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RESEARCH PAPER

Levetiracetam inhibits glutamate transmission through presynaptic P/Q-type calcium channels on the granule cells of the dentate gyrus

Chun-Yao Lee[1], Chih-Chuan Chen[2] and Horng-Huei Liou[1,2]

[1]*Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan, and* [2]*Department of Neurology, National Taiwan University Hospital, Taipei, Taiwan*

Background and purpose: Levetiracetam is an effective anti-epileptic drug in the treatment of partial and generalized seizure. The purpose of the present study was to investigate whether levetiracetam regulates AMPA and NMDA receptor-mediated excitatory synaptic transmission and to determine its site of action in the dentate gyrus (DG), the area of the hippocampus that regulates seizure activities.
Experimental approach: Whole-cell patch-clamp method was used to record the AMPA and NMDA receptor-mediated excitatory postsynaptic currents ($EPSC_{AMPA}$ and $EPSC_{NMDA}$) in the presence of specific antagonists, from the granule cells in the DG in brain slice preparations from young Wistar rats (60–120 g).
Key results: Levetiracetam (100 μM) inhibited both evoked $EPSC_{AMPA}$ and $EPSC_{NMDA}$ to an equal extent (80%), altered the paired-pulse ratio (from 1.39 to 1.25), decreased the frequency of asynchronous EPSC and prolonged the inter-event interval of miniature $EPSC_{AMPA}$ (from 2.7 to 4.6 s) without changing the amplitude, suggesting a presynaptic action of levetiracetam. The inhibitory effect of levetiracetam on evoked $EPSC_{AMPA}$ was blocked by ω-agatoxin TK (100 nM), a selective P/Q-type voltage-dependent calcium channel blocker, but not by nimodipine (10 μM) or ω-conotoxin (400 nM).
Conclusions and implications: These results suggest that levetiracetam modulated the presynaptic P/Q-type voltage-dependent calcium channel to reduce glutamate release and inhibited the amplitude of EPSC in DG. This effect is most likely to contribute to the anti-epileptic action of levetiracetam in patients.
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Keywords: levetiracetam; glutamate; EPSC; dentate gyrus; voltage-dependent calcium channel

Abbreviations: ACSF, artificial cerebrospinal fluid; BMB, bicuculline methobromide; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DG, dentate gyrus; DL-APV, DL-aminophosphonovaleric acid; EPSC, excitatory postsynaptic current; HVA-VDCCs, high-voltage-activated voltage-dependent Ca^{2+} channels; IEI, inter-event interval; PPR, paired-pulse ratio; QX-314, N-(2,6-Dimethylphenylcarbamoylmethyl) triethylammonium bromide; TTX, tetrodotoxin; ω-AGX, ω-agatoxin TK; ω-CTX, ω-conotoxin GVIA

Introduction

Levetiracetam ((S)-α-ethyl-2-exo-pyrrolidine acetamide) is a novel anti-epileptic drug in the treatment of partial and generalized seizure (Cereghino *et al.*, 2000; Weber and Beran, 2004) and is effective in wide-spectrum neuropsychiatric disorders (Muralidharan and Bhagwagar, 2006; Mula *et al.*, 2007). Several pharmacological properties of levetiracetam on ion channels have been reported, including blockade of high-voltage-activated voltage-dependent Ca^{2+} channels (HVA-VDCCs) to reduce neuronal depolarization in dissociated CA1 pyramidal neurons (Niespodziany *et al.*, 2001; Lukyanetz *et al.*, 2002; Pisani *et al.*, 2004), modulation of K^+ channels to stabilize the membrane potential (Madeja *et al.*, 2003; Lee *et al.*, 2008a) and enhancement of GABA- and glycine-gated currents to strengthen inhibitory tones (Rigo *et al.*, 2002), but little or no effects on voltage-dependent Na^+ and T-type Ca^{2+} channels (Zona *et al.*, 2001); channel and receptor nomenclature follows Alexander *et al.* (2008). Besides acting on ion channels, levetiracetam is reported to inhibit epileptiform discharges in human neocortical slices (Gorji *et al.*, 2002) and reduce neurotransmission in rat hippocampal slices (Yang *et al.*, 2007). In addition, levetiracetam is known to specifically bind to SV2A (Lynch *et al.*, 2004), a protein abundantly located on synaptic vesicles and interacting with

Correspondence: Dr Horng-Huei Liou, Department of Pharmacology and Neurology, College of Medicine, National Taiwan University, Room 1141, No 1, Sec 1, Jen-Ai Road, Taipei 100, Taiwan. E-mail: hhl@ntu.edu.tw
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synaptotagmin 1 to enhance neurotransmitter release (Janz *et al*., 1999). As levetiracetam is a novel anti-epileptic drug for partial and generalized seizures, outside the classical categorization, a greater understanding of the mechanisms by which levetiracetam suppresses seizures is relevant to the development of new therapeutic agents.

In the chronic animal model of temporal lobe epilepsy, dramatic pathological alterations in glutamate transmission in dentate gyrus (DG) are observed (Nadler, 2003). Levetiracetam significantly opposes pilocarpine-induced increase of neuronal excitability and counteracts seizure-induced alteration in excitatory amino acid concentration (Löscher *et al*., 1998; Klitgaard *et al*., 2003). These characteristics of levetiracetam indicate that reduction of neuronal excitability in epileptic states by levetiracetam is possibly due to down-regulation of glutamate transmission.

Glutamate is the most important excitatory neurotransmitter in the mammalian CNS. After release from presynaptic nerve terminals, glutamate binds to both pre- and postsynaptic metabotropic and ionotropic glutamate receptors. Activation of glutamate receptors maintains normal brain functions, but excessive levels of glutamate may cause neuronal damage and seizure activities. An elevated glutamate concentration in the hippocampus has been reported, before seizure onset and during seizures, in patients with epilepsy (During and Spencer, 1993). Reducing glutamate release (Cunningham *et al*., 2004) or blocking glutamate receptors (Kleckner *et al*., 1999) can decrease neuronal excitability and is recognized as an important mechanism of anti-epileptic drugs.

Although previous studies have revealed an action of levetiracetam on ion channels (Niespodziany *et al*., 2001; Lukyanetz *et al*., 2002; Madeja *et al*., 2003; Pisani *et al*., 2004; Lee *et al*., 2008a) and synaptic transmission (Gorji *et al*., 2002; Rigo *et al*., 2002; Yang *et al*., 2007), how levetiracetam influences glutamatergic transmission to reduce the excitability is still unclear. The aim of our present study was to investigate the effect of levetiracetam on the glutamatergic transmission system. We utilized whole-cell patch-clamp recording to investigate the action of levetiracetam on the excitatory postsynaptic currents (EPSC) in the granule cells in the DG, which controls the propagation of seizure activities (McNaughton and Barnes, 1997). We assessed the involvement of different VDCCs in the effects of levetiracetam on glutamatergic transmission. Our results suggested that levetiracetam decreased the amplitude of evoked EPSC (eEPSC) through interacting with presynaptic P/Q-type VDCC to reduce glutamate release, which is likely to contribute to the anti-epileptic action of levetiracetam observed in patients.

Methods

Brain slice preparation

All animal care and experimental procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* by the US National Institutes of Health and approved by the Animal Ethics Committee of National Taiwan University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Male Wistar rats (4–6 weeks, 60–120 g) were housed in polycarbonate boxes (four rats per box). They were maintained in a controlled atmosphere with a 12 h dark/light cycle (lights off at 7:00 PM), a temperature of 22 ± 2°C and 50–70% humidity with free access to pelleted feed and fresh tap water. The animals were decapitated under deep anaesthesia with halothane inhalation and the brains quickly moved to ice-cold oxygenated cutting solution containing (in mM): 125 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 5 $MgCl_2$, 26 $NaHCO_3$, 15 glucose and aerated with 95% O_2–5% CO_2 (to adjust pH value to 7.4 by 1N HCl and NaOH). Coronal brain slices (300 μm) containing DG were cut by a microslicer (DTK-1000, Dosaka, Kyoto, Japan) in cutting solution and then transferred to a holding chamber with artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$ and 15 glucose and aerated with 95% O_2–5%CO_2 (to adjust pH value to 7.4 by 1N HCl and NaOH). Brain slices were then maintained at room temperature (23 ± 2°C) for at least 1 h before recording.

Electrophysiological recordings

The brain slices were transferred to the recording chamber, held submerged and superfused continuously with ACSF at a flow rate of 1–2 mL·min^{-1} for recording at room temperature (23 ± 2°C). Evoked AMPA receptor-mediated EPSC ($eEPSC_{AMPA}$) were recorded in the ACSF containing bicuculline methobromide (BMB, 50 μM) and DL-aminophosphonovaleric acid (DL-APV, 50 μM) at a holding potential of –70 mV. Evoked NMDA receptor-mediated EPSC ($eEPSC_{NMDA}$) were recorded in Mg^{2+}-free ACSF in the presence of BMB (50 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and D-serine (10 μM) at a holding potential of –70 mV. Patch electrodes were pulled from standard-walled borosilicate glass capillaries (CSF-150, Warner Instrument, Hamden, CT, USA) by micropipette puller (P97, Sutter Instrument, Novato, CA, USA) with a resistance of 3–8 MΩ, then filled with a CsCl-based internal solution containing (in mM): 140 CsCl, 9 NaCl, 1 MgCl2, 1 EDTA, 10 HEPES, 5 QX-314 (N-(2,6-Dimethylphenylcarbamoylmethyl) triethylammonium bromide), 2 Mg-ATP and 0.3 Na-GTP (pH was adjusted to 7.3 with 1N CsOH). Granule cells in the DG were visually identified by using an upright infra-red microscope fitted with a water-immersion lens (Olympus, Tokyo, Japan). Whole-cell patch-clamp recording was made from these granule cells with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Series resistance and whole-cell capacitance were monitored continuously during all experiments and estimated by compensating (70–80%) for the fast transients evoked at the onset and offset of 8 ms, –5 mV voltage-command steps. The data were discarded if series resistance changed by more than 20%. To evoke EPSC, a stimulation glass pipette filled with 3 M NaCl solution was placed on the inner molecular layer of DG to elicit EPSC with a stimulator (S-48, Grass-Telefactor, W. Warwick, RI, USA) and isolation unit (A.M.P.I., Jerusalem, Israel). The stimulus duration was between 10 and 50 μs and the strength was between 5 and 20 V, with a frequency of 0.2 Hz. To avoid interference due to synaptic fatigue induced from low-frequency stimulation, we delivered continuous stimulation for more than 5 min (>60 stimuli) after accessing the whole-cell configuration to achieve stable eEPSC amplitudes before starting the recording.

eEPSC were quantified by measuring peak amplitudes of average responses. At least 3 min (about 36 events) were recorded to determine the average peak amplitudes in control and drug experiments. The responses of the drug were collected and analysed by stable eEPSC amplitudes after more than 5 min application of the drug (Lee *et al*., 2008b).

To measure paired-pulse ratio (PPR), test and conditioned pulses were delivered at 50 ms intervals. The PPR was then calculated by dividing the second EPSC by the first EPSC. To record AMPA receptor-mediated miniature EPSC ($mEPSC_{AMPA}$) in isolation, tetrodotoxin (TTX, 1 μM), BMB (50 μM) and DL-APV (50 μM) were added to the bath solution. Spontaneous synaptic currents were filtered at 5k Hz, digitized at 10k Hz and recorded directly to a computer hard disk using AxoScope 9.0 software (Axon Instruments). Access resistance was monitored at regular intervals throughout each study and recordings were discarded if it changed by >20%. The amplitude, inter-event interval (IEI) and frequency after application of drugs were then analysed. After an evoked stimulation, large amounts of Ca^{2+} flow through VDCCs into the presynaptic nerve terminals, increasing the probability of synaptic vesicle release. Therefore, after the first stimulation of $eEPSC_{AMPA}$, there were subsequently several small asynchronous EPSC (aEPSC). The aEPSC were collected in the period between 0.15 and 1 s after the first stimulation artefact of $eEPSC_{AMPA}$ and analysed to identify whether levetiracetam acted on presynaptic or postsynaptic sites (Lee *et al*., 2008b).

Data analysis and statistical procedures

Data were acquired by clampex 9.0 software (Axon Instruments) and stored in a hard disk. eEPSC were analysed by clampfit 9.0 (Axon Instruments). $mEPSC_{AMPA}$ were analysed by mini-analysis programme 6.0 (Synaptosoft Inc., Fort Lee, NJ, USA). Statistical differences were established by Student's *t*-test and Kolmogorov–Smirnov's test (K-S test). Data were expressed as mean ± SEM, and $P < 0.05$ was taken to indicate statistical significance.

Materials

All drugs used were bath applied. BMB, calphostin C (Cal-C), D-serine and TTX were purchased from Sigma (St. Louis, MO, USA). CNQX, chelerythrine chloride (CTC), DL-APV, H89 and nimodipine were purchased from Tocris (Bristol, UK). ω-Conotoxin GVIA (ω-CTX) and ω-agatoxin TK (ω-AGX) were purchased from Alomone Labs (Jerusalem, Israel). Levetiracetam was kindly supplied by UCB Pharma (Braine l'Alleud, Belgium). All drugs were dissolved in double distilled water, except CNQX, CTC, H89 and nimodipine, which were dissolved in 100% DMSO (the final concentration of DMSO in ACSF was less than 0.1%).

Results

Levetiracetam inhibited both evoked $EPSC_{AMPA}$ and $EPSC_{NMDA}$

Evoked $EPSC_{AMPA}$ and $EPSC_{NMDA}$ were obtained by whole-cell patch-clamp recordings from the granule cells in DG. The experimental traces show that levetiracetam significantly reduced the amplitude of $eEPSC_{AMPA}$ (Figure 1A) or of $eEPSC_{NMDA}$ (Figure 1B). The mean amplitudes of $eEPSC_{AMPA}$ and of $eEPSC_{NMDA}$ after application of levetiracetam were reduced equally over the concentration range used (10–500 μM, $n = 6$, $P < 0.01$) respectively (Student's *t*-test, Figure 1C). However, there was no significant difference in the effect of levetiracetam concentrations from 100 to 500 μM. Inhibition by levetiracetam was rapid and readily reversible (Figure 1D), with inhibition or recovery taking about 5 min. Series resistance was continuously monitored during the whole experiment to show the stability of the recording (Figure 1D).

Effects of levetiracetam on PPR indicated a presynaptic action

Paired-pulse ratio is a form of short-term plasticity, reflecting presynaptic release probability (Zucker, 1989). To determine the synaptic site of levetiracetam action, we investigated the effect of this compound on the PPR in the granule cells of DG. A typical example of eEPSCs in Figure 2A shows a change in the PPR resulting from the application of levetiracetam. Before levetiracetam perfusion, the PPR was 1.39 ± 0.05 at an interval of 50 ms. After application of levetiracetam (100 μM), the PPR was significantly decreased (Figure 2B, $n = 7$, $P < 0.01$ with Student's *t*-test).

The effect of levetiracetam on the IEI and amplitude of $mEPSC_{AMPA}$

We then examined the effects of levetiracetam (100 μM) on action potential-independent spontaneous $mEPSC_{AMPA}$, recorded in the presence of TTX (1 μM), BMB (50 μM) and DL-APV (50 μM) in DG (Figure 3A). As shown in Figure 3B, the mean IEI of $mEPSC_{AMPA}$ was significantly increased by levetiracetam (100 μM) treatment (Figure 3B, $P < 0.001$ with K-S test, $n = 9$). However, the mean amplitude of $mEPSC_{AMPA}$ was not changed (Figure 3C, $P > 0.05$ with Student's *t*-test, $n = 9$) after levetiracetam (100 μM).

The effect of levetiracetam on the frequency and amplitude of aEPSC

To further confirm the presynaptic effect of levetiracetam, we collected and analysed the aEPSC following the $eEPSC_{AMPA}$ (Figure 4A). Compared with $mEPSC_{AMPA}$, which arises randomly from synapses all over the cell, aEPSC comes from the evoked synapses in distal dendrites, in which most AMPA receptors are located. This characteristic of aEPSC helps to identify the presynaptic effects of levetiracetam (Bekkers and Clements, 1999). The amplitudes of these small EPSC were similar to $mEPSC_{AMPA}$ acquired under TTX perfusion and were analysed in the same way as $mEPSC_{AMPA}$ to identify the pre- or postsynaptic mechanisms. The mean frequency of aEPSC was significantly suppressed after levetiracetam (100 μM) treatment (Figure 4B, $P < 0.05$, Student's *t*-test, $n = 5$), although the mean amplitude of aEPSC was not changed (Figure 4C and D, $P > 0.05$ with K-S test, $n = 5$). This result was compatible with the effect of levetiracetam on $mEPSC_{AMPA}$, suggesting a presynaptic site of the action of levetiracetam.

Only ω-AGX but neither nimodipine nor ω-CTX prevented the effects of levetiracetam

Glutamate release from presynaptic nerve terminals is elicited by Ca^{2+} influx through VDCCs (Wu and Saggau, 1997). To

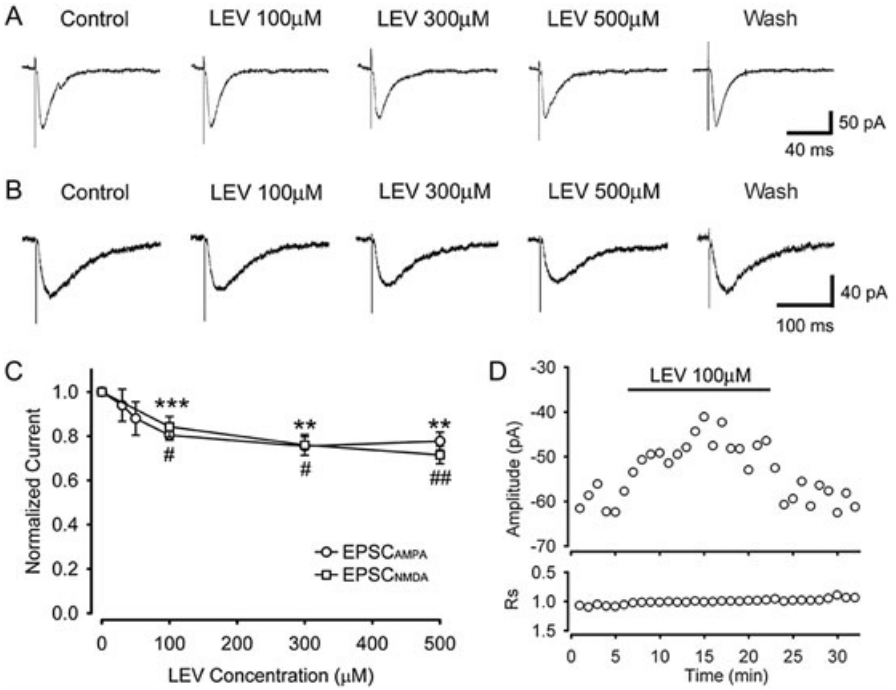


Figure 1 LEV inhibited the amplitudes of $eEPSC_{AMPA}$ and $eEPSC_{NMDA}$ in DG. (A) LEV inhibited the amplitudes of $eEPSC_{AMPA}$ in a concentration-dependent manner. The recording was performed in the presence of BMB (50 μM) and DL-APV (50 μM) with holding potential at −70 mV. (B) LEV inhibited the amplitudes of $eEPSC_{NMDA}$ in a concentration-dependent manner. The recording was performed in the presence of BMB (50 μM), CNQX (10 μM) and D-serine (10 μM) with holding potential at −70 mV in Mg^{2+}-free ACSF. (C) Summary data showing a concentration–response curve obtained at various concentration of LEV on the amplitude of $eEPSC_{AMPA}$ and $eEPSC_{NMDA}$. (**$P < 0.01$, ***$P < 0.001$ for $eEPSC_{AMPA}$ and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for $eEPSC_{NMDA}$, Student's *t*-test). (D) A continuous recording from a DG granule cell, illustrating onset of and recovery from, the action of LEV. The series resistance was continuously monitored during the whole experiment. ACSF, artificial cerebrospinal fluid; BMB, bicuculline methobromide; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DG, dentate gyrus; DL-APV, DL-aminophosphonovaleric acid; $eEPSC_{AMPA}$; evoked AMPA receptor-mediated EPSC; $eEPSC_{NMDA}$, evoked NMDA receptor-mediated EPSC; EPSC, excitatory postsynaptic current; LEV, levetiracetam.

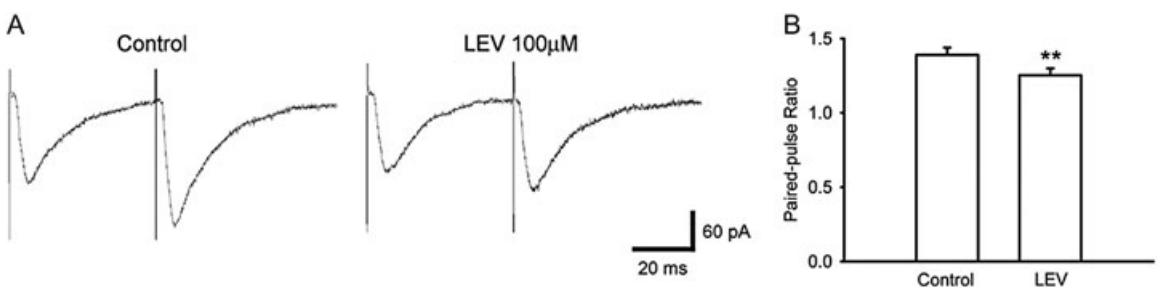


Figure 2 LEV altered the PPR of $eEPSC_{AMPA}$. (A) LEV (100 μM) altered the PPR of $eEPSC_{AMPA}$ at 50 ms interval. The recording was performed in the presence of BMB (50 μM) and DL-APV (50 μM) with holding potential at −70 mV. (B) Summary data showing the average PPR of $eEPSC_{AMPA}$ in control and LEV (100 μM) conditions (**$P < 0.01$, Student's *t*-test). The PPR was decreased after application of LEV (100 μM) ($n = 7$, **$P < 0.01$, Student's *t*-test). BMB, bicuculline methobromide; DL-APV, DL-aminophosphonovaleric acid; $eEPSC_{AMPA}$; evoked AMPA receptor-mediated EPSC; EPSC, excitatory postsynaptic current; LEV, levetiracetam; PPR, paired-pulse ratio.

clarify the presynaptic mechanisms of the reduction of glutamate release by levetiracetam, in terms of the VDCC involved, we used selective VDCC blockers: nimodipine (for L-type), ω-CTX (for N-type) and ω-AGX (for P/Q-type) (Cunningham *et al.*, 2004; Pisani *et al.*, 2004). Figure 5A shows that application of nimodipine (10 μM) caused a decrease in the $eEPSC_{AMPA}$ to 85 ± 5% of the control value ($P < 0.05$ with Student's *t*-test). The combination of levetiracetam (100 μM) and nimodipine (10 μM) further reduced the $eEPSC_{AMPA}$ amplitude (Figure 5A and B; $n = 6$, $P < 0.01$ to control and $P < 0.01$ to nimodipine alone, Student's *t*-test,). Similarly, when N-type VDCCs were blocked by ω-CTX (400 nM), $eEPSC_{AMPA}$ was reduced and combination with levetiracetam (100 μM) further reduced the amplitude of $eEPSC_{AMPA}$ (Figure 5C and D; $n = 6$, $P < 0.001$ to control and $P < 0.01$ to ω-CTX with Student's *t*-test,). Figure 5E shows that ω-AGX (100 nM) blocked the total $eEPSC_{AMPA}$ in DG granule cells ($P < 0.01$, Student's *t*-test), but now the addition of levetiracetam (100 μM) did not further inhibit the amplitude of $eEPSC_{AMPA}$ (Figure 5E and F; $n = 4$, $P < 0.05$ to control, $P > 0.05$ to ω-AGX, Student's *t*-test).

Another set of experiments used these compounds in reverse order, that is, levetiracetam (100 μM) first and then

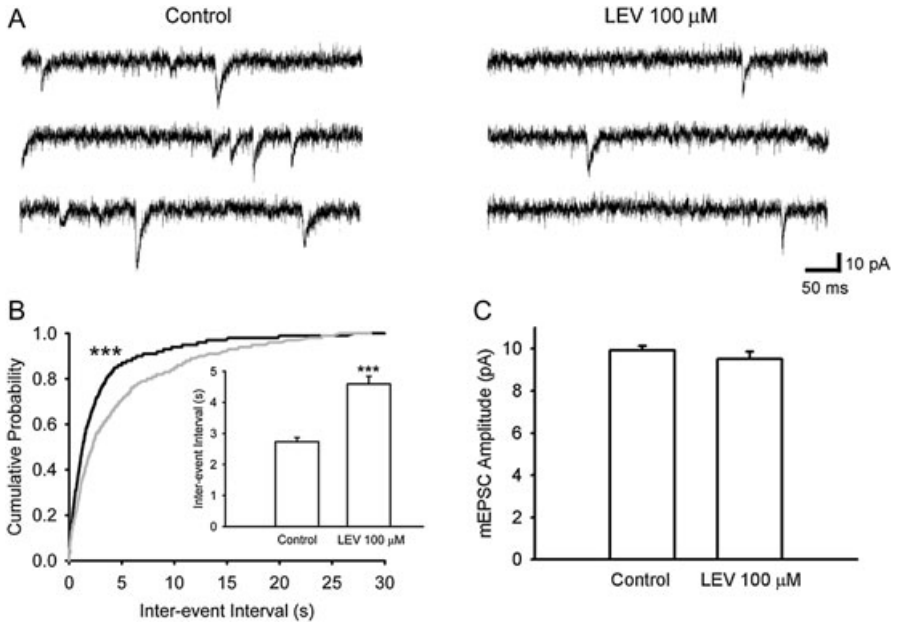


Figure 3 The effect of LEV (100 μM) on $mEPSC_{AMPA}$. (A) LEV (100 μM) inhibited the frequency but not the amplitude of $mEPSC_{AMPA}$. The recording was performed in the presence of BMB (50 μM), DL-APV (50 μM) and TTX (1 μM) with holding potential at –70 mV. (B) Summary data showing the mean IEI of $mEPSC_{AMPA}$ before and after LEV (100 μM) perfusion. LEV (100 μM) shifted the cumulative probability plot of $mEPSC_{AMPA}$ IEI to the right (***$P < 0.001$, K-S test). The inset plot summarizes the mean IEI of $mEPSC_{AMPA}$ before and after LEV (100 μM) perfusion. The mean IEI of $mEPSC_{AMPA}$ was significantly increased by LEV (100 μM, ***$P < 0.001$, Student's *t*-test). (C) Summary data showing the mean amplitude of $mEPSC_{AMPA}$ before and after LEV (100 μM) perfusion. The mean amplitude of $mEPSC_{AMPA}$ was not significantly affected by LEV (100 μM, $P > 0.05$, Student's *t*-test). BMB, bicuculline methobromide; DL-APV, DL-aminophosphonovaleric acid; EPSC, excitatory postsynaptic current; $mEPSC_{AMPA}$, AMPA receptor-mediated miniature EPSC; IEI, inter-event interval; LEV, levetiracetam; TTX, tetrodotoxin.

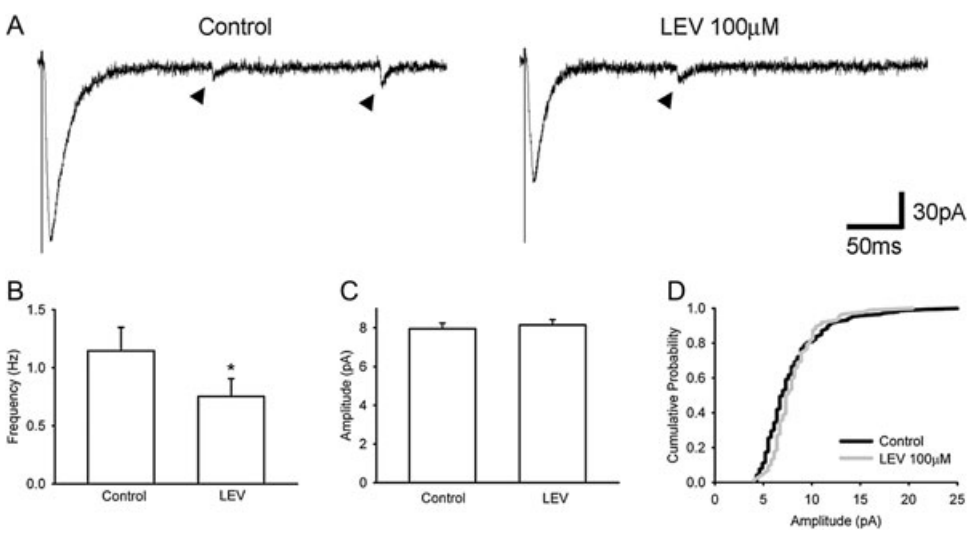
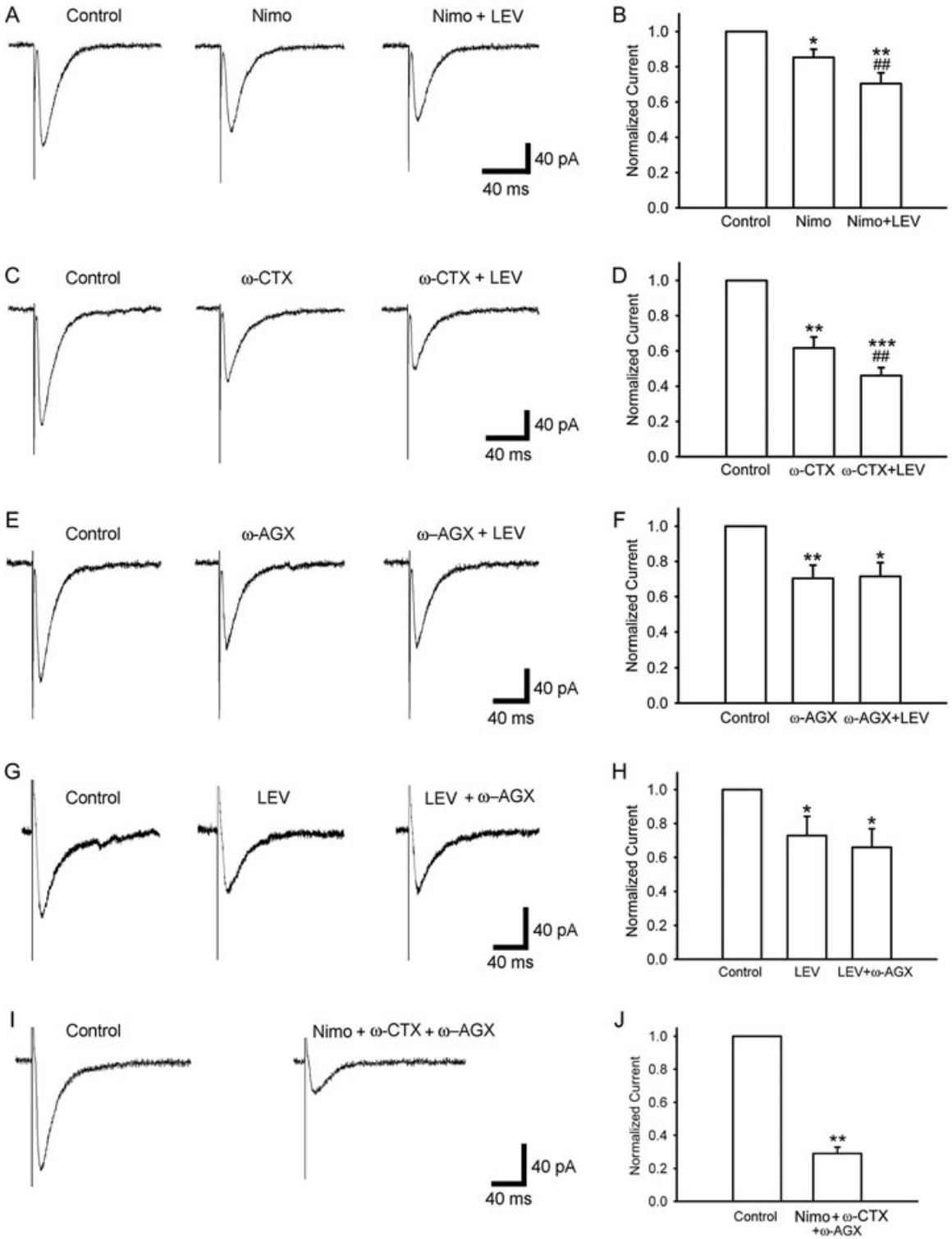


Figure 4 LEV inhibited the frequency of asynchronous excitatory postsynaptic currents (aEPSC). (A) aEPSC (indicated by the black arrows) was recorded between 0.15 and 1 s after the first stimulation artefact of evoked AMPA receptor-mediated EPSC in controlled conditions and during LEV (100 μM) perfusion. The recording was performed in the presence of bicuculline methobromide (50 μM) and DL-aminophosphonovaleric acid (50 μM) with the holding potential at –70 mV. (B) Summary data showing that the average frequency of aEPSC was reduced by LEV (100 μM, *$P < 0.05$, Student's *t*-test). (C) Summary data showing that the average amplitude of aEPSC was not significantly affected by LEV ($P > 0.05$, Student's *t*-test). (D) LEV (100 μM) did not significantly shift the cumulative probability plot of aEPSC amplitude, showing that the amplitude of aEPSC was not reduced by LEV ($P > 0.05$, K-S test).

adding the selective VDCC blocker. As shown in Figure 5G, levetiracetam (100 μM) blocked the total $eEPSC_{AMPA}$ in DG granule cells ($P < 0.05$ with Student's *t*-test); adding ω-AGX (100 nM) did not further inhibit the amplitude of $eEPSC_{AMPA}$ (Figure 5G and H; $n = 6$, $P < 0.05$ to control, $P > 0.05$ to levetiracetam, Student's *t*-test,). Combining all three selective VDCC blockers (nimodipine 10 μM, ω-CTX 400 nM and ω-AGX 100 nM) markedly reduced the amplitude of $eEPSC_{AMPA}$ but did not totally block the response (Figure 5I and J; $n = 3$, $P < 0.05$ to control, Student's *t*-test). The remaining



$eEPSC_{AMPA}$ might be due to the presence of other subtypes of VDCC (Evans and Zamponi, 2006).

We also tested the effects of ω-AGX on the PPR as this point is crucial for the hypothesis that levetiracetam selectively blocks P/Q-type VDCC. In contrast to the effects of levetiracetam, application of ω-AGX (100 nM) significantly increased the PPR over control values (Figure 6, $n = 4$, $P < 0.05$, Student's *t*-test).

Discussion and conclusions

Levetiracetam is a new anti-epileptic drug and its synaptic mechanism(s) are not known. In the present study, we addressed this issue by using whole-cell patch-clamp recordings. Our data demonstrated a presynaptic action of levetiracetam, based on the as following findings. Firstly, levetiracetam (100 μM) inhibited both $eEPSC_{AMPA}$ and $eEPSC_{NMDA}$ with

Figure 5 The effect of LEV (100 μM) in the presence of VDCC blockers. (A) Experimental traces showing nimodipine (Nimo; 10 μM) inhibited $eEPSC_{AMPA}$, and LEV (100 μM) further inhibited $eEPSC_{AMPA}$. The recording was performed in the presence of BMB (50 μM) and DL-APV (50 μM) with holding potential at −70 mV. (B) Nimodipine (10 μM) decreased the amplitude of $eEPSC_{AMPA}$ (*$P < 0.05$ to control). In the presence of nimodipine, LEV (100 μM) further decreased the amplitude of $eEPSC_{AMPA}$ ($n = 6$, **$P < 0.01$ to control and $^{\#\#}P < 0.01$ to nimodipine treatment, Student's *t*-test). (C) Experimental traces showing ω-CTX (400 nM) inhibited $eEPSC_{AMPA}$, and LEV (100 μM) further inhibited $eEPSC_{AMPA}$. The recording was performed in the presence of BMB (50 μM) and DL-APV (50 μM) with holding potential at −70 mV. (D) ω-CTX (400 nM) alone decreased the amplitude of $eEPSC_{AMPA}$ (**$P < 0.01$, Student's *t*-test). In the presence of ω-CTX, LEV (100 μM) further decreased the amplitude of $eEPSC_{AMPA}$ ($n = 6$, ***$P < 0.001$ to control and $^{\#\#}P < 0.01$ to ω-CTX treatment, Student's *t*-test). (E) Experimental traces showing ω-AGX (100 nM) inhibited $eEPSC_{AMPA}$, and LEV (100 μM) did not further inhibit $eEPSC_{AMPA}$. The recording was performed in the presence of BMB (50 μM) and DL-APV (50 μM) with holding potential at −70 mV. (F) ω-AGX (100 nM) decreased the amplitude of $eEPSC_{AMPA}$ (**$P < 0.01$, Student's *t*-test). In the presence of ω-AGX, the amplitude of $eEPSC_{AMPA}$ after LEV treatment was not further decreased ($n = 4$, *$P < 0.05$ to control, $P > 0.05$ to ω-AGX treatment, Student's *t*-test). (G) Experimental traces showing LEV (100 μM) inhibited $eEPSC_{AMPA}$, and ω-AGX (100 nM) did not further inhibit $eEPSC_{AMPA}$. The recording was performed in the presence of BMB (50 μM) and DL-APV (50 μM) with holding potential at −70 mV. (H) Summary data showing that LEV (100 μM) alone decreased the amplitude of $eEPSC_{AMPA}$ (*$P < 0.05$ with Student's *t*-test) and, after adding ω-AGX, the amplitude of $eEPSC_{AMPA}$ was not further decreased ($n = 6$, *$P < 0.05$ to control, $P > 0.05$ to LEV treatment, Student's *t*-test). (I) Experimental traces showing that combining three VDCC blockers (nimodipine 10 μM, ω-CTX 400 nM, ω-AGX 100 nM) inhibited $eEPSC_{AMPA}$. The recording was performed in the presence of BMB (50 μM) and DL-APV (50 μM) with holding potential at −70 mV. (J) Summary data showing the effects of combining three VDCC blockers (nimodipine 10 μM, ω-CTX 400 nM, ω-AGX 100 nM) on the amplitude of $eEPSC_{AMPA}$ ($n = 3$, **$P < 0.01$ to control, Student's *t*-test). BMB, bicuculline methobromide; DL-APV, DL-aminophosphonovaleric acid; $eEPSC_{AMPA}$, evoked AMPA receptor-mediated EPSC; EPSC, excitatory postsynaptic current; LEV, levetiracetam; VDCC, voltage-dependent Ca^{2+} channel; ω-AGX, ω-agatoxin TK; ω-CTX, ω-conotoxin GVIA.

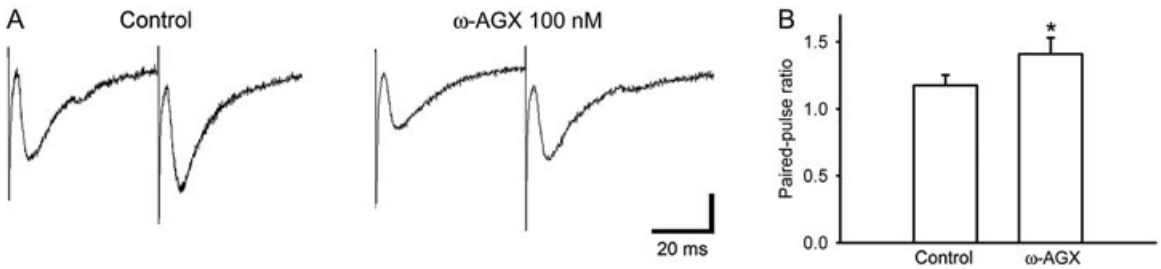


Figure 6 ω-AGX altered the PPR of $eEPSC_{AMPA}$. (A) Experimental traces showing that ω-AGX (100 nM) altered the PPR of $eEPSC_{AMPA}$ at 50 ms interval. The recording was performed in the presence of BMB (50 μM) and DL-APV (50 μM) with holding potential at −70 mV. (B) Summary data showing the average PPR of $eEPSC_{AMPA}$ in control conditions and after adding ω-AGX (100 nM). The PPR was clearly increased after ω-AGX (100 nM) application ($n = 4$, *$P < 0.05$, Student's *t*-test). BMB, bicuculline methobromide; DL-APV, DL-aminophosphonovaleric acid; $eEPSC_{AMPA}$, evoked AMPA receptor-mediated EPSC; EPSC, excitatory postsynaptic current; PPR, paired-pulse ratio; ω-AGX, ω-agatoxin TK.

similar concentration–response patterns indicating a presynaptic inhibition of glutamate release (Perkel and Nicoll, 1993). Then, PPR facilitation is an enhancement of the synaptic release caused by presynaptic activation of VDCC (Inchauspe *et al.*, 2007), Ca^{2+} current facilitation (Müller *et al.*, 2008), accumulation of intracellular Ca^{2+} and other mechanisms (Zucker, 1989). The levetiracetam-induced reduction of $eEPSC_{AMPA}$ was associated with a modification of the PPR, demonstrating that the action of levetiracetam was located at presynaptic sites to regulate transmitter release. Also, the frequency and amplitude of $mEPSC_{AMPA}$ reflects the changes in postsynaptic AMPA receptor-mediated responsiveness to presynaptic glutamate release (Redman, 1990). Levetiracetam reduced the frequency of $mEPSC_{AMPA}$ significantly, supporting a presynaptic action. Finally, levetiracetam induced the same pattern of amplitude and frequency in aEPSC as in $mEPSC_{AMPA}$, further confirming its presynaptic action. The strongest effect of levetiracetam appeared to be on the $mEPSC_{AMPA}$ frequency, consistent with the results of Carunchio *et al.* (2007). Thus, it appears that this parameter would be the most sensitive and appropriate to assess the mechanisms of levetiracetam. Taken together, we concluded that levetiracetam inhibited glutamate release, presynaptically, to suppress synaptic transmission between the perforant pathway and the granule cells in the DG.

Levetiracetam binds specifically to the vesicle protein, SV2A, located in a presynaptic site and involved in neurotransmitter release (Lynch *et al.*, 2004). An *in vitro* extracellular recording has reported the site of action of levetiracetam to be located on the presynaptic nerve terminal in the CA1 region of the hippocampus (Yang *et al.*, 2007). Although earlier work suggested that the site of action of levetiracetam is the presynaptic terminal, the evidence for this suggestion is indirect. To further investigate the presynaptic mechanism of levetiracetam in the DG, the involvement of three types of HVA-VDCCs was assessed with specific channel blockers. Nimodipine, ω-CTX and ω-AGX inhibited the amplitude of $eEPSC_{AMPA}$, indicating that L-, N- and P/Q-type VDCCs all contributed to the Ca^{2+} transient in the presynaptic nerve terminal. Among three HVA-VDCC antagonists, only ω-AGX blocked the inhibitory action of levetiracetam, demonstrating that levetiracetam inhibited glutamate release mainly through affecting P/Q-type VDCCs in the DG (Figure 5). Similar results were also obtained by using these compounds in reversed order (Figure 5G and H), strengthening the hypothesis that the effects of levetiracetam were exerted via the presynaptic nerve terminal and the P/Q-type VDCCs.

Levetiracetam has been found to act on HVA-VDCCs and reduce neuronal excitability in isolated neuronal cells. Niespodziany *et al.* (2001) found levetiracetam inhibited

L- and N-type VDCC in CA1 pyramidal neurons in hippocampal slices. Lukyanetz *et al.* (2002) reported levetiracetam blocked N-type VDCC in isolated CA1 hippocampal neurons. Pisani *et al.* (2004) showed levetiracetam modulated epileptiform activity through both N- and P/Q-type VDCC in isolated neocortical pyramidal neurons. However, these findings could not address the question of the synaptic mechanism of levetiracetam. HVA-VDCCs not only regulate membrane potential and neuronal excitability in isolated neurons, but they also augment synaptic activity and neurotransmitter release in synapses. After depolarization of the presynaptic nerve terminal, Ca^{2+} flows in through HVA-VDCCs, binds to nearby Ca^{2+}-binding proteins, initiates the transmitter releasing machinery and, finally, triggers the release of glutamate (Augustine, 2001). Different subtypes of HVA-VDCCs are co-localized at presynaptic terminals in different proportions in different brain areas. They can coexist at a single release site as a heterogeneous mixture and contribute jointly to the local Ca^{2+} transient that triggers transmitter release (Reid *et al.*, 2003). The P/Q-type VDCC is reported to be the predominant Ca^{2+} channel involved in triggering neurotransmitter release in the perforant path from the entorhinal cortex to the DG (Qian and Noebels, 2001), which is compatible with our pharmacological analysis that levetiracetam preferentially acts on P/Q-type VDCCs to decrease the amount of Ca^{2+} influx, reduce glutamate release and thus modulate synaptic activity in the DG.

Several other anti-epileptic drugs have shown to block P/Q-type VDCCs and reduce glutamate release. Gabapentin and pregabalin block P/Q-type VDCCs to reduce glutamate release, presynaptically, in entorhinal cortical slice preparations (Cunningham *et al.*, 2004). Gabapentin also inhibited synaptic transmission in spinal cord dorsal horn through blockade of P/Q-type VDCCs (Bayer *et al.*, 2004). Lamotrigine reduces Ca^{2+} influx in cortical neurons by inhibiting P/Q-type VDCC (Stefani *et al.*, 1996). From our investigation, the inhibition of $eEPSC_{AMPA}$ by levetiracetam, through an action on P/Q-type VDCCs, may therefore participate in its anti-epileptic property. Anti-epileptic drugs have different effects, depending on the region of the brain, reflecting the diversity of individual synapses in terms of the ion channels, receptors, pre- and postsynaptic cell types and surrounding environment (Craig and Boudin, 2001). The possibility that levetiracetam could act on different subtypes of HVA-VDCC in different brain regions needs further investigation.

Paired-pulse ratio is a form of short-term plasticity, associated with the probability of presynaptic release. Reduction in presynaptic release is typically correlated with an increase in PPR, while enhancement of release is typically correlated with a reduction in PPR (Zucker, 1989). We found levetiracetam decreased the PPR (Figure 2), which was an 'atypical' response, if it reduced presynaptic release. However, Yang *et al.* (2007) reported that with 10 and 80 Hz stimulation (at a stimulation interval of 100 ms and 12.5 ms), treatment with levetiracetam (10 and 100 μM) reduced the $EPSP_2/EPSP_1$ ratio. According to our results and the previous findings, levetiracetam's action on presynaptic VDCCs to regulate the Ca^{2+} pool and reduce glutamate release may possibly be use-dependent (Yang *et al.*, 2007). This possibility is supported by the finding that levetiracetam blocked VDCCs to attenuate the repetitive firing in cultured cortical neurons (Pisani *et al.*, 2004).

It is relevant to note that ω-AGX decreased the amplitude of $eEPSC_{AMPA}$ concomitant with an increase in PPR (Figure 6), while levetiracetam decreased PPR (Figure 2), implying that they cannot be acting by the same mechanism, despite the apparent block of the levetiracetam effect by ω-AGX. This discrepancy in altered PPR, between levetiracetam and ω-AGX, might be explained as follows. Firstly, levetiracetam could be use-dependently modulating presynaptic VDCC to reduce glutamate release, as mentioned earlier (Yang *et al.*, 2007), although our results at this stage do not provide enough data to support this hypothesis, and further investigations are necessary to prove this point of view. Another explanation is that levetiracetam might act on P/Q-type VDCCs by affecting the channel's biophysical properties (open or closed states), which is distinct from the action of ω-AGX (Mori *et al.*, 1996). Finally, an effect independent of inhibiting VDCC could be involved in the mechanism of action of levetiracetam.

A recent study found that levetiracetam modulated AMPA receptor-mediated currents on cultured cortical neurons (Carunchio *et al.*, 2007). According to their results, levetiracetam (200 μM) inhibits both the amplitude (to 0.86) and the frequency (to 0.82) of $mEPSC_{AMPA}$ recording on the cultured cortical neurons. Both the previous and our present results showed a decrease in the frequency of $mEPSC_{AMPA}$, which supported a presynaptic effect. On the other hand, we did not observe any decrease in the amplitude of $mEPSC_{AMPA}$ in our current study. This discrepancy may be due to the heterogeneous composition of AMPA receptors and consequently to the interaction of levetiracetam with some specific receptor subunits. The heterogeneous sensitivity to levetiracetam, observed in different brain areas, indicates that levetiracetam is able to interact with some AMPA subunits that are differently expressed in the brain (Ozawa *et al.*, 1998). Another difference is that we used freshly prepared brain slices with an intact synaptic system, rather than a culture of neuronal cells, in which such synapses might not be found.

Clinical effective doses of levetiracetam (1–3 g per day) result in plasma levels between 35 and 100 μM and peak levels between 90 and 250 μM (Patsalos, 2000). Levetiracetam reached maximum inhibitory effect on eEPSC in the granule cells of the DG at 100 μM, which is close to the plasma levels after clinical effective doses. At these concentrations, levetiracetam may reduce glutamatergic synaptic transmission (to 80%), which is consistent with previous findings that levetiracetam inhibited the calcium current by ~18% (Lukyanetz *et al.*, 2002). It may be an advantage for levetiracetam to exert its anti-epileptic property without significant overall suppression of the CNS, which would be expected from extensive suppression of glutamatergic transmission.

The DG is considered as a gate of the hippocampus and plays a critical role in regulating the propagation of seizure activities from the entorhinal cortex to the hippocampus (McNaughton and Barnes, 1997). It also acts as a frequency-dependent filter for paroxysmal activity spreading from the entorhinal cortex to the hippocampus (Stringer *et al.*, 1989). A decreased excitability of the DG has proved effective in the treatment of epilepsy (Lee *et al.*, 2008b). Levetiracetam tends to inhibit the neuronal excitability in this brain area, which is

crucial to the regulation of seizures, thus preventing the initiation and spread of seizures. The pharmacological profile of levetiracetam in our work is consistent with previous findings that levetiracetam is effective in the chronic animal model of temporal lobe epilepsy *in vivo* (Loscher *et al*., 1998; Klitgaard *et al*., 2003) and *in vitro* (Gorji *et al*., 2002). The efficacy of levetiracetam in this DG-based epilepsy model implies that levetiracetam may directly act on the DG to regulate excitatory synaptic transmission in order to exert its anti-epileptic effects.

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Conflicts of interest

None.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to receptors and channels (GRAC), 3rd edition (2008 revision). *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Augustine GJ (2001). How does calcium trigger neurotransmitter release? *Curr Opin Neurobiol* **11**: 320–326.

Bayer K, Ahmadi S, Zeilhofer HU (2004). Gabapentin may inhibit synaptic transmission in the mouse spinal cord dorsal horn through a preferential block of P/Q-type Ca^{2+} channels. *Neuropharmacology* **46**: 743–749.

Bekkers JM, Clements JD (1999). Quantal amplitude and quantal variance of strontium-induced asynchronous EPSCs in rat dentate granule neurons. *J Physiol* **516**: 227–248.

Carunchio I, Pieri M, Ciotti MT, Albo F, Zona C (2007). Modulation of AMPA receptors in cultured cortical neurons induced by the anti-epileptic drug levetiracetam. *Epilepsia* **48**: 654–662.

Cereghino JJ, Biton V, Abou-Khalil B, Dreifuss F, Gauer LJ, Leppik I (2000). Levetiracetam for partial seizures. Results of a double-blind, randomized clinical trial. *Neurology* **55**: 236–242.

Craig AM, Boudin H (2001). Molecular heterogeneity of central synapses: afferent and target regulation. *Nat Neurosci* **4**: 569–578.

Cunningham MO, Woodhall GL, Thompson SE, Dooley DJ, Jones RS (2004). Dual effect of gabapentin and pregabalin on glutamate release at rat entorhinal stnapses *in vitro*. *Eur J Neurosci* **20**: 1566–1576.

During MJ, Spencer DD (1993). Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. *Lancet* **341**: 1607–1610.

Evans RM, Zamponi GW (2006). Presynaptic Ca^{2+} channels – integration centers for neuronal signaling pathways. *Trends Neurosci* **29**: 617–624.

Gorji A, Hohling JM, Madeja M, Straub H, Kohling R, Tuxhorn I *et al*. (2002). Effect of levetiracetam on epileptiform discharges in human neocortical slices. *Epilepsia* **43**: 1480–1487.

Inchauspe CG, Forsythe ID, Uchitel OD (2007). Changes in synaptic transmission properties due to the expression of N-type calcium channels at the calyx of Held synapse of mice lacking P/Q-type calcium channels. *J Physiol* **584**: 835–851.

Janz R, Goda Y, Geppert M, Missler M, Sudhof TC (1999). SV2A and SV2B function as redundant Ca^{2+} regulators in neurotransmitter release. *Neuron* **24**: 1003–1016.

Kleckner NW, Glazewski JC, Chen CC, Moscrip TD (1999). Subtype-selective antagonism of N-methyl-D-aspartate receptors by felbamate: insights into the mechanism of action. *J Pharmacol Exp Ther* **289**: 886–894.

Klitgaard H, Matagne A, Grimee R, Vanneste-Goemaere J, Margineanu DG (2003). Electrophysiological, neurochemical and regional effects of levetiracetam in the rat pilocarpine model of temporal lobe epilepsy. *Seizure* **12**: 92–100.

Lee CH, Lee CY, Tsai TS, Liou HH (2008a). PKA-mediated phosphorylation is a novel mechanism for levetiracetam, an antiepileptic drug, activating ROMK1 channels. *Biochem Pharm* **76**: 225–235.

Lee CY, Fu WM, Chen CC, Su MJ, Liou HH (2008b). Lamotrigine inhibits postsynaptic AMPA receptor and glutamate release in the dentate gyrus. *Epilepsia* **49**: 889–897.

Löscher W, Hönack D, Rundfeldt C (1998). Antiepileptogenic effects of the novel anticonvulsant levetiracetam (ucb L059) in the kindling model of temporal lobe epilepsy. *J Pharmacol Exp Ther* **284**: 474–479.

Lukyanetz EA, Shkryl VM, Kostyuk PG (2002). Selective blockade of N-type calcium channels by levetiracetam. *Epilepsia* **43**: 9–18.

Lynch BA, Lambeng N, Nocka K, Kensel-Hammes P, Bajjalieh SM, Matagne A *et al*. (2004). The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam. *Proc Natl Acad Sci USA* **101**: 9861–9866.

McNaughton BL, Barnes CA (1997). Physiological identification and analysis of dentate granule cell responses to stimulation of the medial and lateral perforant pathways in the rat. *J Comp Neurol* **175**: 439–454.

Madeja M, Margineanu DG, Gorji A, Siep E, Boerrigter P, Klitgaard H *et al*. (2003). Reduction of voltage-operated potassium currents by levetiracetam: a novel antiepileptic mechanism of action? *Neuropharmacology* **45**: 661–671.

Mori Y, Mikala G, Varadi G, Kobayashi T, Koch S, Wakamori M *et al*. (1996). Molecular pharmacology of voltage-dependent calcium channels. *Jpn J Pharmacol* **72**: 83–109.

Mula M, Pini S, Cassano GB (2007). The role of anticonvulsant drugs in anxiety disorders: a critical review of the evidence. *J Clin Psychopharmacol* **27**: 263–272.

Müller M, Felmy F, Schneggenburger R (2008). A limited contribution of Ca^{2+} current facilitation to paired-pulse facilitation of transmitter release at the rat calyx of Held. *J Physiol* **586**: 5503–5520.

Muralidharan A, Bhagwagar Z (2006). Potential of levetiracetam in mood disorders: a preliminary review. *CNS Drugs* **20**: 969–979.

Nadler JV (2003). The recurrent mossy fiber pathway of the epileptic brain. *Neurochem Res* **28**: 1649–1658.

Niespodziany I, Klitgaard H, Margineanu DG (2001). Levetiracetam inhibits the high-voltage-activated Ca(2+) current in pyramidal neurones of rat hippocampal slices. *Neurosci Lett* **306**: 5–8.

Ozawa S, Kamiya H, Tsuzuki K (1998). Glutamate receptors in the mammalian central nervous system. *Prog Neurobiol* **54**: 581–618.

Patsalos PN (2000). Pharmacolkinetic profile of levetiracetam: toward ideal characteristics. *Pharmacol Ther* **85**: 77–85.

Perkel DJ, Nicoll RA (1993). Evidence for all-or-none regulation of neurotransmitter release: implications for long-term potentiation. *J Physiol* **471**: 481–500.

Pisani A, Bonsi P, Martella G, De Persis C, Costa C, Pisani F *et al*. (2004). Intracellular calcium increase in epileptiform activity: modulation by levetiracetam and lamotrigine. *Epilepsia* **45**: 719–728.

Qian J, Noebels JL (2001). Presynaptic Ca^{2+} channels and neurotransmitter release at the terminal of a mouse cortical neuron. *J Neurosci* **21**: 3721–3728.

Redman S (1990). Quantal analysis of synaptic potential in neurons of the central nerve system. *Physiol Rev* **70**: 165–198.

Reid CA, Bekkers JM, Clements JD (2003). Presynaptic Ca^{2+} channels: a functional patchwork. *Trends Neurosci* **26**: 683–687.

Rigo JM, Hans G, Nguyen L, Rocher V, Belachew S, Malgrange B *et al*. (2002). The anti-epileptic drug levetiracetam reverses the inhibition by negative allosteric modulators of neuronal GABA- and glycine-gated currents. *Br J Pharmacol* **136**: 659–672.

Stefani A, Spadoni F, Siniscalchi A, Bernardi G (1996). Lamotrigine inhibits Ca^{2+} currents in cortical neurons: functional implications. *Eur J Pharmacol* **307**: 113–116.

Stringer JL, Williamson JM, Lothman EW (1989). Induction of paroxysmal discharges in the dentate gyrus: frequency dependence and relationship to afterdischarge production. *J Neurophysiol* **62**: 126–135.

Weber S, Beran RG (2004). A pilot study of compassionate use of levetiracetam in patients with generalised epilepsy. *J Clin Neurosci* **11**: 728–731.

Wu LG, Saggau P (1997). Presynaptic inhibition of elicited neurotransmitter release. *Trends Neurosci* **20**: 204–212.

Yang XF, Weisenfeld A, Rothman SM (2007). Prolonged exposure of levetiracetam reveals a presynaptic effect on neurotransmission. *Epilepsia* **48**: 1861–1869.

Zona C, Niespodziany I, Marchetti C, Klitgaard H, Bernardi G, Margineanu DG (2001). Levetiracetam does not modulate neuronal voltage-gated Na^{+} and T-type Ca^{2+} currents. *Seizure* **10**: 279–286.

Zucker R (1989). Short-term synaptic plasticity. *Annu Rev Neurosci* **12**: 13–31.