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RESEARCH PAPER

ONO-2506 inhibits spike–wave discharges in a genetic animal model without affecting traditional convulsive tests via gliotransmission regulation

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BACKGROUND AND PURPOSE

Anticonvulsants have been developed according to the traditional neurotransmission imbalance hypothesis. However, the anticonvulsive pharmacotherapy currently available remains unsatisfactory. To develop new antiepileptic drugs with novel antiepileptic mechanisms, we have tested the antiepileptic actions of ONO-2506, a glial modulating agent, and its effects on tripartite synaptic transmission.

EXPERIMENTAL APPROACH

Dose-dependent effects of ONO-2506 on maximal-electroshock seizure (MES), pentylenetetrazol-induced seizure (PTZ) and epileptic discharge were determined in a genetic model of absence epilepsy in mice (*Cacna1a*$^{tm2Nobs/tm2Nobs}$ strain). Antiepileptic mechanisms of ONO-2506 were analysed by examining the interaction between ONO-2506 and transmission-modulating toxins (tetanus toxin, fluorocitrate, tetrodotoxin) on release of L-glutamate, D-serine, GABA and kynurenic acid in the medial-prefrontal cortex (mPFC) of freely moving rats using microdialysis and primary cultured rat astrocytes.

KEY RESULTS

ONO-2506 inhibited spontaneous epileptic discharges in *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice without affecting MES or PTZ. Given systemically, ONO-2506 increased basal release of GABA and kynurenic acid in the mPFC through activation of both neuronal and glial exocytosis, but inhibited depolarization-induced releases of all transmitters. ONO-2506 increased basal glial release of kynurenic acid without affecting those of L-glutamate, D-serine or GABA. However, ONO-2506 inhibited AMPA-induced releases of L-glutamate, D-serine, GABA and kynurenic acid.

CONCLUSIONS AND IMPLICATIONS

ONO-2506 did not affect traditional convulsive tests but markedly inhibited epileptic phenomena in the genetic epilepsy mouse model. ONO-2506 enhanced release of inhibitory neuro- and gliotransmitters during the resting stage and inhibited tripartite transmission during the hyperactive stage. The results suggest that ONO-2506 is a novel potential glial-targeting antiepileptic drug.

LINKED ARTICLE

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Abbreviations

ACSF, artificial cerebrospinal fluid; AUC, area under curve; FLC, fluorocitrate; HKMRS, 50 mM K^+ containing modified Ringer's solution; MES, maximal electroshock seizure test; mPFC, medial prefrontal cortex; MRS, modified Ringer's solution; NCE, nanocarbon film electrode; PTZ, pentylenetetrazol-induced seizure test; SNARE, soluble N-ethylmaleimide sensitive factor attachment protein receptors; TeNT, tetanus toxin; TTX, tetrodotoxin; xLC, extreme liquid chromatography

Introduction

Currently, pharmacotherapy using conventional anticonvulsants is ineffective in more than 20% of patients with epilepsy (Blume, 2007). The majority of the established anticonvulsants were developed based on traditional convulsion screening tests, namely maximal electroshock seizure test (MES) (White *et al.*, 2002) and pentylenetetrazol-induced seizure test (PTZ) (White *et al.*, 2002), rather than on mechanism-directed drug design (Gower *et al.*, 1992; Loscher and Honack, 1993; White *et al.*, 2002). Levetiracetam, which has been shown to exhibit little anticonvulsant activity in MES and PTZ (Gower *et al.*, 1992; Loscher and Honack, 1993), provides benefit in some individual patients with refractory epilepsy; however, drug refractoriness or intolerability is still a major problem in anticonvulsant medication (Otoul *et al.*, 2005). Thus, neurocentric pharmacotherapy against epilepsy over several past decades did not provide a major breakthrough in overcoming refractoriness to antiepileptic drugs.

Several groups have proposed that glial mechanisms, including self-reinforcing interplay between dysfunctional energy homeostasis, inflammation and astrocytic signalling, play important roles in epileptogenesis (Tian *et al.*, 2005; Wetherington *et al.*, 2008). Indeed, both pre-clinical and clinical studies have demonstrated the presence of glial abnormalities in epileptic regions (Griffin *et al.*, 1995; Lee *et al.*, 2007; Somera-Molina *et al.*, 2007; Shapiro *et al.*, 2008). Epileptic brain is characterized by profound astrogliotic reaction with morphological and functional changes in astrocytes (Seifert *et al.*, 2006). Accumulating evidence also indicates that deficient tripartite synaptic transmission plays important roles in the pathogenesis of epilepsy (Fellin and Haydon, 2005; Tian *et al.*, 2005; Halassa *et al.*, 2007). The role of Glutamate release from astrocytes has been proposed to play an important role in synchronous discharges triggering epileptiform seizures (Tian *et al.*, 2005). Particularly, several pre-clinical studies have suggested that quinolinic and kynurenic acids, two gliotransmitters which are tryptophan metabolites, have pro-convulsive and anti-convulsive properties, respectively (Vamos *et al.*, 2009; Severino *et al.*, 2011). In spite of these efforts, there are no antiepileptic or anticonvulsive drugs that have been developed based on glial-targeted drug design. This is mainly due to the lack of information on the roles of neuroactive gliotransmitters, tryptophan metabolites and D-serine in epilepsy. In addition, the neurotransmission mechanisms of tryptophan metabolites remain poorly understood, mainly due to the quantification limits of analytical methods used for determination of the concentrations of these gliotransmitters using UV and fluorescence detection.

Various pre-clinical studies demonstrated that arundic acid (ONO-2506), a novel astrocyte-modulating agent, has a wide clinical spectrum against neurological conditions, such as ischemic brain damage (Ohtani *et al.*, 2007), Parkinson's disease (Oki *et al.*, 2008) and Alzheimer's disease (Mori *et al.*, 2006). These studies suggested that ONO-2506 had a neuroprotective action and that astrocytes were a potentially new target for the treatment of various neurological diseases. ONO-2506 is a potent inhibitor of the production and release of S100B protein from astrocytes (Asano *et al.*, 2005) but the effects of ONO-2506 on exocytosis of gliotransmitters have not been studied.

To develop novel antiepileptic drugs, the present study determined the effects of ONO-2506 on MES, PTZ and a genetic model of absence epilepsy (Saito *et al.*, 2009). Furthermore, the present study also determined the effects of ONO-2506 on the release of L-glutamate, D-serine, GABA and kynurenic acid, in the medial prefrontal cortex (mPFC) of freely moving rats and in primary cultured astrocytes by extreme liquid chromatography (xLC) (Yamamura *et al.*, 2009b; Tanahashi *et al.*, 2012) and HPLC equipped with nanocarbon film electrode detection (NCE-HPLC) (Kato *et al.*, 2011).

Methods

Experimental animals

All animal care and experimental procedures described in this report complied with the Ethical Guidelines established by the Institutional Animal Care and Use Committee at Mie University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). A total of 72 rats and 120 mice were used in the experiments described here.

Male Sprague–Dawley rats (neonatal and 8-week-old) were housed under conditions of constant temperature at 22 ± 2°C with 12–12 h light–dark cycle. The established genetic model of absence epilepsy, the conditional *Cacna1a* gene knock-down male mice, *Cacna1a*$^{tm2Nobs/tm2Nobs}$ (7-week-old) and their littermates (C57BL/6 background) were developed and generated as described previously (Saito *et al.*, 2009). The expression of $Ca_v2.1$ ion channel protein and its Ca^{2+} current are reduced in *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice to about 30% relative to the wild type (Saito *et al.*, 2009). The *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice exhibit frequent, spontaneous, absence-like epileptic seizures accompanied by generalized 5–6 Hz spike and wave discharges coupled with immobility. In addition, this mutant mouse exhibits ataxic behaviour and cerebellar atrophy (obvious at 2 months and progressing up to 6 months of age), as well as rare clonic seizures, but shows normal viability (Saito *et al.*, 2009).

Electrocorticography

Each Cacna1a$^{tm2Nobs/tm2Nobs}$ mouse (at 7 weeks of age) was placed in a stereotaxic frame and was kept under anaesthesia using 2.1% isoflurane. For electrocorticographic monitoring of freely moving mice, the recording and reference electrodes were screwed onto the mouse skull over the frontal and occipital regions (Saito *et al.*, 2009; Yamamura *et al.*, 2009a). A radiotelemetry unit (UniMec 1310TM-10101, Unimec, Tokyo, Japan) was placed s.c. in the flank region through a 1 cm skin incision behind the scapula. After recovery from anaesthesia, each mouse was placed in a separate cage that was positioned on the receiver unit (UniMec ITE1000R, Unimec), and standard rodent food and tap water were provided *ad libitum*. The radiotelemetry unit, once turned on by a magnet, sent signals to the receiver unit, which were then sent on the NOTOCORD-hem system (Primetech, Tokyo, Japan) (Yamamura *et al.*, 2009a,b). After baseline recording, the mice were treated with 0 (saline), 25, 50 and 100 mg·kg^{-1} ONO-2506 (i.p.) (n = 8·per group) or 100, 200 and 300 mg·kg^{-1} ethosuximide (i.p.) (n = 5·per group)

MES

Seizures were induced in male mice (at 7 weeks of age) by application of 45 mA current through corneal electrode for 0.2 s using electro-shocker (BH1-730105, BRC, Nagoya, Japan). Mice (n = 6·per group) were treated with saline or ONO-2506 (25, 50, 100 mg·kg^{-1}, i.p.). Based on the antiepileptic effects of ONO-2506, 60 min after systemic ONO-2506 administration, seizures were induced by MES; and the mice were observed throughout the duration of the tonic flexor, tonic extensor and clonic convulsions.

PTZ

Pentylenetetrazole (85 mg·kg^{-1}) was injected s.c. to induce clonic seizures (White *et al.*, 2002). Male mice (7-week-old) were treated with saline or ONO-2506(25, 50, 100 mg·kg^{-1}, i.p.) (n = 6·per group). Sixty minutes after ONO-2506 administration, mice were observed for 20 min after PTZ challenge. The behaviour of PTZ-induced seizures was monitored using the revised Racine's scale for PTZ-induced seizures (Luttjohann *et al.*, 2009).

Primary astrocyte cultures

Cortical astrocyte cultures were prepared from neonatal Sprague–Dawley rats (n = 12) killed by decapitation at 0–24 h of age and removal of the cerebral hemispheres under a dissecting microscope. Cortices were placed into a dish containing 10 mL DMEM (Sigma) at 4°C. Tissue was chopped into very fine pieces using scissors and then triturated briefly with a 1 mL micropipette. The suspension was filtered using 70 µm nylon mesh (BD, Franklin Lakes, NJ) and centrifuged at 100× g for 3 min at 4°C. The pellet was resuspended in 10 mL DMEM supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA). The suspension was centrifuged at 100× g for 3 min at 4°C, and the pellet was resuspended again in 12 mL DMEM with 10% fetal calf serum. The re-suspension step was repeated three times. The cortical suspensions were plated into several 75 cm^3 tissue culture flasks equivalent to the number of animals used. Flasks were kept at 37°C in a CO_2 incubator (95% air and 5% CO_2). The medium was replaced with fresh culture medium (DMEM containing 10% fetal calf serum) after 48 h and subsequently changed every 6 days. After 14 days of culture (DIV14), the contaminating cells were removed by shaking in a standard incubator for 16 h at 200 rpm. The medium was removed and discarded, and the flask contents were harvested and reseeded to yield double the original number of flasks. Astrocytes were removed from flasks by trypsinization and seeded onto translucent PET membrane (8 µm) 24-well plates (BD) at a density of 10^5 cells cm^{-2} for experiments on DIV21 (Tanahashi *et al.*, 2012).

To study the effects of cleavage with synaptobrevin on glial transmitters release, astrocytes were incubated in the fresh culture medium containing tetanus toxin (TeNT; 3 µg·mL^{-1}) for 24 h (pre-incubation with TeNT: this process was omitted in other experiments) (Okada *et al.*, 2001; 2005; Mothet *et al.*, 2005; Tanahashi *et al.*, 2012). To study the effects of fluorocitrate (FLC) on glial transmitters release, astrocytes were incubated in a fresh culture medium containing 1 mM FLC for 8 h (pre-incubation with FLC: this process was omitted in other experiments) (Tanahashi *et al.*, 2012).

The astrocytes were washed three times in artificial cerebrospinal fluid (ACSF) containing, in mM: NaCl 130, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1 and glucose 5.5, and buffered with 20 mM HEPES buffer to pH 7.3, and were then incubated in ACSF buffered with 100% O_2 for 30 min recovery time at 35°C (washed out with ACSF). To determine the release of glial transmitters, astrocytes were incubated in 100 µL ASCF. To study the effects of tetrodotoxin (TTX) on the release of glial transmitters, astrocytes were incubated in ACSF containing 1 µM TTX for 1 h (pre-incubation with TTX: This process was omitted in other experiments) (Tanahashi *et al.*, 2012). After pre-incubation, the transmitter release studies were carried out in quadruplicate at 35°C using the discontinuous method (Tanahashi *et al.*, 2012). To study the effects of ONO-2506 on basal glial transmitter release, the ACSF was switched to ACSF containing ONO-2506 (10, 30 and 100 µM) for 30 min (Tanahashi *et al.*, 2012). To study the effects of ONO-2506 on AMPA-induced glial transmitter release, after the above sampling, the ACSF was switched to ACSF containing 100 µM AMPA with the same ONO-2506 for 30 min (Tanahashi *et al.*, 2012). The released transmitter level in each sample (30 min·per fraction) was measured by liquid chromatography.

Preparation of microdialysis system

Male Sprague–Dawley rats, weighing 250–300 g were anaesthetized (with 1.8% isoflurane) and placed in a stereotaxic frame. Before inserting the microdialysis probe, all rats used in this study were pre-treated with microinjection of 0.3 µL modified Ringer's solution (MRS) with or without 1 ng TeNT (Murakami *et al.*, 2001; Okada *et al.*, 2001; 2005; Tanahashi *et al.*, 2012). The concentric I-shaped direct insertion type dialysis probes (D-I-7-03: 0.2 mm diameter, 2 mm exposed membrane: Eicom, Kyoto, Japan) were implanted in the mPFC (A = +3.2 mm, L = –0.8 mm, V = –6.0 mm relative to bregma) (Ohoyama *et al.*, 2011; Yamamura *et al.*, 2011).

The perfusion experiments commenced 24 h after recovery from isoflurane anaesthesia (Okada *et al.*, 2001). The perfusion rate was set at 2 µL·min^{-1}, using MRS composed of (in mM) 145 Na^+, 2.7 K^+, 1.2 Ca^{2+}, 1.0 Mg^{2+} and 154.4 Cl^-, and buffered with 2 mM phosphate buffer and 1.1 mM Tris buffer

to adjust to pH 7.4 (Ohoyama *et al.*, 2011; Tanahashi *et al.*, 2012; Yamamura *et al.*, 2011). Initially, the perfusate was MRS with or without 1 mM FLC, and the extracellular neurotransmitter levels were measured at least 8 h after starting perfusion (the microdialysis samples were collected in 20 min intervals) (Tanahashi *et al.*, 2012). After the coefficients of variation for extracellular levels of each transmitter became less than 5% over a period of 60 min (stabilization), control data were obtained over another 60 min.

After determination of control data (pre-treatment period), the perfusion medium was switched from MRS to MRS containing 1 μM TTX, or 50 mM K^+ containing MRS (HKMRS) for 20 min (K^+-evoked stimulation). The ionic compositions of these perfusates were modified, and isotonicity was maintained by an equimolar change of Na^+ (Murakami *et al.*, 2001; Okada *et al.*, 2001; 2005; Tanahashi *et al.*, 2012). The detailed study designs were described in Results section. The location of the dialysis probes was verified at the end of each experiment in 200 μm thick brain slices (Vibratome 1000, Technical Products International, St. Louis, MO).

Measurement of kynurenic acid concentration

The concentration of kynurenic acid was determined by HPLC (ECD-300, Eicom) equipped with sputter deposited nanocarbon film electrode detection (NCE-HPLC) (Kato *et al.*, 2011), set at +1600 mV (vs. an Ag/AgCl reference electrode). The analytical column (Triat C18 particle 2.0 μm, 150 × 3.0 mm, YMC, Kyoto, Japan) was maintained at 25°C, and the flow rate of the mobile phase was set at 500 $\mu L \cdot min^{-1}$. The mobile phase was 100 mM phosphate buffer (pH 4.5) containing 5% acetonitrile (v/v). Representative chromatograms of kynurenic acid analysis using NCE-HPLC are presented in Figure 1. The quantification limit for kynurenic acid by the NCE-HPLC method was 2 $fmol \cdot 10\ \mu L^{-1}$ (0.2 nM).

Measurement of L-glutamate, D-serine and GABA concentrations

The concentrations of L-glutamate, D-serine and GABA in dialysed MRS or incubated ACSF were determined by xLC (dual xLC 3185 PU, Jasco, Tokyo) with fluorescence detection (xLC3120FP, Jasco) after derivatization with isobutyryl-L-cysteine and *o*-phthalaldehyde. Derivatizing reagent solutions were prepared by dissolving isobutyryl-L-cysteine (2 mg) and *o*-phthalaldehyde (1 mg) in 0.1 mL ethanol followed by the addition of 0.9 mL 0.2 M sodium borate buffer (pH 9.0). The reagent solutions were filtered with 0.2 μm syringe filters and prepared freshly every second day and stored at 4°C when not in use. Automated pre-column derivatization was carried out by drawing up a 5 μL aliquot of sample, standard or blank solution and 5 μL of derivatizing reagent solution, and holding in the reaction vials 5 min prior to injection (xLC3059AS, Jasco). Derivatized samples (5μL) were injected by the auto sampler (xLC3059AS, Jasco). The excitation and emission wavelengths were 345 and 455 nm respectively. The analytical column (Triat C18, particle 1.9 μm, 50 × 2.0 mm, YMC) was maintained at 50°C, and the flow rate of the mobile phase was set at 500 $\mu L \cdot min^{-1}$. A linear gradient elution program was performed over 10 min with mobile phase A (5 mM citrate buffer, pH 8.0), B (5 mM citrate buffer, pH 2.0) and C (containing 50% acetonitrile and 50% ethanol).

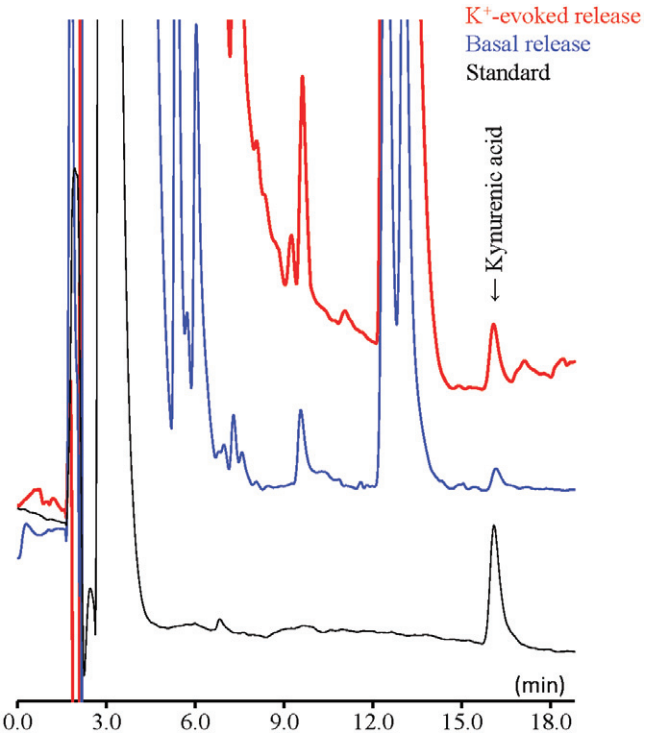

Figure 1

Typical chromatograms of kynurenic acid analysis using NCE-HPLC. The chromatograms were obtained from 10 μL of a standard solution containing 100 $fmol \cdot \mu L^{-1}$ of kynurenic acid, prefrontal perfusate in MRS and HKMRS. The quantification limit for kynurenic acid is 0.2 $fmol \cdot 10\ \mu L^{-1}$.

Data analysis

Values were expressed as means ± SEM. The dose-dependent effects of systemic administration of ONO-2506 on the frequencies and durations of spike and wave discharges in *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice, the duration of tonic flexor, tonic extensor and clonic convulsions induced by MES and the onset latency for clonic convulsion induced by PTZ were analysed by one-way ANOVA with Tukey's multiple comparison test.

Effects of perfusion with FLC (1 mM), TTX (1 μM) and microinjection of TeNT (1 $ng \cdot 0.3\ \mu L^{-1}$) on the basal and K^+-evoked transmitter releases in the mPFC were compared using MANOVA with Tukey's multiple comparison test. The dose-dependent effects of systemic administration of ONO-2506 (30, 100 and 300 $mg \cdot kg^{-1}$, i.p.) on the basal and K^+-evoked transmitter releases in the mPFC were compared using MANOVA with Tukey's multiple comparison test. Effects of perfusion with FLC (1 mM), TTX (1 μM) and microinjection of TeNT (1 $ng \cdot 0.3\ \mu L^{-1}$) on the 300 $mg \cdot kg^{-1}$ ONO-2506-induced transmitter releases in the mPFC were compared using Student's *t*-test.

The concentration-dependent effects of ONO-2506 (0, 10, 30 and 100 μM) on the transmitter releases from cultured astrocytes were compared using repeated-measurements of (repeated ANOVA) with Tukey's multiple comparison test. The effects of FLC (1 mM), TTX (1 μM) and TeNT (3 $\mu g \cdot mL^{-1}$) on the transmitter releases from cultured astrocytes were com-

pared using the Student's t-test. The effects of FLC (1 mM), TTX (1 μM) and TeNT (3 μg·mL^{-1}) on the ONO-2506-induced transmitter releases from cultured astrocytes were compared using repeated measures ANOVA with Tukey's multiple comparison test. The effects of FLC (1 mM), TTX (1 μM) and TeNT (3 μg·mL^{-1}) on 100 μM AMPA-induced transmitter releases from cultured astrocytes were compared using the Student's t-test. The concentration-dependent effects of ONO-2506 (0, 10, 30 and 100 μM) on the 100 μM AMPA-induced transmitter releases from cultured astrocytes were compared using one-way ANOVA with Tukey's multiple comparison test.

Materials

Arundic acid (ONO-2506) was provided by Ono Pharmaceutical Co. (Osaka, Japan). The synaptobrevin inhibitor tetanus toxin (TeNT), the glial toxin fluorocitrate (FLC) and PTZ were from Sigma (St. Louis, MO). The voltage-sensitive Na^+ channel inhibitor tetrodotoxin (TTX) and AMPA were obtained from Wako Chemicals (Osaka). ONO-2506, TeNT, FLC and TTX were dissolved directly in perfusion or incubation medium. Drug and channel nomenclature follows Alexander *et al.*, (2011)

Results

Anti-epileptic properties of ONO-2506 and ethosuximide

The frequency and duration of spike and wave discharges in *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice were 26.8 ± 4.3 counts·h^{-1} and 9.3 ± 1.1 s respectively (n = 13). ONO-2506 administration at 25, 50 and 100 mg·kg^{-1}, i.p. (n = 8·group^{-1}) dose-dependently reduced both the frequency [ANOVA: $F(3,21) = 15.7$ ($P < 0.01$)] and duration [ANOVA: $F(3,21) = 186.1$ ($P < 0.01$)] of spike and wave discharges (Figure 2A) in *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice. The suppressive effects on the frequency of spike and wave discharges occurred 30 min after ONO-2506 administration (Figure 2E). Especially, ONO-2506 (25 mg·kg^{-1}, i.p.) shortened the duration of spike and wave discharges but did not affect the frequency (Figure 2A). Similar to ONO-2506, ethosuximide at 100, 200 and 300 mg·kg^{-1} i.p. (n = 5·per group) also dose-dependently decreased both the frequency [ANOVA: $F(3,12) = 32.3$ ($P < 0.01$)] and duration [ANOVA: $F(3,12) = 10.1$ ($P < 0.01$)] of spike and wave discharges (Figure 2B).

Anti-convulsive properties of ONO-2506

Based on the above results, 60 min after ONO-2506 administration at 25, 50 and 100 mg·kg^{-1} (i.p.), each mouse was exposed to MES or PTZ. The durations of tonic flexor, tonic extensor and clonic convulsions induced by MES (45 mA × 0.2 s) were 1.8 ± 0.2, 15.9 ± 1.2 and 7.3 ± 0.9 s, respectively, in the control experiment. At each dose used, ONO-2506 did not affect the seizure activities induced by MES (Figure 2C).

In control mice, PTZ (85 mg·kg^{-1}, s.c.) induced seizures in all animals without any mortality, with onset latency for clonic seizures: score II (facial jerking with muzzle or muzzle and eye) or score III (neck jerks) of the revised Racine's scale (Luttjohann *et al.*, 2009), of 6.8 ± 1.9 min. At each of the stated concentrations, ONO-2506 did not affect the PTZ-induced seizure activities (Figure 2D).

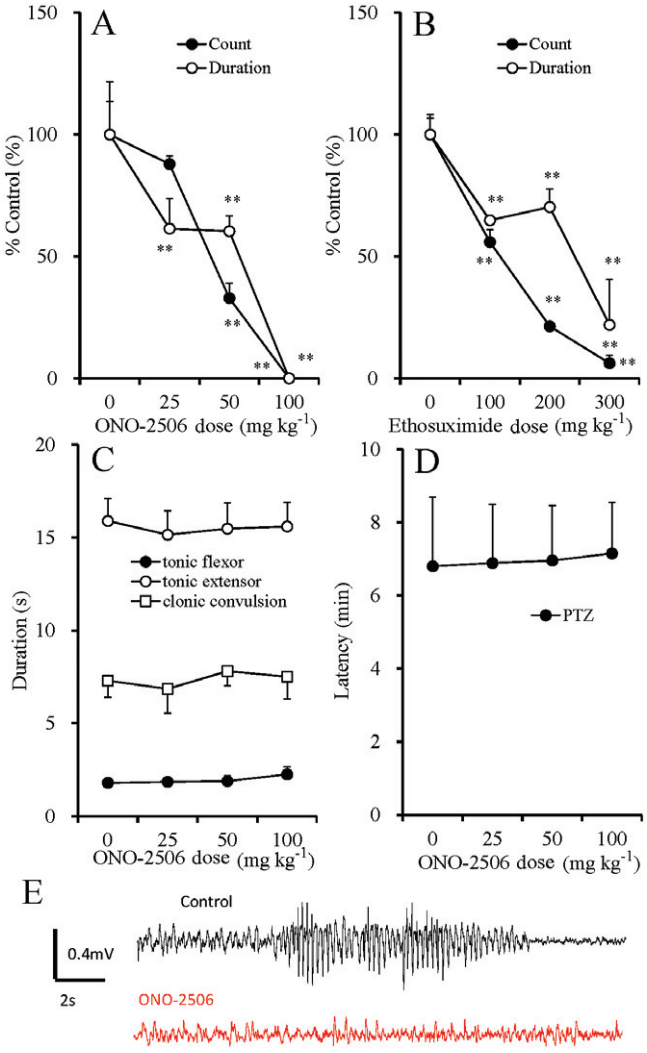

Figure 2

Effects of ONO-2506 on frequency and duration of spike and wave discharges, MES and PTZ in Cacna1a$^{tm2Nobs/tm2Nobs}$ mice (A) Dose-dependent effects of systemic administration of ONO-2506 (0, 25, 50 and 100 mg·kg^{-1}, i.p.) (n = 8·per group) on the frequency and duration of spike and wave discharges in Cacna1a$^{tm2Nobs/tm2Nobs}$ mice. (B) Dose-dependent effects of systemic administration of ethosuximide (0, 100, 200 and 300 mg·kg^{-1}, i.p.) (n = 5·per group) on the frequency and duration of spike and wave discharges in Cacna1a$^{tm2Nobs/tm2Nobs}$ mice. (C) Lack of dose-dependent effects of systemic administration of ONO-2506 (0, 25, 50 and 100 mg·kg^{-1}, i.p.) (n = 6·per group) on the duration of tonic flexor, tonic extensor and clonic convulsion induced by MES. (D) Lack of dose-dependent effects of systemic administration of ONO-2506 (0, 25, 50 and 100 mg·kg^{-1}, i.p.) (n = 6·per group) on the onset latency for clonic seizures induced by PTZ: score II (facial jerking with muzzle or muzzle and eye) or score III (neck jerks) of the revised Racine's scale (Luttjohann *et al.*, 2009). The putative dose-dependent effects of ONO-2506 on convulsions and spike and wave discharges and of ethosuximide on spike and wave discharges were analysed by ANOVA with Tukey's multiple comparison test. **$P < 0.01$ significantly different from control. (E) Typical EEG of Cacna1a$^{tm2Nobs/tm2Nobs}$ mice without (control) and with 100 mg·kg^{-1} ONO-2506 (ONO-2506).

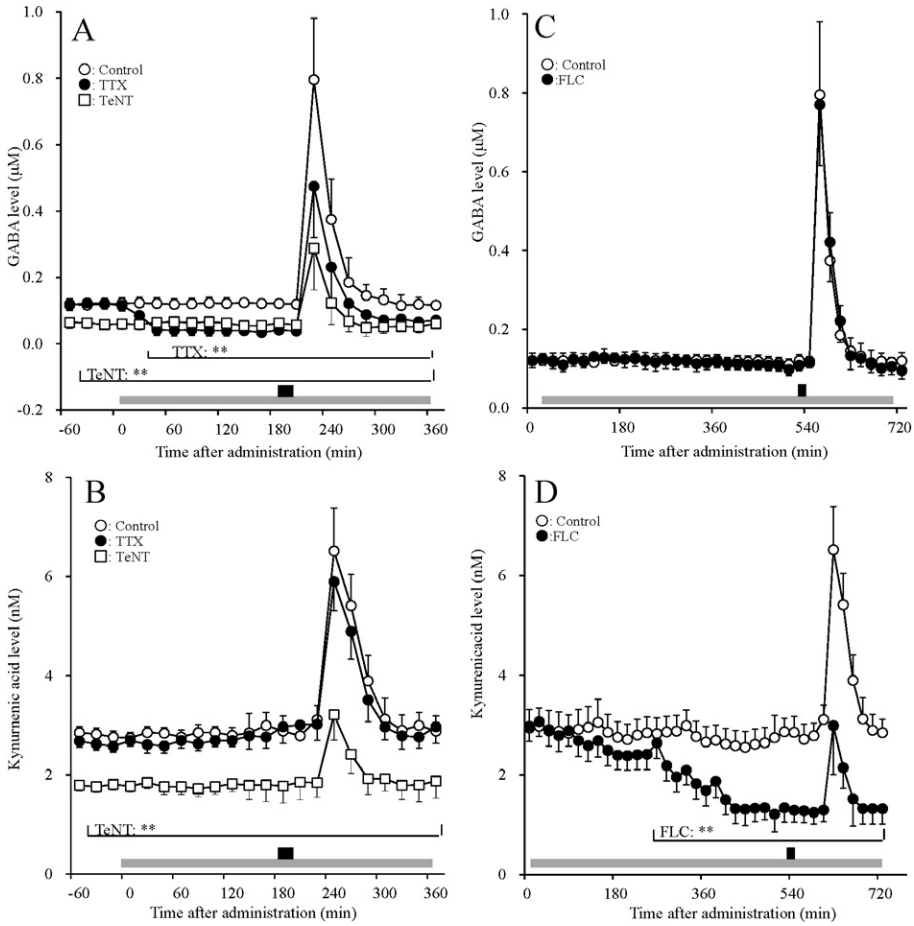

Figure 3

Effects of TTX, FLC and TeNT on basal and K^+-evoked release of GABA and kynurenic acid in the mPFC. Effects of perfusion with 1 μM TTX and microinjection of 1 ng·0.3 μL^{-1} TeNT on the basal and K^+-evoked release of (A) GABA and (B) kynurenic acid in the mPFC. Effects of perfusion with 1 mM FLC on the basal and K^+-evoked release of (C) GABA and (D) kynurenic acid in the mPFC. Data are means ± SEM (n = 6) of extracellular transmitter levels. The effects of microinjection of TeNT and perfusion with TTX and FLC were compared using MANOVA with Tukey's multiple comparison test. *P < 0.05; **P < 0.01 significantly different from control.

In vivo microdialysis study

The basal extracellular concentrations of L-glutamate, D-serine and GABA in the mPFC were 2.08 ± 0.26, 1.12 ± 0.19 and 0.12 ± 0.02 μM, respectively (not corrected for *in vitro* dialysis probe recovery). The basal extracellular level of kynurenic acid in the mPFC was 2.96 ± 0.38 nM (not corrected for *in vitro* dialysis probe recovery).

Effects of TTX, FLC and TeNT on basal and K^+-evoked release of L-glutamate and D-serine in the mPFC

Perfusion experiments were conducted to study the SNARE-associated transmitter release in the mPFC, 24 h after microinjection of TeNT (1 ng·0.3 μL^{-1}). The extracellular transmitter levels were measured at least 8 h after starting perfusion, as described recently (Tanahashi *et al.*, 2012). Microinjection of TeNT (1 ng·0.3 μL^{-1}) reduced the basal extracellular levels of GABA (Student's t-test, P < 0.01) and kynurenic acid (Student's t-test, P < 0.01) in the mPFC (Figure 3A and B). Next, we studied the effects of TTX and FLC on basal transmitter release. After stabilization, the perfusion medium was switched from MRS to MRS containing 1 mM FLC or 1 μM TTX. Perfusion with 1 μM TTX decreased the basal extracellular GABA level [repeated ANOVA: $F(6,30)$ = 292.0 (P < 0.01)] (Figure 3A) but had no effect on kynurenic acid (Figure 3B) in the mPFC. In contrast to TTX, perfusion with 1 mM FLC reduced the frontal extracellular kynurenic acid level [repeated ANOVA: $F(26,130)$ = 263.1 (P < 0.01)] (Figure 3D) without affecting that of GABA (Figure 3C). The stability of the inhibitory effects of FLC on kynurenic acid release was observed at 4 h after FLC perfusion starting (Figure 3D).

Next, we studied the effects of TTX and FLC on depolarization-induced transmitter release. The perfusion medium was switched from MRS containing 1 mM FLC or 1 μM TTX to 50 mM K^+ containing MRS (HKMRS) with the same agent (1 μM TTX or 1 mM FLC). We also studied the effects of TeNT microinjection on depolarization-induced transmitter release by replacing the perfusion medium from

MRS to HKMRS. The 50 mM K⁺-evoked stimulation (perfusion with HKMRS for 20 min) increased the extracellular levels of GABA and kynurenic acid in the mPFC (Figure 3). Especially, the magnitude of K⁺-evoked releases of GABA and kynurenic acid reached peak levels at 20 and 40 min after the start of K⁺-evoked stimulation, respectively (Figure 3). Perfusion with TTX (1 μM) reduced K⁺-evoked GABA release [MANOVA: $F_{TTX}(1,10) = 13.5$ ($P < 0.01$), $F_{Time}(6,5) = 378.6$ ($P < 0.01$), $F_{TTX \times Time}(6,60) = 6.5$ ($P < 0.01$)] but not kynurenic acid (Figure 3A and B). Microinjection of TeNT (1 ng·0.3 μL^{-1}) reduced K⁺-evoked releases of GABA [MANOVA: $F_{TeNT}(1,10) = 34.8$ ($P < 0.01$), $F_{Time}(6,5) = 563.5$ ($P < 0.01$), $F_{TeNT \times Time}(6,60) = 26.5$ ($P < 0.01$)] and kynurenic acid [MANOVA: $F_{TeNT}(1,10) = 19.3$ ($P < 0.01$), $F_{Time}(6,5) = 168.1$ ($P < 0.01$), $F_{TeNT \times Time}(6,60) = 18.9$ ($P < 0.01$)] (Figure 3A and B). Perfusion with 1 mM FLC reduced K⁺-evoked releases of kynurenic acid [MANOVA: $F_{FLC}(1,10) = 23.1$ ($P < 0.01$), $F_{Time}(6,5) = 173.5$ ($P < 0.01$), $F_{FLC \times Time}(6,60) = 21.9$ ($P < 0.01$)] (Figure 3D) but had no effect on GABA (Figure 3C).

We reported previously that the extracellular levels of L-glutamate and D-serine in the mPFC were not altered after either TeNT microinjection (1 ng·0.3 μL^{-1}) or TTX perfusion (1 μM); but were reduced after perfusion with FLC (1 mM). At 6 h after starting the FLC perfusion, the inhibitory effects of FLC on release of L-glutamate and D-serine reached a plateau (Tanahashi *et al.*, 2012). In the same study, 100 mM K⁺-evoked stimulation increased the extracellular levels of L-glutamate and D-serine in the mPFC. The 100 mM K⁺-evoked L-glutamate release was inhibited by TeNT microinjection (1 ng·0.3 μL^{-1}), perfusion with 1 μM TTX and 1 mM FLC. ; however, the 100 mM K⁺-evoked D-serine release was inhibited by TeNT microinjection and FLC perfusion but not by perfusion with TTX (Tanahashi *et al.*, 2012). In the present study, perfusion with 1 mM FLC, but not with TeNT or TTX, reduced the extracellular levels of both L-glutamate and D-serine in the mPFC (data not shown), similar to our previous study (Tanahashi *et al.*, 2012). We also studied the effects of TeNT, TTX and FLC on depolarization-induced transmitter release by switching the perfusion medium from MRS to 50 mM K⁺ containing MRS for 20 min. The 50 mM K⁺-evoked stimulation increased L-glutamate release but had no effect on extracellular D-serine level. The 50 mM K⁺-evoked L-glutamate release in the mPFC was reduced by TeNT, TTX and FLC, similar to the 100 K⁺-evoked L-glutamate release (Tanahashi *et al.*, 2012).

Dose-dependent effects of ONO-2506 on basal and K⁺-evoked transmitter releases in the mPFC

We also studied the effects of systemic ONO-2506 on basal transmitter release. After stabilization, the rats were injected i.p. with ONO-2506 (0, 30, 100 or 300 mg·kg^{-1}, i.p.). This resulted in a dose-dependent increase in the basal release of GABA [MANOVA: $F_{Dose}(3,20) = 3.8$ ($P < 0.05$), $F_{Time}(9,12) = 35.7$ ($P < 0.01$), $F_{Dose*Time}(27\ 180) = 19.2$ ($P < 0.01$)] (Figure 4A) and kynurenic acid [MANOVA: $F_{Dose}(3,20) = 3.2$ ($P < 0.05$), $F_{Time}(9,12) = 78.4$ ($P < 0.01$), $F_{Dose \times Time}(27\ 180) = 4.7$ ($P < 0.01$)] (Figure 4B), but not L-glutamate or D-serine (data not shown). Low-dose ONO-2506 (30 mg·kg^{-1}) did not alter the extracellular levels of GABA or kynurenic acid, whereas the higher doses (100 and 300 mg·kg^{-1}) increased those levels (Figure 4A and B).

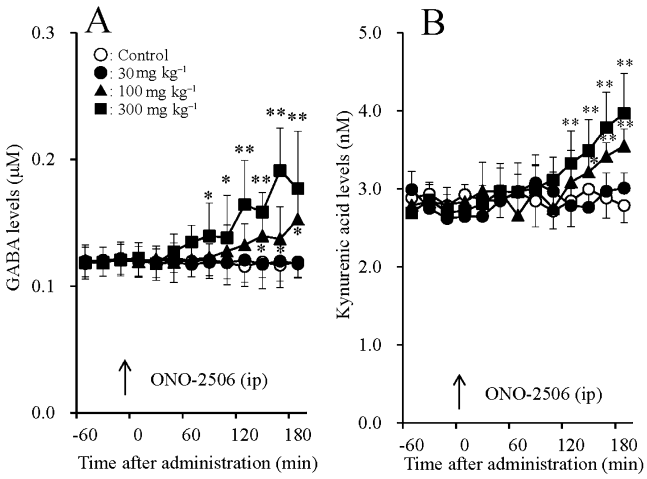

Figure 4

Effects of systemic administration of ONO-2506 on basal release of GABA and kynurenic acid in the mPFC. Dose-dependent effects of ONO-2506 (30, 100 and 300 mg·kg^{-1}) on the extracellular levels of (A) GABA and (B) kynurenic acid in the mPFC. Ordinates: extracellular levels (nM or μM, $n = 6$) of GABA and kynurenic acid in the mPFC, abscissas: time after administration (min) of ONO-2506 (i.p.). The dose-dependent effects of ONO-2506 on extracellular levels of GABA and kynurenic acid in the mPFC were analysed by MANOVA with Tukey's multiple comparison test. *$P < 0.05$; **$P < 0.01$ significantly different from pre-treatment period.

After this experiment (4 h after ONO-2506 injection), we investigated the effects of ONO-2506 on depolarization-induced transmitter release. The perfusion medium was switched from MRS to HKMRS for 20 min (K⁺-evoked stimulation). K⁺-evoked stimulation increased the extracellular concentrations of GABA, L-glutamate and kynurenic acid (Figure 5); but not those of D-serine (data not shown).

Systemic administration of ONO-2506 (30, 100 and 300 mg·kg^{-1}, i.p.) dose-dependently decreased K⁺-evoked releases of L-glutamate [MANOVA: $F_{Dose}(3,20) = 5.4$ ($P < 0.01$), $F_{Time}(9,12) = 273.8$ ($P < 0.01$), $F_{Dose \times Time}(27\ 180) = 16.4$ ($P < 0.01$)] (Figure 5A), GABA [MANOVA: $F_{Dose}(3,20) = 4.2$ ($P < 0.05$), $F_{Time}(9,12) = 276.2$ ($P < 0.01$), $F_{Dose \times Time}(27\ 180) = 12.7$ ($P < 0.01$)] (Figure 5B) and kynurenic acid [MANOVA: $F_{Dose}(3,20) = 3.8$ ($P < 0.05$), $F_{Time}(9,12) = 333.6$ ($P < 0.01$), $F_{Dose \times Time}(27\ 180) = 82.4$ ($P < 0.01$)] (Figure 5C).

Effects of TTX, TeNT and FLC on ONO-2506-induced transmitter release in the mPFC

To determine the mechanisms of ONO-2056-induced releases of GABA and kynurenic acid, differences in areas under curve (AUC; range: 120–180 min) of extracellular levels of GABA and kynurenic acid in the mPFC, after after systemic administration of ONO-2506, between pre-treatment with and without TTX, TeNT or FLC were analysed by Student's *t*-test. Two hours after perfusion with 1 μM TTX, each rat was given 300 mg·kg^{-1} ONO-2506, i.p.. Perfusion with 1 μM TTX decreased the ONO-2506-induced GABA release (Figure 6A) without affecting that of kynurenic acid (Figure 6B). Eight

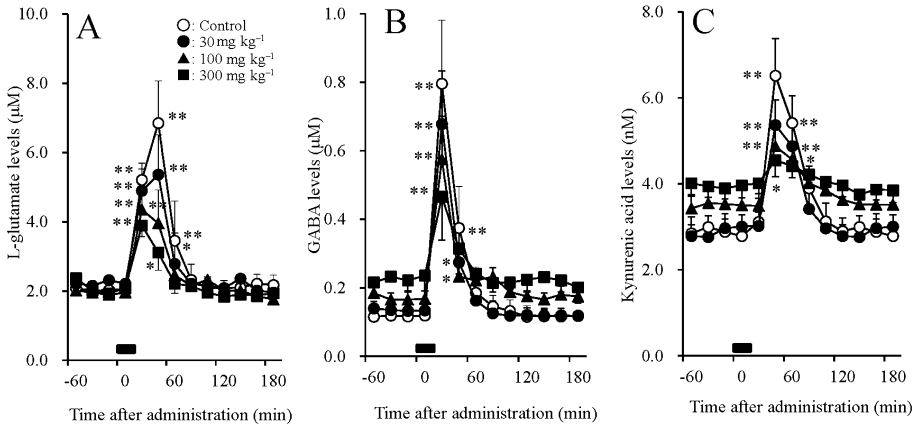

Figure 5

Effects of systemic administration of ONO-2506 on K^+-evoked releases of L-glutamate, GABA and kynurenic acid in the mPFC. Dose-dependent effects of ONO-2506 (30, 100 and 300 mg·kg^{-1}) on K^+-evoked releases of (A) L-glutamate, (B) GABA and (C) kynurenic acid in the mPFC. Four hours after systemic administration of ONO-2506, the perfusate was switched from MRS to HKMRS for 20 min (K^+-evoked stimulation). Ordinates: extracellular levels of L-glutamate (μM, $n = 6$), GABA (μM, $n = 6$) and kynurenic acid (nM, $n = 6$) in the mPFC, abscissas: time after K^+-evoked stimulation (min). The dose-dependent effects of ONO-2506 on K^+-evoked releases in the mPFC were analysed using MANOVA with Tukey's multiple comparison test. *$P < 0.05$; **$P < 0.01$ significantly different from pre-treatment period.

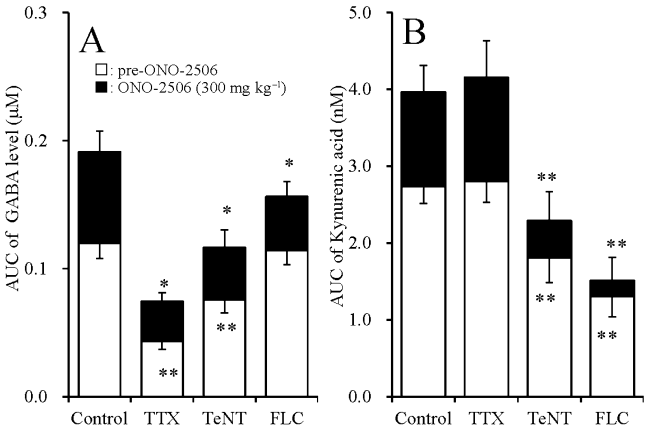

Figure 6

Effects of TTX, TeNT and FLC on ONO-2506-induced releases of GABA and kynurenic acid in the mPFC. Effects of TTX, TeNT and FLC on the AUC values of 300 mg·kg^{-1} ONO-2506-induced releases of (A) GABA and (B) kynurenic acid in the mPFC. Ordinates: AUC values of GABA (μM, $n = 6$) and kynurenic acid (nM, $n = 6$) in the mPFC. ONO-2506-induced release was calculated by subtraction of the mean of basal release from the extracellular level after ONO-2506 administration from 120 to 180 min. Data are means ± SEM ($n = 6$). *$P < 0.05$; **$P < 0.01$ significantly different from control, by Student's *t*-test.

hours after perfusion with 1 mM FLC (Tanahashi *et al.*, 2012), each rat was given 300 mg·kg^{-1} ONO-2506, i.p.. Perfusion with 1 mM FLC decreased ONO-2506-induced release of GABA and kynurenic acid (Figure 6A and B). Microinjection of TeNT (1 ng·0.3 μL^{-1}) also decreased ONO-2506-induced releases of GABA and kynurenic acid (Figure 6A and B).

Cultured astrocytes

Effects of ONO-2506 on transmitter release from primary cultured astrocytes

To study the effects of ONO-2506 on glial transmitter release, astrocytes were pre-incubated with ACSF then cultured with ACSF containing ONO-2506 (0, 10, 30 and 100 μM). At baseline, glial cells released L-glutamate, D-serine and kynurenic acid but not GABA. ONO-2506 concentration-dependently increased glial releases of kynurenic acid [repeated ANOVA, $F(3,15) = 20.6$ ($P < 0.01$)] (Figure 7) but had no effect on L-glutamate or D-serine (data not shown).

Next, we studied the effects of TeNT on ONO-2506-induced release of kynurenic acid from astrocytes. Astrocytes were pre-incubated with TeNT (3 μg·mL^{-1}) for 24 h then cultured in ASCF containing 10, 30 and 100 μM ONO-2506. Pre-incubation with TeNT (3 μg·mL^{-1}) for 24 h decreased glial release of kynurenic acid (Student's *t*-test: $P < 0.01$) (Figure 7). After incubation with TeNT, ONO-2506 had no effect on glial release of kynurenic acid (Figure 7).

We also studied the effects of FLC and TTX on ONO-2506-induced release of kynurenic acid. Astrocytes were first incubated with FLC or TTX, then with ASCF containing 1 mM FLC or 1 μM TTX and 10, 30 and 100 μM ONO-2506. Pre-incubation with 1 mM FLC for 8 h decreased glial release of kynurenic acid (Student's *t*-test: $P < 0.01$) (Figure 7). Furthermore, pre-incubation with 1 mM FLC ameliorated the effect of ONO-2506 on glial release of kynurenic acid from astrocytes (Figure 7); however, ONO-2506 concentration-dependently increased glial kynurenic acid release when incubated with 1 μM TTX [repeated ANOVA, $F(3,15) = 39.3$ ($P < 0.01$)]. Furthermore, pre-incubation with 1 μM TTX for 2 h had no effect on glial release of kynurenic acid (Figure 7).

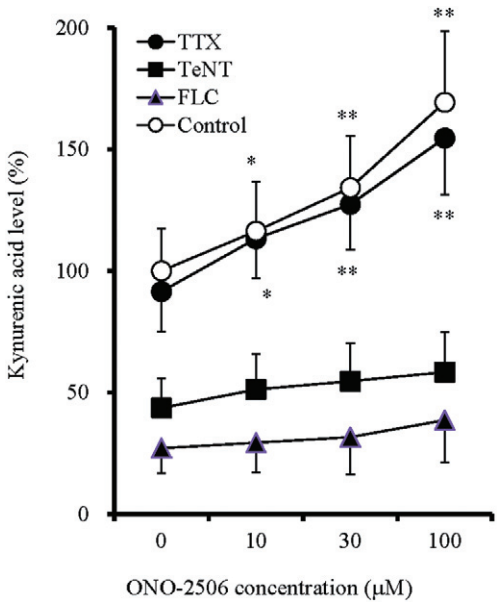

Figure 7

Effects of TTX, TeNT and FLC on ONO-2506-induced kynurenic acid release from primary cultured astrocytes. Effects of pre-treatment without (control) or with 1 μM TTX, 3 μg·mL^{-1} TeNT and 1 mM FLC on ONO-2506-induced kynurenic acid release from astrocytes. After incubation with TTX for 2 h, TeNT for 24 h or FLC for 8 h, ONO-2506 was added to the incubation medium at 10, 30 or 100 μM. Ordinates: % control of kynurenic acid levels (%, n = 6), abscissas: ONO-2506 concentration (μM). *P < 0.05; **P < 0.01 significantly different from 0 μM, repeated ANOVA with Tukey's multiple comparison test.

Effects of TTX, TeNT and FLC on AMPA-induced transmitter release from primary cultured astrocytes

To study the effects of TTX, FLC and TeNT on AMPA-induced glial transmitter release, pre-incubation of primary astrocytes with ACSF containing TTX (1 μM for 2 h), FLC (1 mM for 8 h) or TeNT (3 μg·mL^{-1} for 24 h), 100 μM AMPA was added to the ACSF. Both pre-incubation with TeNT and FLC decreased AMPA-evoked glial releases of GABA (Student's t-test: P < 0.01) (Figure 8A), kynurenic acid (Student's t-test: P < 0.01) (Figure 8B), L-glutamate and D-serine (data not shown) (Tanahashi *et al.*, 2012); whereas pre-incubation with 1 μM TTX for 2 h had no effect.

Effects of ONO-2506 on AMPA-induced transmitter release from primary cultured astrocytes

Finally, we studied the effects of ONO-2506 on 100 μM AMPA-induced glial release of L-glutamate, D-serine, GABA and kynurenic acid. After incubation of primary astrocytes in ACSF containing ONO-2506 (10, 30 and 300 μM), they were incubated in ACSF containing AMPA (100 μM) with the same concentration of ONO-2506. AMPA increased the release of L-glutamate, D-serine, GABA and kynurenic acid. ONO-2506 concentration-dependently decreased 100 μM AMPA-induced glial releases of L-glutamate [one-way ANOVA: $F_{Dose}(3,44)$ = 6.4 (P < 0.01)], D-serine [one-way ANOVA: $F_{Dose}(3,44)$ = 9.3 (P < 0.01)], GABA [one-way ANOVA: $F_{Dose}(3,44)$ = 8.5 (P < 0.01)] and kynurenic acid [one-way ANOVA: $F_{Dose}(3,44)$ = 31.9 (P < 0.01)] from primary cultured astrocytes (Figure 9).

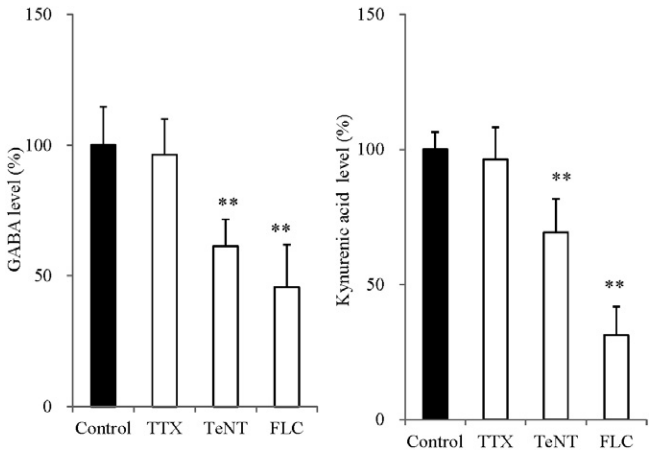

Figure 8

Effects of TTX, TeNT and FLC on AMPA-induced releases of GABA and kynurenic acid from primary cultured astrocytes. Effects of TTX (1 μM for 2 h), TeNT (0.3 ng·mL^{-1} for 24 h) and FLC (1 mM for 8 h) on 100 μM AMPA-induced releases of (A) GABA and (B) kynurenic acid from astrocytes. Data are means ± SEM of % control values (%) (n = 6). *P < 0.05; **P < 0.01 significantly different from control; Student's t-test.

Discussion and conclusions

Antiepileptic profile of ONO-2506

Many of the conventional anticonvulsants are effective in standard models that have been traditionally used for identification of new antiepileptic drugs, namely MES and PTZ tests (White *et al.*, 2002), whereas ONO-2506 was ineffective in these two standard convulsion models. In contrast to these traditional tests, ONO-2506 displayed protective actions against spontaneous absence seizures in *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice (Saito *et al.*, 2009). Therefore, ONO-2506 is not an anticonvulsant but an antiepileptic drug. This antiepileptic profile of ONO-2506 is somewhat similar to that of levetiracetam (De Smedt *et al.*, 2007). Levetiracetam did not affect MES and only weakly inhibited PTZ (Gower *et al.*, 1992; Loscher and Honack, 1993; Mandhane *et al.*, 2007)but it was effective against absence epilepsy in rats in two genetic models (Strasbourg and WAG/Rij rats) (Gower *et al.*, 1995; Bouwman and van Rijn, 2004). Indirect comparisons based on meta-analysis indicated that add-on therapy with levetiracetam has a favourable response and/or withdrawal rate relative to several other antiepileptic drugs in patients with partial epilepsy with the doses used in clinical trials (Otoul *et al.*, 2005). Therefore, the present results suggest that ONO-2506 is a novel candidate antiepileptic drug.

ONO-2506 was discovered through screening tests carried out by Ono Pharmaceutical Co., searching for an agent that could inhibit astrocytic S100B (Asano *et al.*, 2005). S100B is

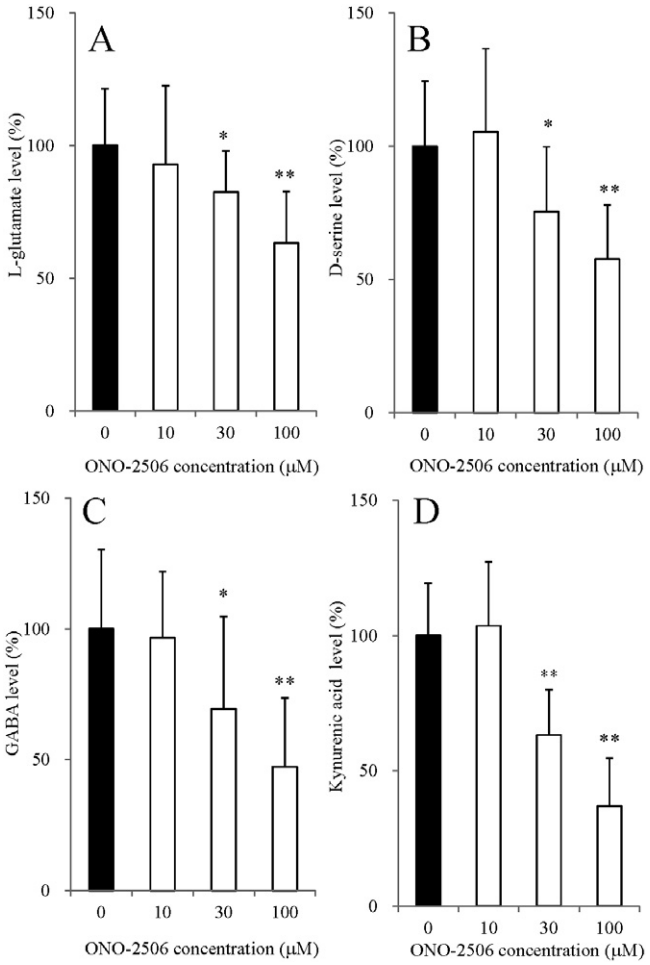

Figure 9

Concentration-dependent effects of ONO-2506 on AMPA-induced releases of L-glutamate, D-serine, GABA and kynurenic acid from primary cultured astrocytes. Concentration-dependent effects of ONO-2506 on 100 μM AMPA-induced releases of (A) L-glutamate, (B) D-serine, (C) GABA and (D) kynurenic acid from primary cultured astrocytes. Data are means ± SEM of % control values (%) ($n = 6$). *$P < 0.05$; **$P < 0.01$ significantly different from control; one-way ANOVA with Tukey's multiple comparison test.

an acidic calcium-binding protein produced mainly by astrocytes, with two opposing effects, trophic (it stimulates neurite outgrowth and survival of neurons at nanomolar concentrations) and toxic (it stimulates the expression of proinflammatory cytokines such as IL-6 and induces apoptosis at micromolar concentration) (Rothermundt *et al.*, 2003). Epileptic patients are reported to overexpress S100B in the epileptic focus regions (Griffin *et al.*, 1995; Lee *et al.*, 2007). Therefore, ONO-2506 is a candidate anti-epileptogenesis agent acting through the suppression of over-expression of S100B induced by epileptic seizure; however, the mechanism of the anti-absence action of ONO-2506 remains to be clarified.

Several studies using the Strasbourg and WAG/Rij rats, the most widely used rodent models, emphasized that absence epilepsy has a cortical origin (Meeren *et al.*, 2002; Pinault, 2003; Gurbanova *et al.*, 2006; van Luijtelaar, 2011) and is classified as a cortico-thalamo-cortical type epilepsy (Meeren *et al.*, 2002; Pinault, 2003; Gurbanova *et al.*, 2006; Blumenfeed and Coulter, 2007; van Luijtelaar, 2011). Furthermore, a recent study using WAG/Rij rats, suggested that a deficit in gliotransmission in the frontal cortex played important roles in the pathogenesis of absence epilepsy (Sitnikova *et al.*, 2011). Therefore, to clarify the antiepileptic mechanism of ONO-2506, the present study determined the effects of ONO-2506 on gliotransmission in frontal cortex using microdialysis and primary cultured cortical astrocytes.

Mechanisms of gliotransmitter release in cultured astrocytes

Various transmitters, such as L-glutamate, D-serine, GABA and kynurenic acid, are released from astrocytes (Hamilton and Attwell, 2010; Parpura and Zorec, 2010; Potter *et al.*, 2010; Lee *et al.*, 2011). Our previous study demonstrated that basal and 100 μM AMPA-evoked releases of L-glutamate and D-serine from astrocytes were inhibited by the glial toxin, FLC and the synaptobrevin inhibitor, TeNT, but such release was insensitive to the voltage-gated sodium channel inhibitor, TTX (Tanahashi *et al.*, 2012), similar to the present results. The present study also identified the glial release mechanism of kynurenic acid from primary cultured astrocytes. The release of kynurenic acid from unstimulated astrocytes was inhibited by FLC and TeNT, but not by TTX. Furthermore, the 100 μM AMPA-evoked releases of GABA (basal GABA release from astrocytes could not be detected) and kynurenic acid from astrocytes were also inhibited by FLC and TeNT, but not by TTX. The expression of SNARE, including synaptobrevin, in cultured astrocytes has been demonstrated by immunohistochemistry, and glial D-serine release seems to be regulated by a Ca^{2+}-dependent SNARE complex (Schell *et al.*, 1995; Mothet *et al.*, 2005; Tanahashi *et al.*, 2012). Cleavage with synaptobrevin by TeNT inhibited both basal and AMPA-stimulated release of L-glutamate, D-serine, GABA and kynurenic acid from cultured astrocytes. Therefore, the release of L-glutamate, D-serine, GABA and kynurenic acid were, at least in part, regulated by glial exocytosis mechanisms (Tanahashi *et al.*, 2012).

Mechanisms of transmitter release in frontal cortex

Contrary to primary cultured astrocytes, the microdialysis study demonstrated different mechanisms among L-glutamate, D-serine, GABA and kynurenic acid. The basal releases of L-glutamate and D-serine were reduced by FLC, but neither by TTX nor TeNT; however, the basal GABA release was decreased by TTX and TeNT, but not by FLC. Therefore, the origin of the basal GABA release in the mPFC is neuronal exocytosis (Murakami *et al.*, 2001; Okada *et al.*, 2001; 2005; Tanahashi *et al.*, 2012), whereas the origin of basal L-glutamate and D-serine release in the mPFC are from glial cells and by leakage from synaptic clefts (Timmerman and Westerink, 1997; Tanahashi *et al.*, 2012).

The K^+-evoked L-glutamate release was reduced by TTX, TeNT and FLC; whereas that of GABA was reduced by TTX and TeNT but not by FLC. This discrepancy suggests that the depolarization-induced GABA release derived mainly from

neuronal exocytosis, whereas the depolarization-induced L-glutamate release was from both neuronal and glial exocytosis. The frontal extracellular D-serine level was increased by 100 mM K^+-evoked stimulation, but not by 50 mM K^+-stimulation. The 100 mM K^+-evoked D-serine release was decreased by TeNT and FLC but insensitive to TTX (Tanahashi *et al.*, 2012). Therefore, the 100 mM K^+-evoked D-serine release was by glial exocytosis but the threshold of neuronal hyperexcitation for D-serine exocytosis is probably higher than that for L-glutamate.

Our NCE-HPLC method detected kynurenic acid at high oxidation potential (higher than 1500 mV vs. Ag/AgCl reference electrode). However, conventional electrodes including glass-carbon or graphite cannot detect kynurenic acid, since the materials of these conventional electrode become unstable upon the application of high oxidation potential (Niwa *et al.*, 2006). In contrast, the quantification limit of our NCE-HPLC method for kynurenic acids was 2 fmol·10 μL^{-1} (0.2 nM) because the NCE exhibited excellent electrochemical performance including stability and low background noise level at the oxidation potential region of kynurenic acid compared with other conventional glass-carbon or graphite electrodes for HPLC (Niwa *et al.*, 2006; Kato *et al.*, 2008). Furthermore, the NCE-HPLC also demonstrated that TeNT and FLC, but not TTX, markedly decreased the basal and K^+-evoked releases of kynurenic acid in the mPFC. These results suggest that the frontal release of kynurenic acid is mainly by glial exocytosis.

Effects of ONO-2506 on gliotransmitter release and mechanisms of antiepileptic action

Systemic administration of ONO-2506 dose-dependently increased the extracellular levels (basal release) of inhibitory transmitters, GABA and kynurenic acid without affecting those of excitatory transmitters, L-glutamate or D-serine. Furthermore, ONO-2506 dose-dependently suppressed the frequency and duration of spike and wave discharges in *Cacna1a*$^{tm2Nobs/tm2Nob}$. These results suggest that the antiepileptic action of ONO-2506 was mediated by enhanced inhibitory transmission by GABA and kynurenic acid (Meldrum, 2007; Vamos *et al.*, 2009; Potter *et al.*, 2010).

Recent findings suggest that GABAergic transmission in the thalamo-cortical network plays important roles in the pathophysiology of absence epilepsy (Errington *et al.*, 2011). Enhancement of GABAergic transmission inhibited convulsive epileptic seizures (White *et al.*, 2002; Meldrum, 2007) but systemic administration of GABA-mimetic antiepileptic drugs, such as vigabatrin and tiagabine, exacerbated absence seizures in absence epilepsy animal models (Depaulis and van Luijtelaar, 2006). In contrast, local administration of GABA-mimetic agents into the hippocampus inhibited spike and wave discharges of WAG/Rij rats (Tolmacheva and van Luijtelaar, 2007). Similar to GABA-mimetic agents, systemic administration of phenytoin aggravated absence seizures, whereas local cortical administration of phenytoin was effective in reducing spike and wave discharges of Strasbourg and WAG/Rij rats (Gurbanova *et al.*, 2006). Therefore, over-inhibition in the thalamocortical network via over-enhancement of GABAergic transmission probably activates a cortico-thalamo-cortical origin of spike–wave discharges, whereas selective inhibition of cortico-thalamo-cortical network transmission is probably involved in the anti-absence action. To clarify our hypothesis, we plan to study the effects of local administration of GABAergic agonist into the frontal cortex and thalamus on absence epilepsy in *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice

In contrast to GABA, selective dysfunction of kynurenic acid synthesis has been demonstrated in the frontal cortex of WAG/Rij rats and i.c.v. administration of kynurenic acid concentration-dependently reduced WAG/Rij seizures (Peeters *et al.*, 1994; Kaminski *et al.*, 2003). Therefore, an important component of the anti-absence mechanism of ONO-2506 was its stimulatory effects on kynurenic acid release during the resting stage. The ONO-2506-induced GABA release was inhibited by TTX, TeNT and FLC, whereas the ONO-2506-induced kynurenic acid release was inhibited by TeNT and FLC but not by TTX. These results suggest that the ONO-2506-induced GABA release in the mPFC is mediated through enhancement of both neuronal and glial exocytosis mechanisms. In contrast to GABA, ONO-2506-induced kynurenic acid release in the mPFC is probably produced by enhancement of glial exocytosis mechanisms.

Evidence suggests that activation of AMPA glutamate receptors plays an important role in the pathophysiology of absence epilepsy seizures, since i.c.v. administration of AMPA concentration-dependently enhanced WAG/Rij seizures (Peeters *et al.*, 1994). ONO-2506 also concentration-dependently reduced AMPA-induced releases of L-glutamate, D-serine, GABA and kynurenic acid from astrocytes. Indeed, ONO-2506 inhibited the frequency (a measure of excitability) and duration (measure of endogenous stopping mechanism) of spike and wave discharges in *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice. Furthermore, ONO-2506 concentration-dependently reduced K^+-evoked releases of L-glutamate, GABA and kynurenic acid in the frontal cortex. These inhibitory effects of ONO-2506 on K^+-evoked and AMPA-induced transmitter release suggest that ONO-2506 inhibits the propagation of epileptic discharges in *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice. The antiepileptic mechanisms of ONO-2506 include the combination of enhancement of both neuronal and glial inhibitory transmitter release during the resting stage and prevention of both neuronal and glial transmitter release during hyperexcitation.

In conclusion, the present study demonstrated that ONO-2506, a glial modulating agent, inhibited the spontaneous absence epileptic seizures of *Cacna1a*$^{tm2Nobs/tm2Nobs}$ mice without affecting MES or PTZ convulsion tests. ONO-2506 dose-dependently increased the basal release of inhibitory transmitters, GABA and kynurenic acid in the mPFC without affecting the levels of excitatory transmitter, L-glutamate; however, ONO-2506 dose-dependently inhibited the K^+-evoked releases of L-glutamate, GABA and kynurenic acid in the mPFC. ONO-2506 concentration-dependently increased the basal kynurenic acid release without affecting those of L-glutamate, D-serine or GABA from primary cultured astrocytes; whereas ONO-2506 inhibited AMPA-induced releases of all transmitters in a concentration-dependent manner. These results suggest that ONO-2506 enhances both neuronal and glial inhibitory transmitter releases without affecting excitatory transmitter release at the resting stage but inhibits both neuronal and glial transmitter releases induced by hyperactivation. Based on these properties, we conclude

that ONO-2506 is a potential antiepileptic agent, targeting glial cells.

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Conflict of interest

The authors state no conflict of interest.

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