimulatory effects of VPA on basal 5-HT release and the biphasic concentration-dependent effects of VPA (elevation by 1500 μM VPA and inhibition by 6000 μM VPA) on basal DA release also were not affected by BoNT/B (Figure 8).

After inhibition of syntaxin with BoNT/C, the stimulatory effects of low concentrations of CBZ on basal releases of 5-HT and DA were abolished, whereas the high concentrations of CBZ decreased them ($P<0.01$) (Figure 8). Under the same conditions, the stimulatory effect of low concentrations of VPA on basal release was abolished. High concentrations of VPA increased basal 5-HT release ($P<0.05$), but this stimulatory effect was reduced by BoNT/C ($P<0.01$). Contrary to 5-HT, high concentrations of VPA reduced basal DA release ($P<0.05$), under the inhibition of syntaxin (Figure 8). Under the same conditions, low concentrations of ZNS did not affect and increased ($P<0.01$) basal 5-HT and DA release, respectively, but the low concentrations of ZNS-mediated stimulatory response of DA release was reduced by BoNT/C ($P<0.01$) (Figure 8). The high concentrations of ZNS decreased ($P<0.01$) and did not affect basal releases of 5-HT and DA, respectively (Figure 8).

Interaction between AEDs and BoNTs on Ca^{2+} -evoked monoamine release

The microinjection of 0.3 mg BoNT/C reduced the Ca^{2+} -evoked releases of DA and 5-HT ($P<0.01$), whereas 0.3 ng BoNT/B did not affect Ca^{2+} -evoked releases (Figure 9).

After inhibition of synaptobrevin with BoNT/B, the low concentrations of CBZ, VPA and ZNS increased Ca^{2+} -evoked releases of 5-HT and DA ($P<0.01$), whereas the high

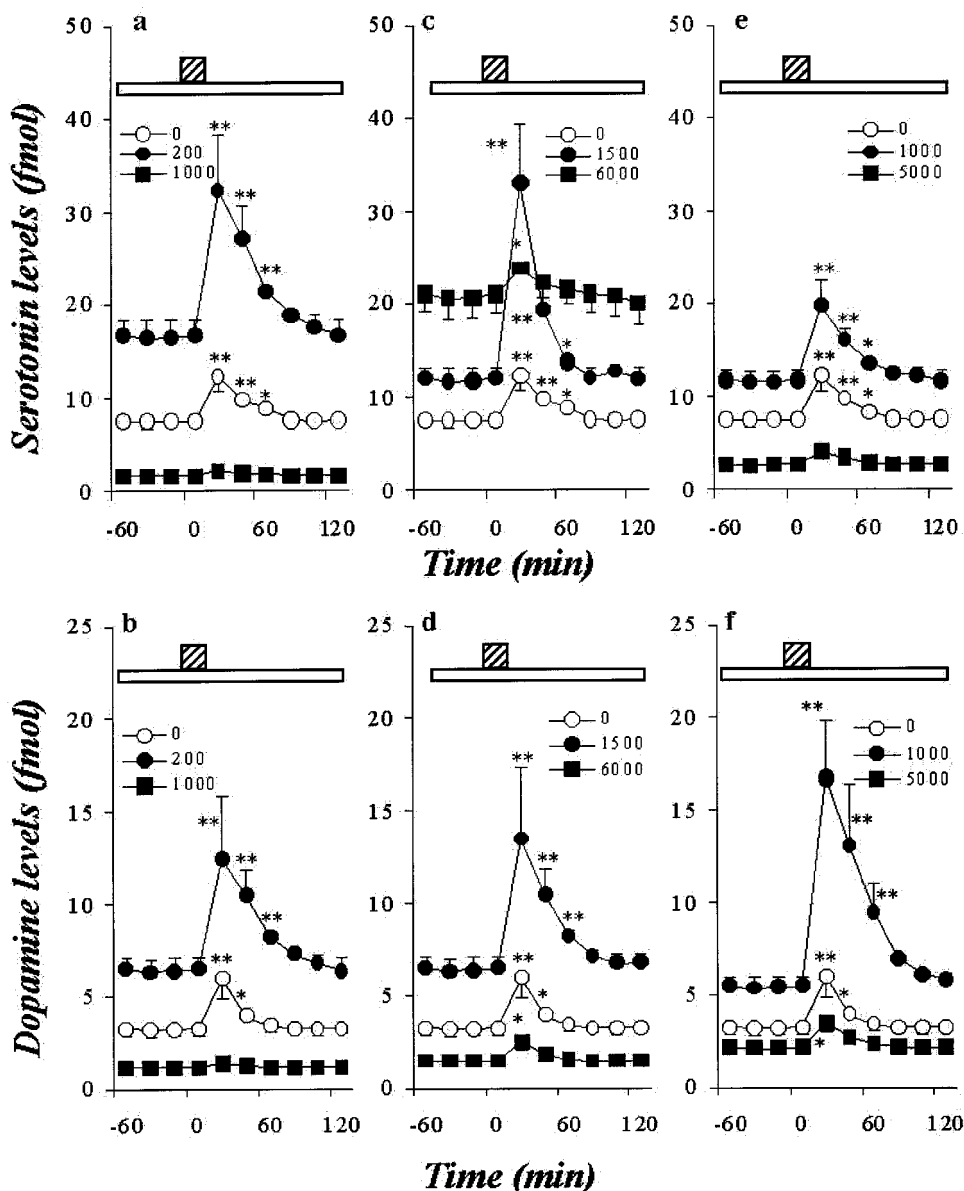


Figure 4 Effects of AEDs on hippocampal Ca^{2+} -evoked releases of DA and 5-HT. The effects of CBZ, VPA and ZNS on the hippocampal Ca^{2+} -evoked 5-HT release are shown in a, c and e, respectively. The effects of CBZ, VPA and ZNS on the hippocampal Ca^{2+} -evoked DA release are shown in b, d and f, respectively. The ordinates indicate the mean \pm s.d. ($n=6$) of extracellular levels of 5-HT or DA (fmol sample^{-1}), and abscissas show the time in minutes (min). The open bars indicate perfusion with AEDs, and striped bars indicate the Ca^{2+} -evoked stimulation for 20 min. The effects of CBZ, VPA and ZNS on hippocampal Ca^{2+} -evoked releases of DA and 5-HT were compared using one-way ANOVA and Tukey's multiple comparison (* $P < 0.05$; ** $P < 0.01$).

concentrations of CBZ, VPA and ZNS decreased them ($P < 0.01$) (Figure 9). After inhibition of syntaxin with BoNT/C, neither low nor high concentrations of CBZ, VPA and ZNS affected Ca^{2+} -evoked releases of 5-HT and DA (Figure 9).

Interaction between AEDs and BoNTs on K^{+} -evoked monoamine release

The microinjection of 0.3 ng BoNT/B and BoNT/C reduced the K^{+} -evoked releases of DA and 5-HT (Figure 10). The inhibitory effect of BoNT/B on K^{+} -evoked releases of 5-HT

and DA was more predominant than that of BoNT/C ($P < 0.01$) (Figure 10).

Inhibition of syntaxin with BoNT/C did not affect the concentration-dependent inhibitory effects of CBZ, VPA and ZNS on K^{+} -evoked releases of 5-HT and DA (Figure 10). Inhibition of synaptobrevin with BoNT/B abolished the inhibitory effects of low concentrations of CBZ, VPA and ZNS on K^{+} -evoked releases of 5-HT and DA ($P < 0.01$) (Figure 10). The high concentrations of CBZ, VPA and ZNS decreased on K^{+} -evoked releases of 5-HT and DA ($P < 0.01$), but these inhibitory effects were reduced by BoNT/B ($P < 0.05$) (Figure 10).

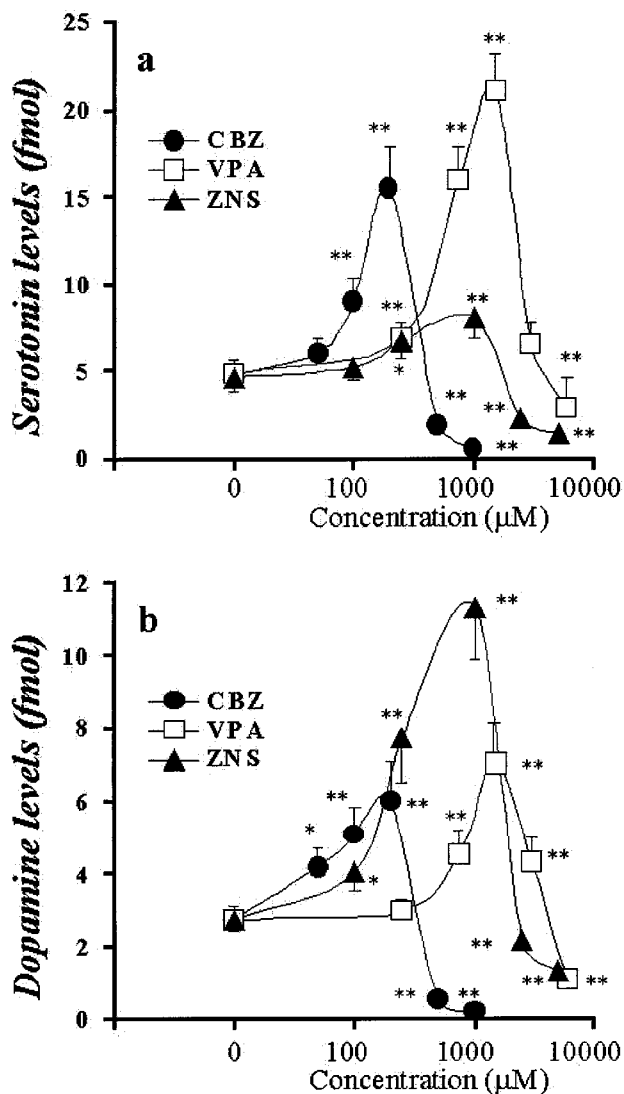


Figure 5 Concentration-dependent effects of AEDs on Ca^{2+} -evoked releases of hippocampal DA and 5-HT. The concentration-dependent effects of CBZ, VPA and ZNS on Ca^{2+} -evoked releases of hippocampal 5-HT and DA are shown in a and b, respectively. The ordinates indicate the mean \pm s.d. ($n=6$) of levels of Ca^{2+} -evoked releases of DA and 5-HT (fmol sample^{-1}), and the abscissas indicate the AEDs concentration (μM). The concentration-dependent effects of CBZ, VPA and ZNS on Ca^{2+} -evoked releases of hippocampal DA and 5-HT were compared using one-way ANOVA and Tukey's multiple comparison (* $P<0.05$; ** $P<0.01$).

Discussion

Exocytosis mechanisms of hippocampal DA and 5-HT

Different exocytosis mechanisms are implicated in both spontaneous and depolarization-induced neurotransmitter release (Yoshihara *et al.*, 1999; Kawata *et al.*, 2001; Okada *et al.*, 2001). The mechanism for depolarization-induced neurotransmitter release has been considered to occur as follows: after arrival of an action potential at the presynaptic terminal leading to Ca^{2+} entry through specific channels and thus increasing abruptly its levels to submillimolar concentration (Roberts *et al.*, 1990). On the other hand, spontaneous

fusion of the synaptic vesicle at the resting state generates miniature synaptic potentials. The frequency of these events changes when the cytosolic Ca^{2+} level is altered in the micromolar range (Delaney & Tank, 1994; Xu *et al.*, 1998). Recent studies have indicated that hippocampal 5-HT release was regulated by the two functional complexes, N-type VSCC/PKC/syntaxin and P-type VSCC/PKA/synaptobrevin (Kawata *et al.*, 2001; Okada *et al.*, 2001). This study demonstrated that hippocampal basal and K^{+} -evoked DA release is regulated by the similar mechanisms of 5-HT exocytosis, since the basal and K^{+} -evoked releases of hippocampal DA were predominantly inhibited by inhibitors of syntaxin (BoNT/C) and synaptobrevin (BoNT/B), respectively. Furthermore, we have already demonstrated that the Ca^{2+} -evoked releases of striatal DA and hippocampal 5-HT were inhibited by N-type VSCC inhibitor, ω -conotoxin GVIA selectively but was unaffected by P-type VSCC inhibitor, ω -agatoxin IVA (Okada *et al.*, 1998c; Kawata *et al.*, 2001). Taken together with this evidence, the present results, the Ca^{2+} -evoked releases of hippocampal DA and 5-HT were inhibited by BoNT/C but not by BoNT/B, suggests that the Ca^{2+} -evoked releases of DA and 5-HT may require specific exocytosis mechanisms, only N-type VSCC/PKC/syntaxin. To clarify this hypothesis, we are determining the exocytosis mechanisms of Ca^{2+} -evoked hippocampal monoamine release.

Effects of CBZ and ZNS on monoamine release

It has been well established that therapeutic-relevant doses of CBZ and ZNS inhibit VGSC activity (McLean & MacDonald, 1986; Mattson, 1997) and this could decrease basal monoamine release (Westerink *et al.*, 1988; 1989). Both our previous and present studies have demonstrated that the therapeutic-relevant concentration of CBZ (from 17 to 42 μM) and ZNS (47 to 330 μM) that inhibit VGSC activity, increased basal and Ca^{2+} -evoked release of DA and 5-HT (Okada *et al.*, 1992; Kawata *et al.*, 1999; 2001). In contrast, the therapeutic-relevant concentration of CBZ and ZNS decreased K^{+} -evoked release of DA, 5-HT and glutamate without affecting basal glutamate release (Okada *et al.*, 1998b; Kawata *et al.*, 1999; 2001). On the other hand, higher than plasma-concentration of CBZ and ZNS associated with their antiepileptic action decreased basal, Ca^{2+} - and K^{+} -evoked releases of DA, 5-HT and K^{+} -evoked glutamate (Okada *et al.*, 1992; 1995; 1997a, d; 1998a, b; Kawata *et al.*, 1999; 2001) without affecting basal glutamate release (Okada *et al.*, 1998b). This contradiction between the effects of CBZ and ZNS on basal, Ca^{2+} - and K^{+} -evoked monoamine release may be caused by the sensitivities of two functional complex pathways for monoamine release, N-type VSCC/PKC/syntaxin and P-type VSCC/PKA/synaptobrevin.

In this study, the stimulatory effects of therapeutic-relevant concentrations of CBZ and ZNS on Ca^{2+} -evoked monoamine release, which was sensitive to BoNT/C but insensitive to BoNT/B, were inhibited by BoNT/C, whereas under the condition of microinjection of BoNT/C, higher than therapeutic-relevant concentrations of CBZ and ZNS did not affect Ca^{2+} -evoked monoamine release. These results suggest that therapeutic-relevant concentrations of CBZ and ZNS enhance syntaxin-related monoamine exocytosis mechanism; however, higher than therapeutic-relevant concentrations of CBZ and ZNS inhibit it.

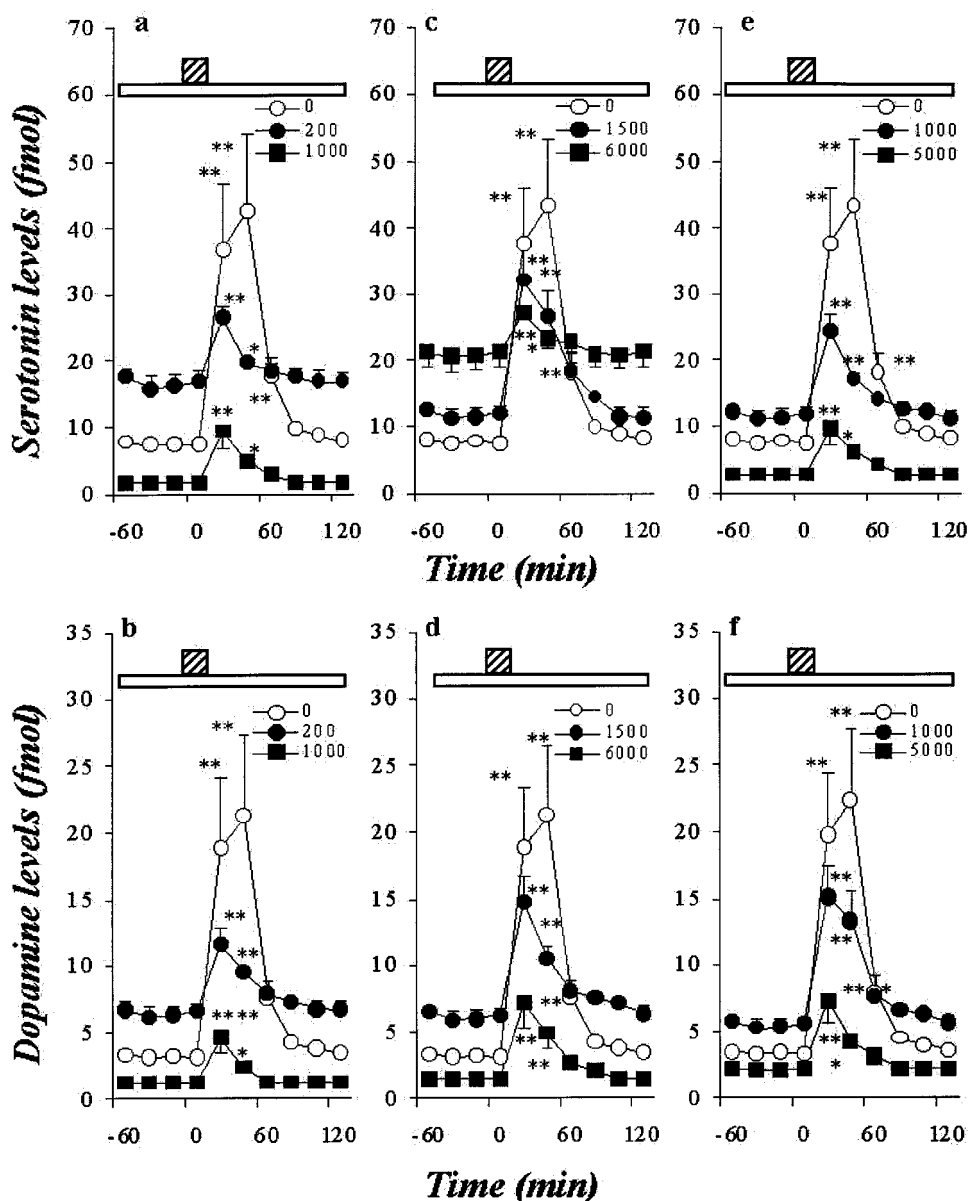


Figure 6 Effects of AEDs on hippocampal K⁺-evoked releases of DA and 5-HT. The effects of CBZ, VPA and ZNS on hippocampal K⁺-evoked 5-HT release are shown in a, c and e, respectively. The effects of CBZ, VPA and ZNS on the hippocampal K⁺-evoked DA release are shown in b, d and f, respectively. The ordinates indicate the mean \pm s.d. ($n = 6$) of extracellular levels of 5-HT or DA (fmol sample⁻¹), and abscissas show the time in minutes (min). The open bars indicate perfusion with AEDs, and striped bars indicate the K⁺-evoked stimulation for 20 min. The concentration-dependent effects of CBZ, VPA and ZNS on hippocampal K⁺-evoked releases of DA and 5-HT were compared using one-way ANOVA and Tukey's multiple comparison (* $P < 0.05$; ** $P < 0.01$).

The concentration-dependent biphasic effects of CBZ and ZNS on basal monoamine release were not affected by inhibition of synaptobrevin with BoNT/B. The inhibition of syntaxin with BoNT/C abolished the stimulatory effects of therapeutic-relevant concentrations of CBZ on monoamine basal release. Under the same conditions, higher than therapeutic-relevant concentrations of CBZ remained able to reduce basal monoamine release. These results suggest that the major mechanisms of stimulatory effects of therapeutic-relevant concentrations of CBZ on basal monoamine release were stimulation of syntaxin-related monoamine exocytosis. Contrary to therapeutic-relevant concentration, the major

mechanisms of inhibitory effects of higher than therapeutic-relevant concentrations of CBZ on basal monoamine release may be inhibition of syntaxin-related monoamine exocytosis; however, the present study could not deny the possibility that the higher than therapeutic-relevant concentrations of CBZ inhibit other basal monoamine exocytosis mechanisms, i.e. adenosine receptor, N-type VSCC and VGSC (Okada *et al.*, 1997d; Kawata *et al.*, 2001). The inhibition of syntaxin with BoNT/C abolished the stimulatory effects of therapeutic-relevant concentrations of ZNS on 5-HT basal release, but higher than therapeutic-relevant concentrations of ZNS reduced it, similar to CBZ. However, under the same

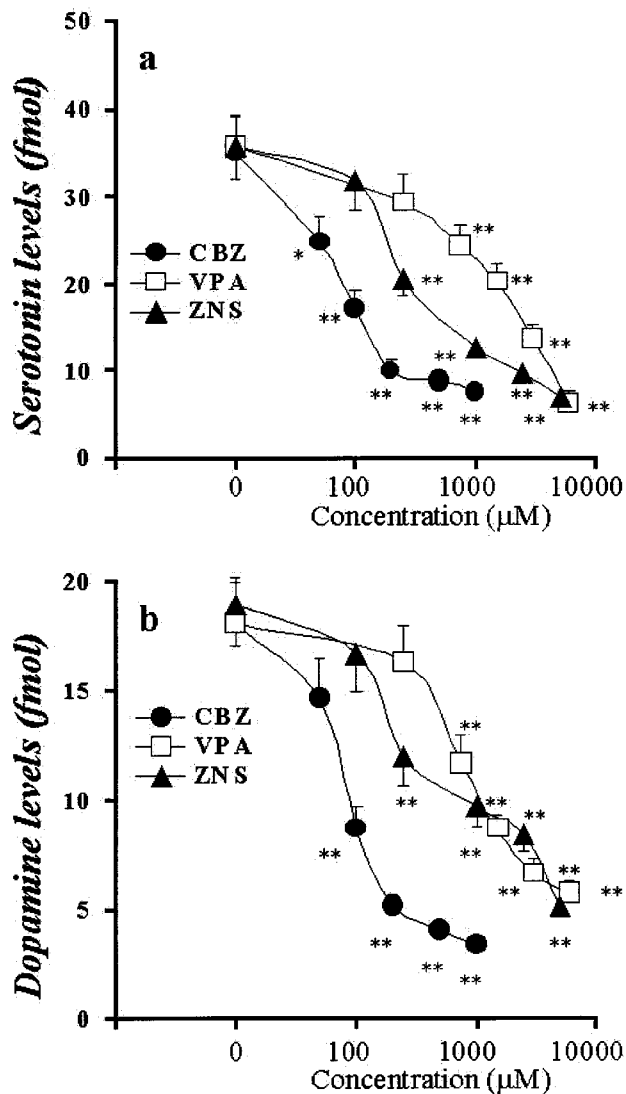


Figure 7 Concentration-dependent effects of AEDs on K^+ -evoked releases of hippocampal DA and 5-HT. The concentration-dependent effects of CBZ, VPA and ZNS on K^+ -evoked releases of hippocampal 5-HT and DA are shown in a and b, respectively. The ordinates indicate the mean \pm s.d. ($n=6$) of levels of K^+ -evoked releases of DA and 5-HT (fmol sample $^{-1}$), and the abscissas indicate the concentration of AEDs (μ M). The concentration-dependent effects of CBZ, VPA and ZNS on hippocampal K^+ -evoked releases of DA and 5-HT were compared using one-way ANOVA and Tukey's multiple comparison (* $P < 0.05$; ** $P < 0.01$).

conditions, the basal DA release was increased and unaffected by within and higher than therapeutic-relevant concentrations of ZNS, respectively. These different results between ZNS and CBZ on basal DA release, under the conditions of inhibition of syntaxin activity, might be modulated by inhibition of monoamine oxidase activity induced by ZNS (Okada *et al.*, 1992; 1995).

The concentration-dependent inhibitory effects of CBZ and ZNS on K^+ -evoked monoamine release were not affected by BoNT/C. In contrast to BoNT/C, under the inhibition of synaptobrevin using BoNT/B, they were inhibited, but the higher than therapeutic-relevant concentrations of CBZ and ZNS could reduce it. Therefore, these results suggest that

both CBZ and ZNS inhibit the synaptobrevin-related exocytosis mechanism concentration-dependently. Furthermore, higher than therapeutic-relevant concentrations of CBZ and ZNS may inhibit other K^+ -evoked monoamine release mechanisms, i.e. P-type VSCC or VGSC (Kawata *et al.*, 1999; 2001).

CBZ reduces Ca^{2+} influx (Kito *et al.*, 1994; 1996; Yoshimura *et al.*, 1995), but the effects of CBZ on VSCC subtypes have not yet been clarified. Kito *et al.* (1994; 1996) demonstrated, using a patch-clamp technique, the lack of effect of CBZ on various subtypes of Ca^{2+} channels in the human neuroblastoma NB1 cell; however, Yoshimura *et al.* (1995) demonstrated that CBZ inhibited N-type VSCC function in cultured bovine adrenal medullary cells. Therefore, taken together with these previous demonstrations, present results suggest that CBZ affects, at least partially, the N-type VSCC/PKC/syntaxin pathway biphasic concentration-dependently without Ca^{2+} inflow through N-type VSCC. On the other hand, the effect of ZNS on VSCC has been studied less extensively. It remains uncertain whether ZNS has the same mechanism of action as CBZ. Although ZNS inhibited T-type VSCC activity (Suzuki *et al.*, 1992), which does not affect monoamine release (Kato *et al.*, 1992), detailed voltage-clamp investigations regarding the effects of ZNS on other subtypes of VSCC has not been demonstrated.

Nevertheless, the present study demonstrated that therapeutic-relevant concentrations of CBZ and ZNS enhanced syntaxin-related exocytosis mechanism, whereas higher than therapeutic-relevant concentrations of CBZ and ZNS reduced both syntaxin- and synaptobrevin-related exocytosis mechanisms.

Effects of VPA on monoamine release

VPA affected basal DA release biphasic concentration-dependently, similar to CBZ and ZNS, whereas VPA increased basal 5-HT release concentration-dependently. Contrary to basal release, both Ca^{2+} -evoked release of DA and 5-HT were enhanced and reduced by therapeutic- and higher than therapeutic-relevant concentrations of VPA, respectively (biphasic concentration-dependently). The stimulatory effects of VPA on basal and Ca^{2+} -evoked releases of DA and 5-HT were inhibited by BoNT/C but unaffected by BoNT/B, whereas the inhibitory effects of higher than therapeutic-relevant concentrations of VPA were reduced by BoNT/C but were not affected by BoNT/B. Therefore, therapeutic- and higher than therapeutic-relevant concentrations of VPA enhance and inhibit syntaxin-related exocytosis mechanism, similar to CBZ and ZNS. It remains uncertain why higher than therapeutic-relevant concentrations of VPA increase and decrease basal release of DA and 5-HT, respectively. In our previous study, VPA increased 5-HT levels in hippocampal tissue, whereas DA levels increased and decreased by therapeutic- and higher than therapeutic-relevant doses of VPA, respectively (Mizuno *et al.*, 1994). Although VPA acts on the syntaxin-related exocytosis mechanism biphasic concentration-dependently similar to CBZ and ZNS, VPA may have another effect on serotonergic transmission, i.e. enhancement of 5-HT synthesis or turnover (Mizuno *et al.*, 1994). On the other hand, the concentration-dependent inhibitory effect of VPA on K^+ -evoked monoamine release, which was low-sensitive to BoNT/C and high-

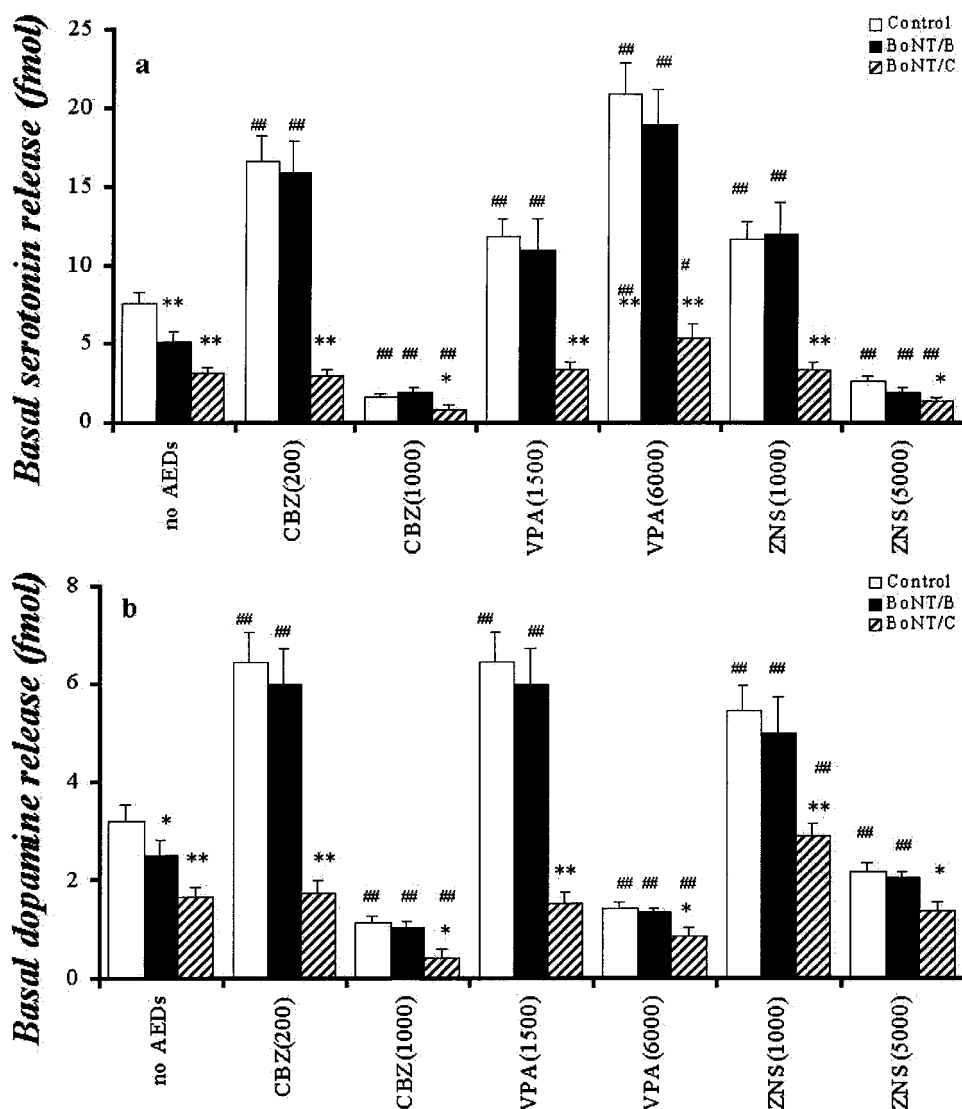


Figure 8 Interaction between AEDs and BoNTs on hippocampal basal releases of DA and 5-HT. The interactions between AEDs and BoNTs on hippocampal basal release of 5-HT and DA are shown in a and b, respectively. The ordinates indicate the mean \pm s.d. ($n=6$) of levels of basal releases of DA and 5-HT (fmol sample⁻¹). The interaction between AEDs and BoNTs on hippocampal basal releases of DA and 5-HT were compared using two-way ANOVA and Tukey's multiple comparison (control vs * $P < 0.05$; ** $P < 0.01$, no AEDs vs # $P < 0.05$; ## $P < 0.01$).

sensitive to BoNT/B, was not affected by BoNT/B. Therefore, these results suggest that VPA inhibits synaptobrevin-mediated exocytosis concentration-dependently, similar to CBZ and ZNS.

Mechanisms of antiepileptic action of CBZ, VPA and ZNS

The occurrence of an epileptic seizure may be generated by the relative imbalance between excitatory and inhibitory neurotransmission, resulting in neurones showing increased excitability and abnormally frequent patterns of discharge (Hirose *et al.*, 2000). Glutamate is the principal excitatory neurotransmitter, and excessive release of glutamate may produce seizures in epileptic patients (During *et al.*, 1995), and in animal models of epilepsy (Ueda & Tsuru, 1994). We have already demonstrated that therapeutic-relevant concen-

trations of CBZ and ZNS reduce both hippocampal K⁺-evoked glutamate release and spreading depression induced glutamate release, without affecting basal release (Okada *et al.*, 1998b). In addition, enhancement of monoaminergic transmission reduced seizure threshold (Wahnschaffe & Loscher, 1991; Wada *et al.*, 1993). Taken together with our previous results, the present study indicates that the mechanisms of antiepileptic action of therapeutic-relevant concentrations of CBZ, VPA and ZNS may be produced by the combination of effects resulting from enhancement of basal release of inhibitory neurotransmitter without affecting basal glutamate release, and reduction of neurotransmitter release at the event of neuronal excitation (i.e. during seizures).

In this study we determined the concentration-dependent different action of CBZ, VPA and ZNS on the basal, Ca²⁺- and K⁺-evoked hippocampal release of DA and 5-HT, in this

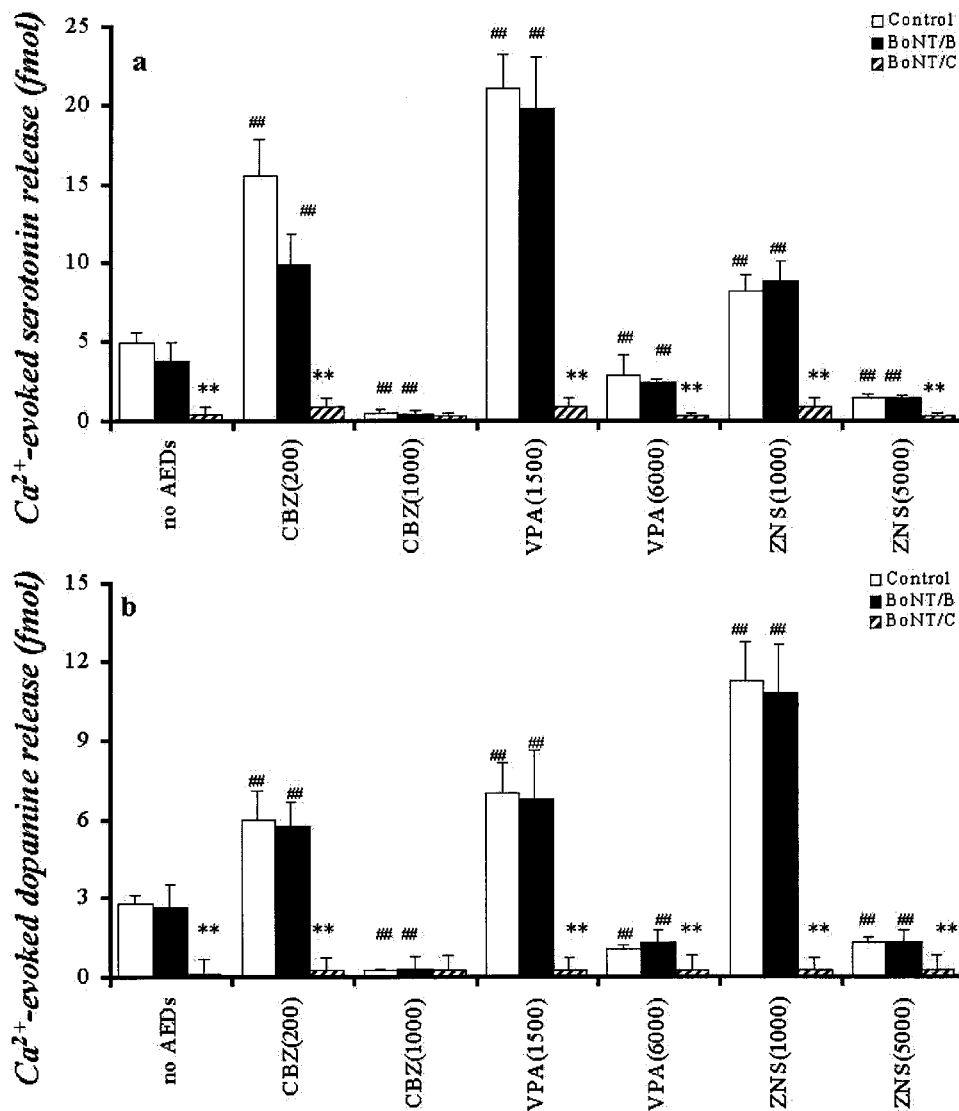


Figure 9 Interaction between AEDs and BoNTs on hippocampal Ca²⁺-evoked releases of DA and 5-HT. The interactions between AEDs and BoNTs on hippocampal Ca²⁺-evoked release of 5-HT and DA are shown in a and b, respectively. The ordinates indicate the mean \pm s.d. ($n=6$) of levels of Ca²⁺-evoked releases of DA and 5-HT (fmol sample⁻¹). The interaction between AEDs and BoNTs on hippocampal Ca²⁺-evoked releases of DA and 5-HT were compared using two-way ANOVA and Tukey's multiple comparison (control vs * $P<0.05$; ** $P<0.01$, no AEDs vs # $P<0.05$; ## $P<0.01$).

study. In conclusion, higher than therapeutic-relevant concentrations of CBZ, VPA and ZNS inhibited the activities of both synaptobrevin- and syntaxin-related monoamine release mechanisms, which resulted in inhibition of monoamine exocytosis. In contrast, the low concentrations of CBZ, VPA and ZNS enhanced the syntaxin-related exocytosis mechanism at the resting stage, and synaptobrevin-related exocytosis mechanism at the neuronal depolarizing stage, i.e. during K⁺-evoked stimulation. CBZ, VPA and ZNS possibly exert their antiepileptic action, at least partially, by combined effect on the enhancement of syntaxin-related monoamine exocytosis mechanism (enhancement of basal release) during neuronal resting stage, which elevates seizure threshold, and inhibition of synaptobrevin-related monoamine exocytosis mechanism during neuronal depolarization. It can now be proposed that at least partially, therapeutic-relevant concentrations of CBZ, VPA and ZNS exert their antiepileptic

action by combined effects on modulation of two functional pathways for exocytosis, namely the N-type VSCC/PKC/syntaxin and the P-type VSCC/PKA/synaptobrevin, during both resting and depolarizing stages, respectively.

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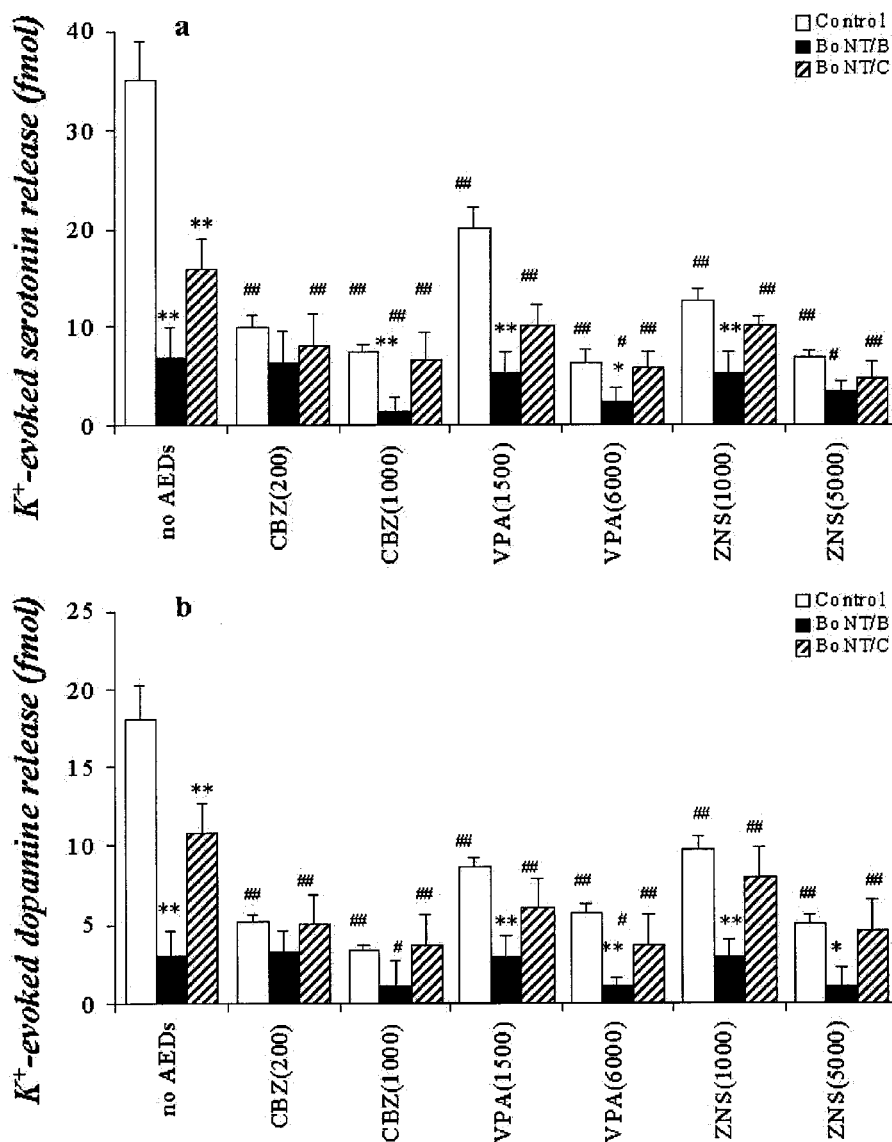


Figure 10 Interaction between AEDs and BoNTs on hippocampal K⁺-evoked releases of DA and 5-HT. The interactions between AEDs and BoNTs on hippocampal K⁺-evoked release of 5-HT and DA are shown in a and b, respectively. The ordinates indicate the mean \pm s.d. ($n=6$) of levels of K⁺-evoked releases of DA and 5-HT (fmol sample⁻¹). The interaction between AEDs and BoNTs on hippocampal K⁺ (control vs * $P < 0.05$; ** $P < 0.01$, no AEDs vs # $P < 0.05$; ## $P < 0.01$).

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