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The anti-epileptic drug levetiracetam reverses the inhibition by negative allosteric modulators of neuronal GABA- and glycine-gated currents

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1 In this study *in vitro* and *in vivo* approaches were combined in order to investigate if the anti-epileptic mechanism(s) of action of levetiracetam (LEV; Keppra®) may involve modulation of inhibitory neurotransmission.

2 GABA- and glycine-gated currents were studied *in vitro* using whole-cell patch-clamp techniques applied on cultured cerebellar granule, hippocampal and spinal neurons. Protection against clonic convulsions was assessed *in vivo* in sound-susceptible mice. The effect of LEV was compared with reference anti-epileptic drugs (AEDs): carbamazepine, phenytoin, valproate, clonazepam, phenobarbital and ethosuximide.

3 LEV contrasted the reference AEDs by an absence of any direct effect on glycine-gated currents. At high concentrations, beyond therapeutic relevance, it induced a small reduction in the peak amplitude and a prolongation of the decay phase of GABA-gated currents. A similar action on GABA-elicited currents was observed with the reference AEDs, except ethosuximide.

4 These minor direct effects contrasted with a potent ability of LEV ($EC_{50} = 1-10$ μM) to reverse the inhibitory effects of the negative allosteric modulators zinc and β-carbolines on both $GABA_A$ and glycine receptor-mediated responses.

5 Clonazepam, phenobarbital and valproate showed a similar ability to reverse the inhibition of β-carbolines on GABA-gated currents. Blockade of zinc inhibition of GABA responses was observed with clonazepam and ethosuximide. Phenytoin was the only AED together with LEV that inhibited the antagonism of zinc on glycine-gated currents and only clonazepam and phenobarbital inhibited the action of DMCM.

6 LEV (17 mg kg^{-1}) produced a potent suppression of sound-induced clonic convulsions in mice. This protective effect was significantly abolished by co-administration of the β-carboline FG 7142, from a dose of 5 mg kg^{-1}. In contrast, the benzodiazepine receptor antagonist flumazenil (up to 10 mg kg^{-1}) was without any effect on the protection afforded by LEV.

7 The results of the present study suggest that a novel ability to oppose the action of negative modulators on the two main inhibitory ionotropic receptors may be of relevance for the anti-epileptic mechanism(s) of action of LEV.

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Abbreviations: βCCB, *n*-butyl-β-carboline-3-carboxylate; AED, antiepileptic drug; DGC, dentate granule cells; DIV, days *in vitro*; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; FG 7142, *n*-methyl-β-carboline-3-carboxamide; LEV, levetiracetam; TLE, temporal lobe epilepsy

Introduction

Levetiracetam (LEV; Keppra®) is a novel antiepileptic drug (AED) devoid of anticonvulsant activity in the two classical screening models for AEDs, the maximal electroshock and pentylenetetrazole seizure tests, in both mice and rats (Löscher & Hönack, 1993; Klitgaard *et al*., 1998). Likewise, testing in other seizure paradigms in rodents only showed a weak anti-convulsant activity in threshold tests involving acute electrical or chemical stimulation (Löscher & Hönack, 1993) and only modest protection was observed against acute seizures induced by submaximal stimulation (Gower *et al*., 1992). This contrasts with a potent seizure protection in animal models of chronic epilepsy, involving genetically determined epileptic (Gower *et al*., 1992; 1995) and kindled animals (Klitgaard *et al*., 1998; Löscher & Hönack, 1993). This highly selective action in animals with 'epileptogenic brains' markedly distinguishes LEV from classical and other new AEDs which have nearly equipotent effects in normal and genetic/kindled animals (Klitgaard *et al*., 1998). Furthermore, this selective effect of LEV appears to derive from a novel electrophysiological mode of action which

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involves inhibition of epileptiform patterns of neuronal hypersynchronization (Margineanu & Klitgaard, 2000) without interference with normal neuronal excitability (Birnstiel *et al*., 1997). Another feature that appears to distinguish LEV from other AEDs is a potent ability to inhibit the development of kindling in experimental animals (Löscher *et al*., 1998). Indeed, this suggests that LEV may possess anti-epileptogenic properties in humans. The molecular mechanism(s) behind the unique profile of LEV still remains obscure although a brain-specific binding site has been described for LEV (Noyer *et al*., 1995).

The main molecular mechanisms accepted as accounting for the anti-epileptic action of classical AEDs relate to inhibition of neuronal excitatory (i) Na^+ channels; (ii) low-voltage activated (T-type) Ca^{2+} channels or (iii) facilitation of inhibitory GABAergic neurotransmission (Macdonald & Kelly, 1995). Recent studies have shown that LEV is devoid of impact on the voltage-activated tetrodotoxin-sensitive inward Na^+ current in cultured rat cortical neurons and on the low-voltage-activated (T-type) Ca^{2+} current in pyramidal neurons from rat hippocampal slices (Zona *et al*., 2001). This suggests that inhibition of voltage-operated Na^+ or T-type Ca^{2+} channels is not involved in the anti-epileptic mechanism of LEV. In contrast, conflicting results exist with respect to the effect of LEV on the GABAergic system. The seizure protection afforded by LEV in audiogenic-susceptible mice was unaffected by the benzodiazepine receptor antagonist flumazenil (Klitgaard *et al*., 1998). Likewise, a lack of effect on GABA levels and the enzymatic activities of GABA transaminase and glutamic acid decarboxylase was reported from a neurochemical study on mouse brain (Sills *et al*., 1997) and LEV was without effect on GABA transport and metabolism in rat astrocyte cultures (Fraser *et al*., 1999). This contrasts with one report showing that systemic administration of high doses of LEV induces short-lasting alterations in GABA metabolism and turnover in some brain regions (Löscher *et al*., 1996). Thus, it still remains to be determined to what extent facilitation of GABAergic inhibition may contribute to the anti-epileptic mechanism of LEV.

Besides GABA, glycine is another major inhibitory neurotransmitter. Receptors with recognition sites for these two amino acids ($GABA_A$ and the strychnine-sensitive glycine receptors) constitute the two main inhibitory receptor systems in the mammalian central nervous system (Mehta & Ticku, 1999; Rabow *et al*., 1995; Barry *et al*., 1999; Betz *et al*., 1999), the former being mainly associated with the telencephalon and the latter with the spinal cord. Whereas a vast number of studies have associated activation of the $GABA_A$ receptor with anti-epileptic effects (for reviews, see Bernard *et al*., 2000; Gale, 1992), only scarce information exists with respect to the glycine receptor, probably due to its anatomical location in the spinal cord. However, glycine receptors are also found in the upper part of the neuraxis, particularly in the hippocampus where they seem to participate in neurotransmission (Fatima-Shad & Barry, 1995; McMahon & Chattipakorn, 2000; Ye *et al*., 1999; Yoon *et al*., 1998), thus involving them in the control of neuronal excitability. Other evidence for a potential relevance of the glycine receptor for antiepileptic drug effects relates to the experimental drug milacemide. This compound was shown to suppress seizures in experimental animals and to possess anti-epileptic activity in humans, an effect which was believed to derive, at least partly, from its conversion to glycine within the brain (Roba *et al*., 1986). This suggests that both $GABA_A$ and strychnine-sensitive glycine receptors may mediate a cellular inhibition relevant for an anti-epileptic action of AEDs.

In the present study, we examined to what extent the anti-epileptic effect of LEV may relate to direct and/or indirect effects on the two main ionotropic inhibitory receptor systems in the brain, i.e. the $GABA_A$ and the strychnine-sensitive glycine receptors. First, GABA- and glycine-gated currents were studied *in vitro* using whole-cell patch-clamp techniques applied on cultured hippocampal, cerebellar granule and spinal neurons. Secondly, the modulation of LEV's seizure protection by ligands interacting with the $GABA_A$ or the strychnine-sensitive glycine receptors was assessed *in vivo* in sound-susceptible mice.

Methods

Neuronal cell cultures

Spinal cord neurons, hippocampal neurons and cerebellar granule cells were obtained, respectively, from 13- and 16-day-old mouse embryos and from 7-day-old rat pups using methods fully described previously (Withers & St John, 1997; Lefebvre *et al*., 1987; Leprince *et al*., 1989). Briefly, spinal cords, hippocampi or cerebella were carefully dissected and freed of meninges. They were then cut into small fragments which were incubated in trypsin (0.25%) and deoxyribonuclease (0.1%) in $Ca^{2+}-Mg^{2+}$ free salt solution for 25 min at 37°C. This was followed by a wash with their culture medium which consisted, for spinal cord and hippocampal neurons, of Dulbecco's modified minimum essential medium (Gibco, Gent, Belgium) supplemented with glucose (6 g l^{-1}, final concentration), 5% (v v^{-1}) foetal calf serum (FCS, Gibco), 10% (v v^{-1}) horse serum (HS, Gibco) and the N1 supplement (insulin 5 μg ml^{-1}, transferrin 5 μg ml^{-1}, progesterone 20 nM; putrescine 100 μM, selenium 30 nM) (Bottenstein & Sato, 1979). For cerebellar granule cells, this culture medium consisted of a minimum essential medium (Gibco; for which the potassium concentration was raised to 25 mM and the sodium concentration decreased in equimolar amount) supplemented with glucose (6 g l^{-1}, final concentration), pyruvate (1 mM), bovine insulin (5 μg ml^{-1}) and 10% (v v^{-1}) horse serum. Dissociation was obtained by up and down aspirations through the large tip of a 5 ml plastic pipette put on the bottom of a conical glass tube. The resulting cell suspension was filtered through a 15 and a 40 μm nylon sieve, respectively for cerebellar granule cells and for spinal cord or hippocampal neurons. Fifty microliters of the cell suspension was seeded on polyornithine (0.1 mg ml^{-1} in distilled water) coated glass coverslips (10 mm diameter) in the centre of 35 mm plastic Petri dishes (NUNC, Roskilde, Denmark) at a concentration of 2.5×10^6 cerebellar and 1.25×10^6 spinal cord and hippocampal cells per ml. The medium was renewed once weekly and cells were used for electrophysiological recordings after 3–5 days *in vitro* (DIV) for cerebellar granule cells and spinal cord neurons and after 7–14 DIV for hippocampal neurons, except when stated otherwise.

Drugs

LEV was synthesized in the chemical laboratories of UCB S.A. GABA, strychnine, bicuculline, picrotoxin, sodium valproate, carbamazepine and ethosuximide were purchased from Sigma (U.S.A.), methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), *n*-butyl-β-carboline-3-carboxylate (βCCB), *n*-methyl-β-carboline-3-carboxamide (FG7142) and 4-chlorodiazepam were from RBI (U.S.A.). Sodium phenobarbital was from Federa (Belgium), phenytoin from Bios (Belgium), both clonazepam and flumazenil from Hoffman-La Roche (Switzerland), glycine from UCB Pharma (Belgium) and pentylenetetrazol from Acros Organics (Belgium).

Electrophysiology

Solutions and electrodes For recording of membrane currents, the cells were transferred to the stage of an inverted Hoffman contrast microscope and maintained at room temperature (20–25°C) in a recording chamber which was continuously perfused permitting application of drugs.

The extracellular perfusion solution contained (mM): NaCl, 116.0; D-glucose, 11.1; KCl, 5.4; $CaCl_2 \times 2H_2O$, 1.8; $MgCl_2 \times 6H_2O$, 2.0; HEPES, 10.0; pH 7.2. The reagents diluted in the external solution were applied by a rapid microperfusion system (SPS-8, List-Medical, Germany).

Recording electrodes were made from borosilicate capillaries (Hilgenberg, Germany) using a Flaming-Brown microelectrode puller (Model P97, Sutter Instruments Co). Recording pipettes (10–20 MΩ) were filled with a solution containing (mM): KCl, 130.0; $CaCl_2 \times 2H_2O$, 1.0; D-glucose, 11.1; EGTA, 10.0; Na- and Mg-ATP, 5.0; HEPES, 10.0; pH 7.4.

Data recording and processing Voltage-clamp recordings were performed with a Bio-Logic RK-400 patch-clamp amplifier using the tight-seal whole-cell recording configuration (Hamill *et al*., 1981). Series resistances were in the range 10–20 MΩ and were electronically compensated (up to 85–90%). Current traces were digitized and stored on a Pentium personal computer. Control of drug application, data acquisition and data analysis were achieved using an ITC-16 acquisition board (Instrutech Corporation, U.S.A.) and the TIDA for Windows software (HEKA Elektronik, Pfölz, Germany). Curve fitting was made using built-in features of TIDA software (single exponential fitting for the determination of the decay time constant of desensitizing currents) or GraphPAD Prism software (U.S.A.) (sigmoidal dose-response curves).

Seizure testing in mice

Animals Genetically sound-susceptible female mice weighing 20–30 g were used. The animals were kept on a 1200 to 1200 light/dark cycle with lights on at 0600 h and were housed at a temperature maintained at 20–21°C and at humidity of about 40%. The mice were housed in groups of 10 per cage ($38 \times 26 \times 14$ cm) and had free access to standard pellet food and water before random assignment to experimental groups of 10 animals. All experimental protocols were approved by the local Ethical Committee for the use of laboratory animals in Belgium.

Audiogenic seizures Sound-susceptible mice were first subjected to a preselection test and only mice in which a tonic convulsion was provoked by an acoustic stimulus were retained. Audiogenic seizures were induced the following day by a 90 dB, 10 to 20 kHz acoustic stimulus for 30 s and the incidence of wild running, clonic and tonic convulsions was recorded. LEV and the reference AEDs clonazepam, phenobarbital, valproate, carbamazepine, phenytoin and ethosuximide were all administered i.p. 30 min before testing, at a dose inducing 60–100% protection against sound-induced clonic convulsions (dose-response curves were determined in preliminary experiments). The effect of FG 7142 (i.p., 15 min before testing) and flumazenil (p.o., 15 min before testing) was determined on the seizure protection afforded by LEV and the reference AEDs.

Solutions and dosing LEV, sodium valproate, ethosuximide and sodium phenobarbital were all dissolved in 0.9% saline. Carbamazepine, phenytoin, clonazepam, flumazenil and FG 7142 were all suspended in 0.9% saline containing 0.1% Tween 80. The injection volume was 10 ml kg^{-1} body weight.

Statistical analysis

Except when stated otherwise, the results are expressed as mean and standard error of the mean (s.e.mean).

For the *in vitro* experiments, statistical significance of the data was assessed from a Student's *t*-test or analysis of variance (ANOVA) followed by Dunnett's multiple comparisons post-tests when significance was reached.

For the *in vivo* experiments, a Fisher test was applied for determining a significant effect on the protection afforded by LEV and the reference AEDs against clonic convulsions in sound-susceptible mice.

In all experiments, differences were considered statistically significant when $P<0.05$.

Results

Characterization of inhibitory amino acid-induced currents in cultured CNS neurons

In our recording conditions, cultured cerebellar granule cells (3–23 DIV), hippocampal (4–21 DIV) and spinal cord (3–5 DIV) neurons had respective mean membrane potentials of -51.0 ± 0.8 mV ($n=258$), -42.3 ± 0.6 mV ($n=328$) and -60.2 ± 0.8 mV ($n=299$). All patch-clamp experiments were performed in the whole-cell configuration. When recorded in the current-clamp mode, all cultured neurons displayed action potentials and fast inactivating inward and delayed rectifying outward currents when they were voltage-clamped. In the voltage-clamp mode at a holding potential of -70 mV, we recorded inward currents elicited by bath application (10 s) of GABA on cerebellar granule cells or hippocampal neurons and of glycine on spinal cord neurons or hippocampal neurons. These inward currents reversed around 0 mV, consistent with them being carried mainly by Cl^- ions, the calculated Nernst equilibrium potential of chloride being 0.6 mV. GABA currents were blocked in the presence of either bicuculline methiodide or picrotoxin, and glycine responses were inhibited by strychnine (see effects of

half-maximal inhibitory concentrations, IC_{50} of these drugs in Figures 3, 4 and 6), indicating the activation of ionotropic $GABA_A$ and glycine receptors, respectively. No cross activation of $GABA_A$ and glycine receptors were observed in any cell type as bicuculline did not affect glycine currents nor did strychnine modify GABA responses (data not shown). NMDA receptors were not involved in glycine-induced responses of hippocampal or spinal cord neurons as these responses were insensitive to MK801 (data not shown).

The inhibitory amino acids-mediated currents were only studied in those cells showing sodium-like inward and potassium-like outward voltage-activated currents, with a membrane potential more hyperpolarized than -30 mV and which did not show any rundown of their transmitter response in successive applications. The plot of relative current amplitudes (I/I_{max}; I_{max} being the current elicited by 1 mM transmitter) as a function of GABA or glycine concentrations were best fitted by sigmoidal curves yielding half-maximal effective concentrations (EC_{50}) for GABA of 34.5 ± 15.0 μM ($n=7$) in cerebellar granule cells and of 12.2 ± 3.9 μM ($n=6$) in hippocampal neurons and for glycine of 77.7 ± 13.6 μM ($n=8$) in spinal cord neurons and of 108.1 ± 3.0 μM ($n=10$) in hippocampal neurons.

Levetiracetam (LEV) and other reference anti-epileptic drugs (AEDs) are weak modulators of ionotropic inhibitory receptors

LEV only induces a minor modulation of GABA responses, which is development- and cell type-dependent In cultures of cerebellar granule cells, LEV, up to a concentration of 1 mM, marginally but significantly affected the peak amplitude of the current induced by 50 μM GABA. At least in young (<5 DIV) cultures, a slowing of the decay phase of the current (desensitization) was noticeable (Figure 1A,B). When individual current traces were fitted to an exponential of the form: $I_{GABA} = a - b \times e^{-\frac{t}{\tau}}$, a doubling of the time constant τ could be measured. This effect was only significant for concentrations of LEV above 100 μM and seemed dose-dependent ($EC_{50} = 203.7 \pm 6.8$ μM; $n=5-28$). In older (>5 DIV) cerebellar cultures, 100 μM LEV-induced inhibition of GABA currents peak amplitude was comparable ($88.8 \pm 2.6\%$ of control; $n=39$) to the one observed in younger cultures (86.5 ± 1.9; $n=10$). In contrast, the enhancement of the decay time constant was significantly smaller ($P<0.01$; two-way ANOVA) in the older cultures ($134.3 \pm 9.2\%$ of control at 100 μM LEV; $n=29$) than in the younger ones ($176.6 \pm 21.2\%$; $n=28$). Since the $GABA_A$ receptor subunit composition changes as cerebellar granule cells become older in culture (Gao & Fritschy, 1995; Kaneda *et al*., 1995; Mathews *et al*., 1994), this developmental stage-dependent difference in the effect of LEV suggests that the stoechiometry of the $GABA_A$ receptor influences the reported effect of LEV. This is further supported by observations in another cell type having a different $GABA_A$ receptor subunit composition, namely hippocampal neurons in culture (Figure 1C,D). In this cell type, LEV had a more pronounced effect on the peak amplitude ($78.2 \pm 3.1\%$ of control ($n=24$) at 100 μM LEV) but induced less slowing of the desensitization of the GABA currents ($134.2 \pm 11.8\%$ of control ($n=16$) at 100 μM LEV) compared to cerebellar granule neurons.

Figure 2A,B compare the effect of a clinically relevant concentration of LEV (100 μM) with six reference AEDs (10 μM carbamazepine (Holopainen *et al*., 2001), 1 μM clonazepam (Gibbs *et al*., 1997), 100 μM phenobarbital (Bruckner & Heinemann, 2000), 50 μM phenytoin (Jahromi *et al*., 2000; White *et al*., 1997), 1 mM valproate (Bruckner & Heinemann, 2000), 1 mM ethosuximide (Leresche *et al*., 1998)) on the peak amplitude and the decay time constant of currents elicited by 20 μM GABA in cultured hippocampal neurons. In these experimental conditions (EC_{50} GABA), all AEDs revealed a marginal reduction in the amplitude of GABA-gated currents, while almost all of them, except ethosuximide, also enhanced the decay time constant of the GABA responses.

LEV is the only AED without impact on glycine-induced currents in spinal cord neurons The potential modulation by LEV on glycine currents was determined by co-application with 100 μM glycine onto spinal cord and hippocampal neurons in culture. As shown in Figure 1E–H, LEV neither affected the amplitude of glycine currents nor did it change the time constant of the desensitization phase of the current, even up to a high concentration of 1 mM both in spinal cord and hippocampal neurons. In contrast, all tested reference AEDs significantly decreased the amplitude of glycine-gated currents in spinal cord neurons (Figure 2C).

LEV reverses the inhibition of GABA-gated currents induced by β-carbolines and zinc

A potential interaction between LEV and negative allosteric modulators of the $GABA_A$ receptor was studied on GABA-induced currents in cultured hippocampal and cerebellar granule neurons. Data from these experiments are summarized in Figures 3 and 4. $GABA_A$ receptor negative allosteric modulators were applied at their IC_{50} which were determined in separate experiments and the reversibility of the effects of the different modulators that were used was always assessed (data not shown). LEV was ineffective in opposing the competitive inhibitory effect of bicuculline methiodide (Figures 3A,B, 4A,B) and in decreasing the inhibition of GABA responses induced by picrotoxin, the prototypic $GABA_A$ receptor channel blocker (Figures 3C,D, 4C,D). In contrast, LEV reversed the inhibitory effect of DMCM on GABA-elicited currents in hippocampal neurons (Figure 3E,F). The EC_{50} of LEV that antagonizes DMCM inhibition was 3.1 ± 0.1 μM ($n=5-20$). This effect was also observed with other β-carbolines, namely FG 7142 (Figure 3G,H) and βCCB–a putatively endogenous compound–(data not shown), and on cerebellar granule neurons (Figure 4E,F). In this latter cell type, LEV also reversed the inhibitory effect of Ro 5-4864, an atypical and controversial negative allosteric modulator of the $GABA_A$ receptor related to the benzodiazepine family (Gee *et al*., 1988; Lan *et al*., 1989; Puia *et al*., 1989). The amplitude of GABA currents was $60.7 \pm 2.7\%$ of control currents ($n=8$) at 25 μM of Ro 5-4864, while in the presence of 100 μM LEV, it returned to control levels ($96.8 \pm 6.6\%$, $n=6$).

Zinc is the only known physiological inhibitor of ligand-gated chloride channels which, moreover, has been postulated to be implied in the pathogenesis of epilepsy (Banerjee *et al*., 1999; Coulter, 2000). Therefore, we also

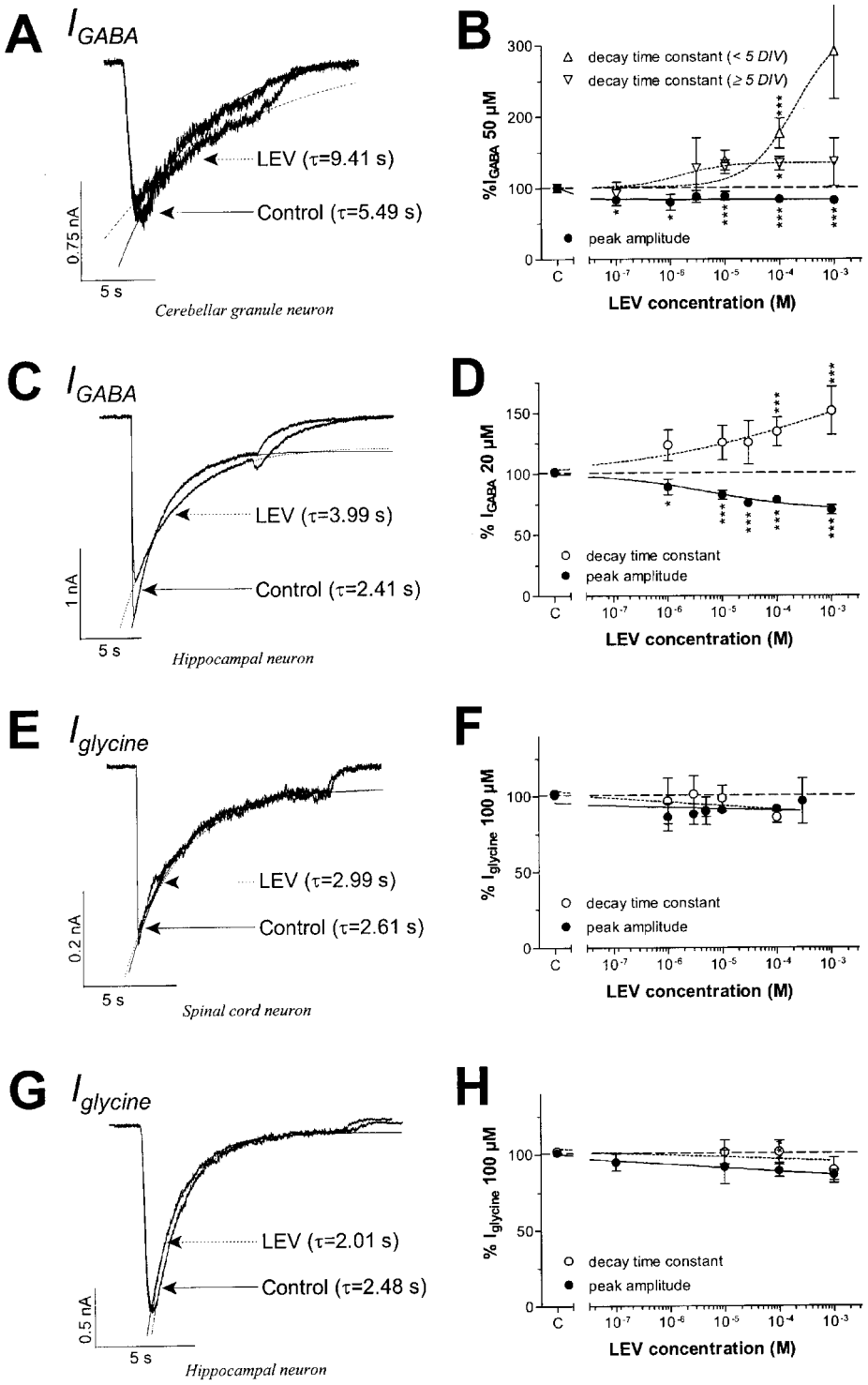

Figure 1 Effect of levetiracetam (LEV) on GABA- and glycine-induced currents. *Left panel*: Three to twenty-three days *in vitro* (DIV) cerebellar granule neurons (A), 7–16 DIV hippocampal neurons (C and G) and 3–5 DIV spinal cord neurons (E) were perfused for 10 s with GABA (20 or 50 μM) or glycine (100 μM) as indicated, alone or in combination with LEV (25 s, 100 μM). A 60 s period was allowed for the washout of drugs. For each recording, a curve fitting procedure was applied to the desensitization phase using a single exponential (see text) and allowing to calculate a decay time constant (τ). *Right panel*: Currents evoked by 50 μM GABA for cerebellar granule neurons (B) and by 20 μM for hippocampal neurons (D) or by 100 μM glycine for spinal cord neurons (F) or for hippocampal neurons (H) (concentrations which elicit a half-maximal response in each cell types) were recorded in the presence of increasing LEV concentrations. Results are expressed as percentage of inhibitory amino acid-induced currents peak amplitudes or decay time constants in the absence of LEV (mean$\pm$s.e.mean, $n=7-135$ for A, 6–71 for D, 5–20 for F and 6–17 for H). ***$P<0.001$ and *$P<0.05$ using ANOVA followed by Dunnett's multiple comparisons post-tests.

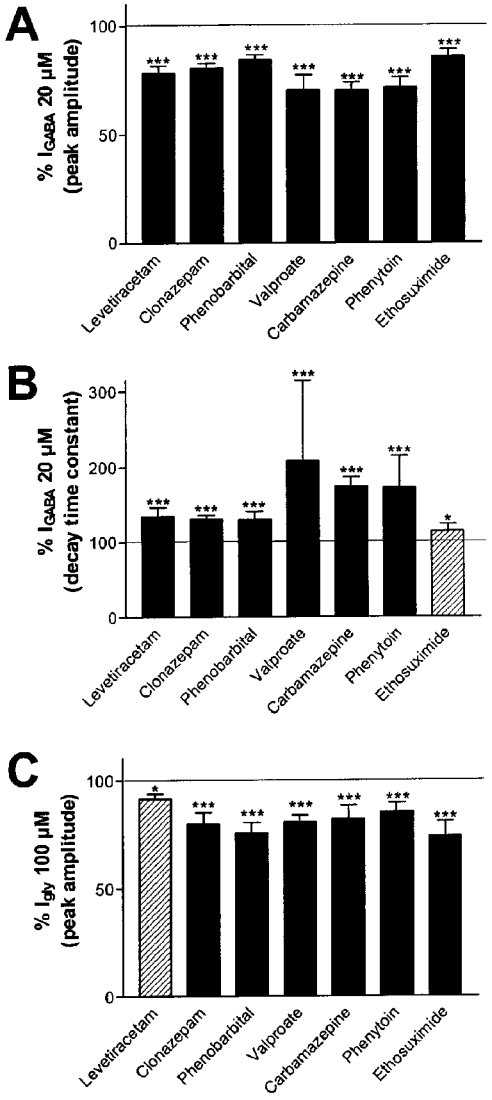

Figure 2 Effect of reference anti-epileptic drugs (AEDs) on GABA- and glycine-induced currents. Currents evoked by 20 μM GABA for hippocampal neurons (A and B) or by 100 μM glycine for spinal cord neurons (C) were recorded in the presence of reference AEDs (100 μM levetiracetam, 10 μM carbamazepine, 1 μM clonazepam, 100 μM phenobarbital, 50 μM phenytoin, 1 mM valproate and 1 mM ethosuximide). Results are expressed as percentage of inhibitory amino acid-induced currents peak amplitudes (A and C) or decay time constants (B) in the absence of AEDs (mean$\pm$s.e.mean, $n=5-12$). ***$P<0.001$ and *$P<0.05$ using Student's t-test analysis. It has to be mentioned that the marginal significance ($P<0.05$) reported in (C) for LEV, which contrasts with Figures 1G and H ($P>0.05$), is due to the use of a different statistical test.

measured the interaction between LEV and zinc on GABA currents in various experimental paradigms. Figure 3I,J show that, in hippocampal neurons, LEV completely reversed the inhibition by zinc of GABA-evoked currents. This effect was rather 'on-off' than truly dose-dependent, affording complete reversal at 30 μM. This antagonism by LEV of zinc-induced inhibition of GABA responses was not observed in cerebellar granule cells (Figure 4G,H), further suggesting that LEV's modulation of $GABA_A$ receptor function depends on the subunit composition of the receptor.

Finally, LEV also antagonized the inhibitory effect on GABA responses in cerebellar granule neurons of a still unidentified endogenous astroglia-derived negative allosteric modulator of the $GABA_A$ receptor (Rigo *et al*., 1994) (91.5$\pm$5.2% of control currents ($n=4$) in the presence of the astroglia-derived factor and 100 μM LEV compared to 52.9$\pm$7.0% ($n=4$) with this factor alone).

Compared to LEV, only clonazepam showed an ability to reverse both DMCM- and zinc-induced inhibition of GABA responses (Figure 5A,B). This AED is known to interact with the $GABA_A$ receptor (Macdonald & Kelly, 1995; Moshe, 2000). Phenobarbital and valproate, which are also modulators of the $GABA_A$ receptor, also opposed DMCM inhibition and, surprisingly, ethosuximide also reversed zinc inhibition.

LEV reverses inhibition of glycine-elicited currents induced by β-carbolines and zinc

Given the antagonism of LEV towards zinc and β-carboline inhibition of GABA responses and the fact that zinc and β-carbolines are known to modulate glycine receptors (Harvey *et al*., 1999; Smart *et al*., 1994; Rigo *et al*., 1998), we explored the interaction between LEV and negative allosteric modulators of the glycine receptor on glycine-gated currents. As for $GABA_A$ receptor modulators, glycine receptor inhibitors were applied at their IC_{50} determined in separate experiments.

As shown in Figure 6A,B, LEV did not modify the inhibition by strychnine, a competitive antagonist at the glycine site of the glycine receptor, of glycine-gated currents in cultured spinal cord neurons. In contrast, LEV completely abolished the inhibitory effects of the two other tested negative allosteric modulators of the glycine receptor. Figure 6C, D clearly demonstrate the potent and effective antagonism of LEV against DMCM-induced inhibition of glycine currents. This effect was maximal from LEV concentrations as low as 1 μM. This antagonism was also observed against βCCB-induced inhibition of glycine currents (data not shown). Compared to the effect on the $GABA_A$ receptor, LEV's reversal of zinc inhibition of glycine receptor-mediated responses was as potent, i.e. as complete, but with a greater efficacy i.e. with an EC_{50} of 0.7$\pm$0.1 μM ($n=5-1$) for spinal cord neurons and of 0.04$\pm$0.02 μM ($n=5-23$) for hippocampal neurons (Figure 6E–H).

As shown in Figure 7, clonazepam and phenobarbital mimicked LEV in antagonizing DMCM inhibition of glycine currents. In contrast, phenytoin was the only reference AEDs able to oppose zinc-induced inhibition.

FG 7142, but not flumazenil, antagonized the seizure protection afforded by LEV

The ability of LEV (17 mg kg^{-1} i.p) to protect against clonic convulsions in audiogenic susceptible mice was abolished by a co-administration with 10 mg kg^{-1} of the β-carboline FG 7142 (Figure 8A). In contrast, co-administration of a dose of 5 mg kg^{-1} of the benzodiazepine receptor antagonist flumazenil was without any effect on the protection afforded by LEV (Figure 8B).

Co-administration of FG 7142 (5 mg kg^{-1}) also abolished the protective effect of clonazepam, valproate, phenobarbital

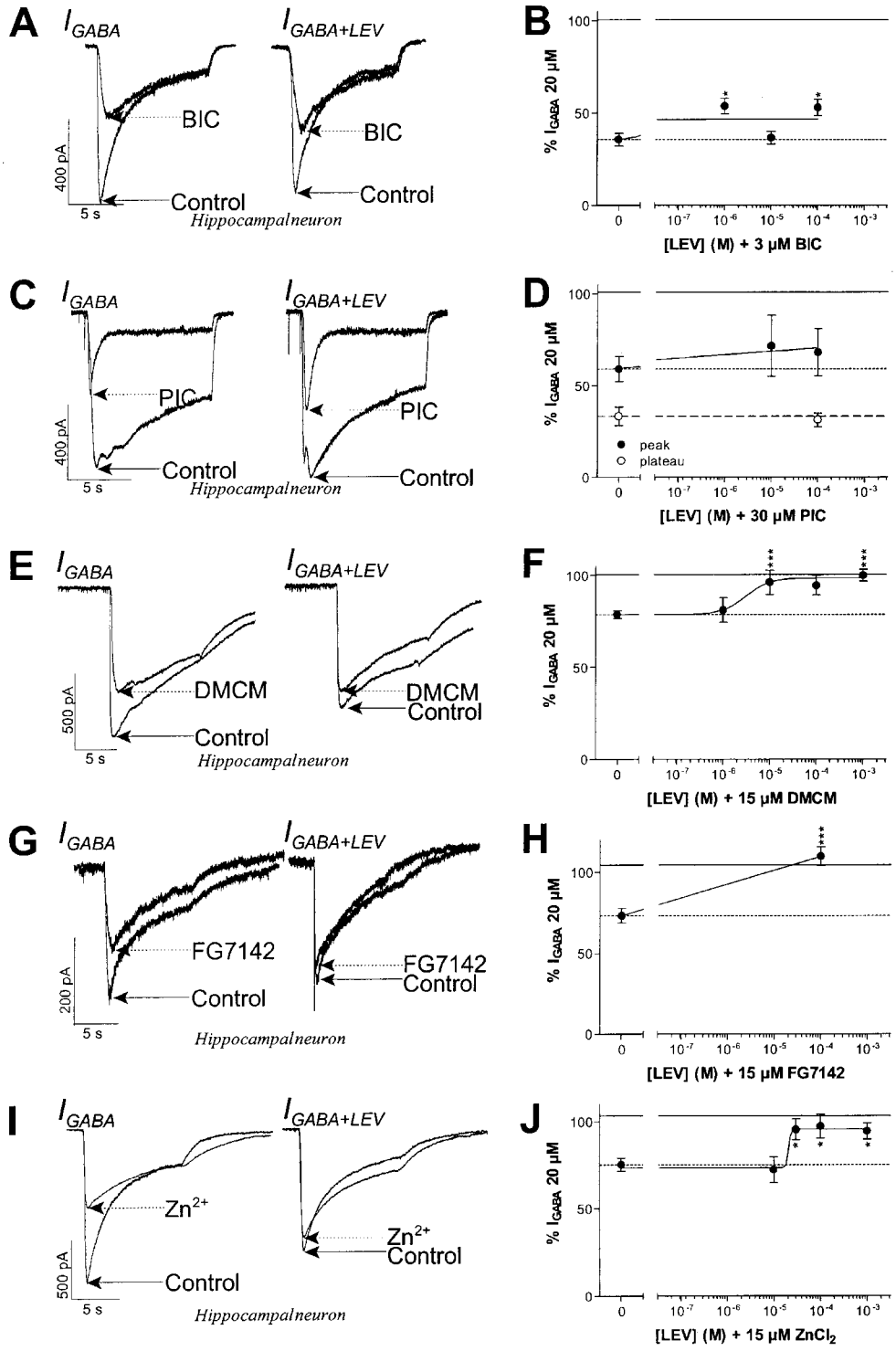

Figure 3 Interaction between LEV and negative allosteric modulators of the $GABA_A$ receptor (hippocampal neurons). *Left panel*: Seven to sixteen DIV hippocampal neurons were perfused for 10 s with 20 μM GABA alone (left traces) or in combination with LEV (right traces) (25 s, 100 μM) (controls) and negative allosteric modulators of the $GABA_A$ receptor (25 s, half-maximal inhibitory concentrations: 3 μM bicuculline (BIC), 30 μM picrotoxin (PIC), 15 μM DMCM, 15 μM FG7142 and 15 μM zinc). A 60 s period was allowed for the washout of drugs (90 s in the case of picrotoxin or β-carbolines). *Right panel*: Currents elicited by 20 μM GABA in the presence of the indicated negative allosteric modulator of the $GABA_A$ receptor were recorded for increasing LEV concentrations. Results are expressed as percentage of GABA-induced currents peak amplitudes in the absence of any drug (solid horizontal line) (mean $\pm$ s.e.mean, $n = 4-16$ for B, 5–41 for D, 5–34 for F, 16–33 for H and 6–36 for J). The horizontal dashed line represents the level of the GABA response in the presence of the $GABA_A$ receptor inhibitor alone. The curve fitting procedure yielded a half-maximal effective LEV concentration of 3.1 ± 0.2 μM for DMCM- and 20.1 ± 19.8 μM for zinc-induced inhibitions of GABA currents. $^{***}P<0.01$ and $^{*}P<0.05$ using ANOVA followed by Dunnett's multiple comparisons post-tests (Student's *t*-test analysis was used for FG7142).

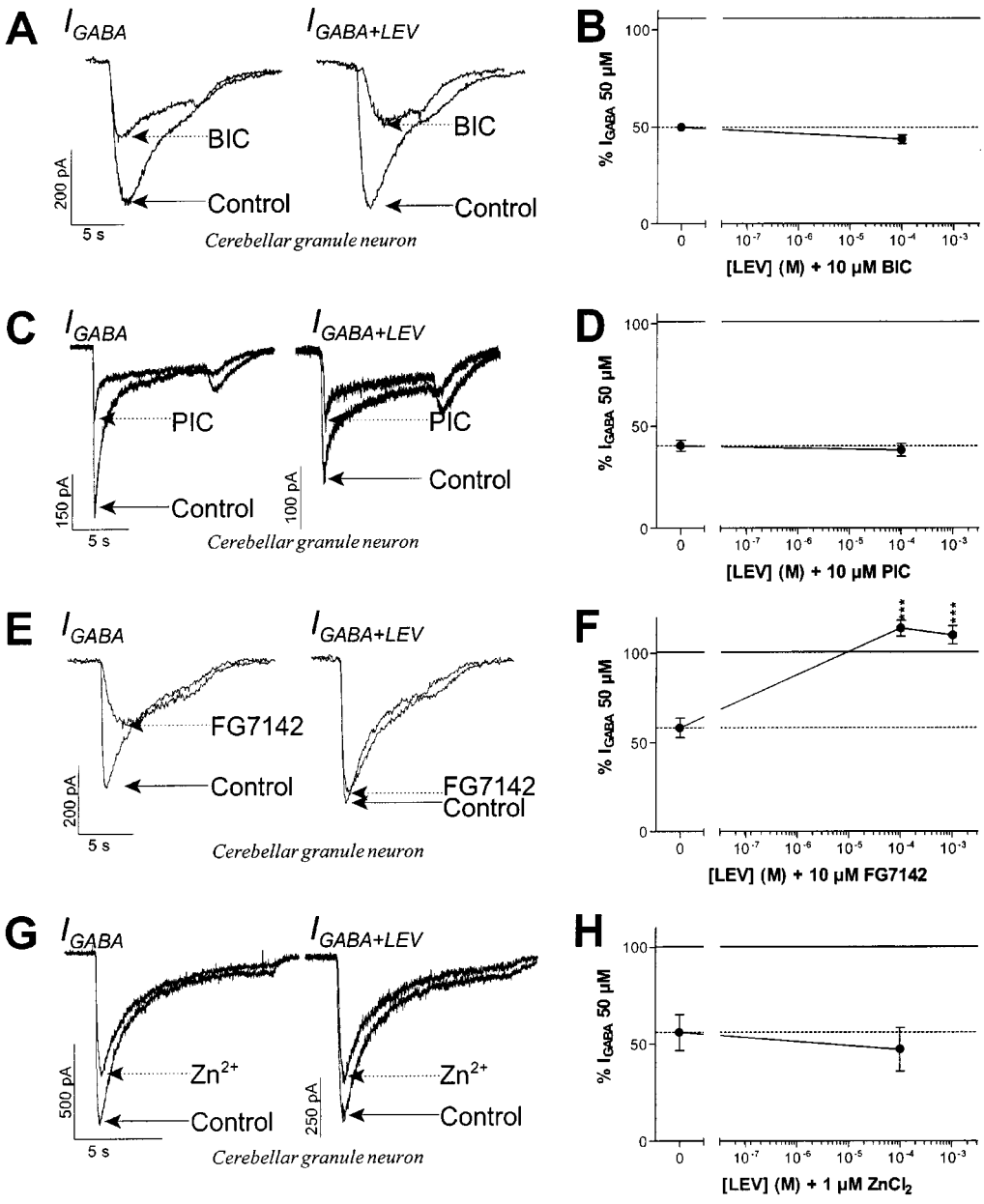

Figure 4 Interaction between LEV and negative allosteric modulators of the $GABA_A$ receptor (cerebellar granule neurons). *Left panel*: Three to twenty-three DIV cerebellar granule neurons were perfused for 10 s with 50 μM GABA alone (left traces) or in combination with LEV (right traces) (25 s, 100 μM) (controls) and negative allosteric modulators of the $GABA_A$ receptor (25 s, half-maximal inhibitory concentrations: 10 μM bicuculline (BIC), 10 μM picrotoxin (PIC), 10 μM FG7142 and 1 μM zinc). A 60 s period was allowed for the washout of drugs (90 s in the case of picrotoxin or FG7142). *Right panel*: Currents evoked by 50 μM GABA in the presence of the indicated negative allosteric modulator of the $GABA_A$ receptor were recorded for increasing LEV concentrations. Results are expressed as in Figure 3 (mean$\pm$s.e.mean; $n=3-6$ for B, 6–13 for D, 4–26 for F and 4–6 for H). ***$P<0.001$ using ANOVA followed by Dunnett's multiple comparisons post-tests.

and ethosuximide against sound-induced clonic convulsions, whereas even a high dose of FG 7142 (50 mg kg^{-1}) was without impact on the protection obtained with carbamazepine and phenytoin (Figure 8A). Flumazenil completely abolished the protective effect of clonazepam, whereas it was without any effect on the protection afforded by valproate and phenobarbital (Figure 8B). Surprisingly, a decrease of the protection induced by carbamazepine, phenytoin and ethosuximide was also observed after a co-administration with flumazenil.

Discussion and conclusions

The present *in vitro* findings reveal that LEV behaves as a null allosteric modulator of the $GABA_A$ and of the glycine receptors that suppresses the inhibitory effect of some $GABA_A$ and glycine receptors negative allosteric modulators, namely β-carbolines and zinc, but does not affect the inhibition afforded by classical antagonists of these receptors. A lack of ability of LEV to antagonize the inhibition by picrotoxin and bicuculline substantiates previous reports

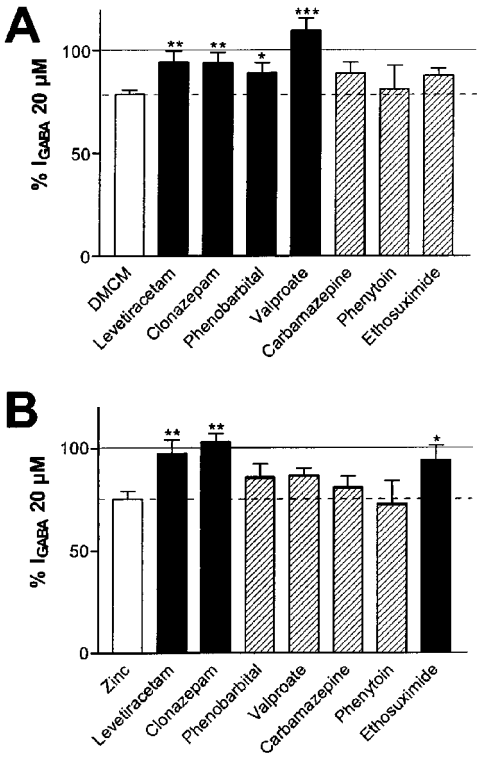

Figure 5 Comparison of the potencies of reference AEDs in reversing the inhibitory effects of DMCM (A) and zinc (B) on GABA-induced currents in 7–14 DIV cultured hippocampal neurons. Currents evoked by 20 μM GABA in the presence of 15 μM DMCM or 15 μM $ZnCl_2$ were recorded with the different reference AEDs (10 μM LEV, 10 μM carbamazepine, 1 μM clonazepam, 100 μM phenobarbital, 50 μM phenytoin, 1 mM valproate and 1 mM ethosuximide). Results are expressed as in Figure 3 (mean $\pm$ s.e.mean; $n=5-20$). AEDs, which exhibit significant reversal ability towards DMCM- or zinc-induced inhibition of GABA-gated currents are represented by solid bars. $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$ using Student's *t*-test analysis.

which suggest that the reduction by LEV of epileptiform activity induced by bicuculline *in vivo* is not associated with direct $GABA_A$ receptor effects (Margineanu & Wulfert, 1997). Furthermore, no direct effect of LEV on glycine-gated currents were observed and effects on GABA responses were marginal at clinically relevant concentrations. These results reveal a unique pharmacological profile compared to the tested reference AEDs. The *in vitro* interaction of LEV with negative allosteric modulators of inhibitory receptors was confirmed *in vivo* by the ability of a β-carboline to antagonize the seizure protection afforded by LEV.

The selective protection by LEV in animal models of chronic epilepsy distinguishes it from all other known AEDs. Indeed, when tested against a series of different chemoconvulsants in rodents, LEV only produced seizure protection against pilocarpine-, kainic acid- and DMCM-induced seizures (Klitgaard *et al*., 1998). The effect against pilocarpine- and kainic acid-induced seizures coincides with LEV's activity in models of chronic epilepsy since these two chemoconvulsants induce seizures that resemble partial seizures in epilepsy patients (Nadler, 1981; Turski *et al*., 1983). In contrast, the observation that LEV protects against DMCM-induced seizures was surprising and opposed to a general absence of activity against other GABAergic chemoconvulsants (Klitgaard *et al*., 1998). The fact that DMCM and other β-carbolines are negative allosteric modulators of both GABA and strychnine-sensitive glycine receptors (Rigo *et al*., 1998) stimulated the present study to explore potential indirect effects of LEV on both these inhibitory receptor systems.

Pharmacokinetic data from the four phase III studies performed so far with LEV as adjunctive therapy in adults with refractory partial epilepsy reveal that clinically effective doses (1000–3000 mg per day) result in trough plasma levels between 35 and 100 μM and in peak plasma concentrations between 90 and 250 μM (unpublished results of UCB Pharma; Patsalos, 2000). In the present study, LEV only showed a significant effect on the decay time of the GABA-gated current at 100 μM and above. Moreover, this effect is likely to depend on the subunit composition of the GABA receptor as suggested by the differences observed between young (<5 DIV), old ($\geqslant 5$ DIV) cerebellar granule and hippocampal neurons. Furthermore, it was not specific to LEV since it was observed with almost all tested AEDs. This appears to exclude that a direct facilitation by LEV of GABAergic inhibition is involved in its anti-epileptic activity in humans. Together with reports showing an absence of effect on both voltage-operated Na^+ and T-type Ca^{2+} currents (Zona *et al*., 2001), this suggests that LEV does not induce a conventional modulation of any of the three main mechanisms involved in the anti-epileptic action of classical AEDs. Instead, the present study revealed that LEV was the only AED showing a consistent suppression of the inhibition by both β-carbolines and zinc on GABA- and glycine-gated currents. This coincided with an ability of the β-carboline FG 7142, but not the benzodiazepine receptor antagonist flumazenil, to eliminate the seizure protection afforded by LEV *in vivo*. Taken together, these results suggest that LEV's ability to abolish the inhibitory action of zinc and other negative allosteric modulators on these inhibitory receptor systems may contribute to its anti-epileptic action in humans.

To what extent can it be substantiated that the ability of LEV to reverse the inhibition by β-carbolines and zinc on inhibitory responses may account for at least a part of its anti-epileptic action? Even if some of them have been suggested as endogenous (Braestrup *et al*., 1980; Medina *et al*., 1989; Peña *et al*., 1986), β-carbolines are unlikely to be important pathogenic agents causing epilepsy. In contrast, the data on zinc are remarkable when confronted to the possibility that the hyperexcitability of the epileptic hippocampus may be associated with circuit and cellular alterations involving changes both in the subunit expression pattern of $GABA_A$ receptors in dentate granule cells (DGC) and in sprouting of zinc-containing DGC axons back onto the inner molecular layer of the dentate gyrus. Indeed, a 'sprouted mossy fibre/zinc-sensitive GABA receptor' hypothesis was recently proposed (Coulter, 1999; 2000). This theory is based on two principal epileptogenic phenomena. Firstly, it appears that the brains of humans with temporal lobe epilepsy (TLE) and animals mimicking TLE (pilocarpine-treated animals with spontaneous seizures and kindled animals) all exhibit a circuit reorganization of sprouted, zinc-containing, mossy fibre terminals which innervate new targets in the inner molecular layer of the dentate gyrus, including inhibitory interneurons. Secondly, observations in tissue specimens from

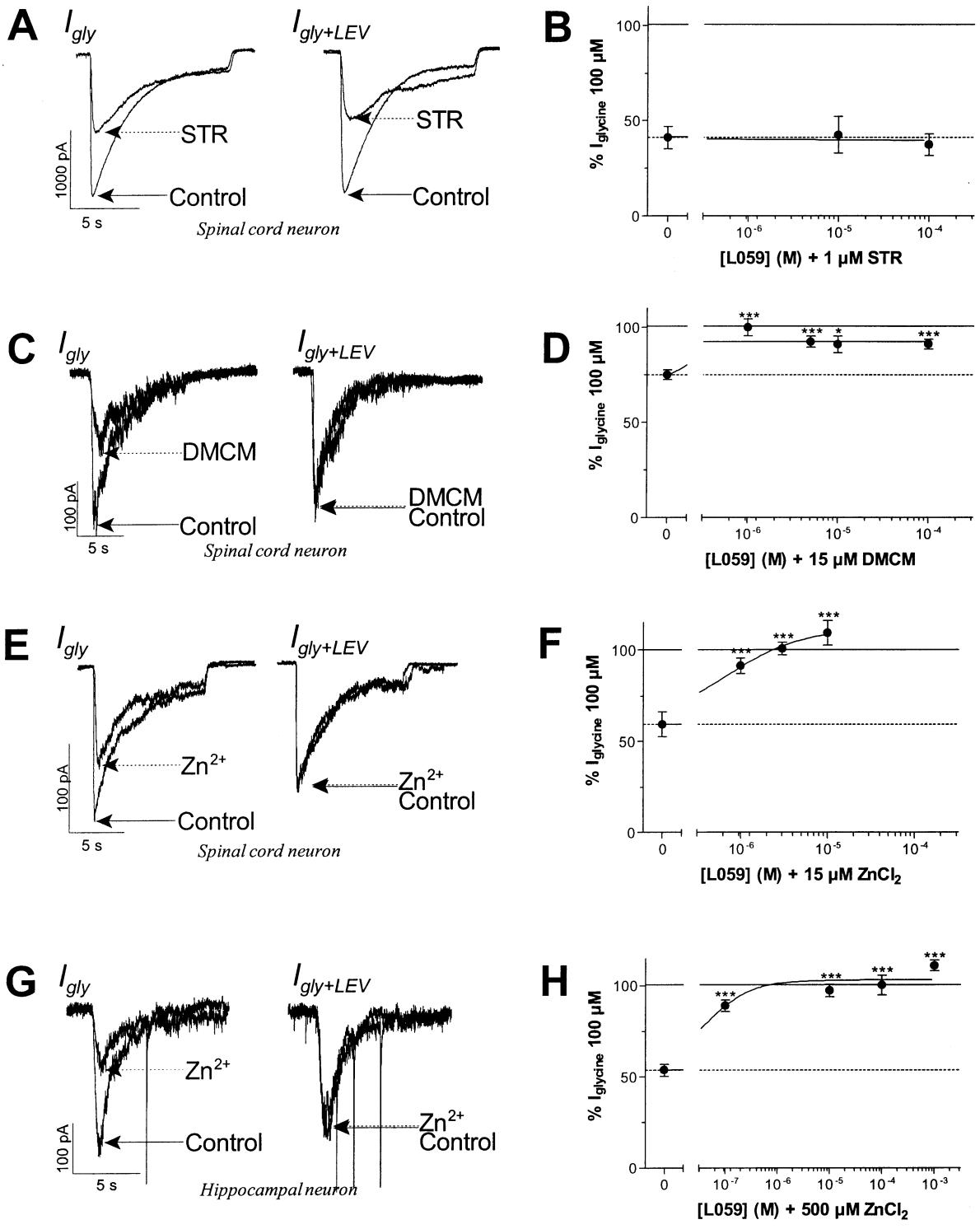

Figure 6 Interaction between LEV and negative allosteric modulators of the glycine receptor. *Left panel*: Three to five DIV spinal cord neurons (A, C and E) and 4–21 DIV hippocampal neurons (G) were perfused for 10 s with 100 μM glycine alone (left traces) or in combination with LEV (25 s, 10 μM) (right traces) (controls) and negative allosteric modulators of the glycine receptor (25 s, half-maximal inhibitory concentrations: 1 μM strychnine (STR), 15 μM DMCM and 15 or 500 μM zinc). A 60 s period was allowed for the washout of drugs (90 s in the case of DMCM). *Right panel*: Currents evoked by 100 μM glycine in cultured spinal cord or hippocampal neurons in the presence of the indicated negative allosteric modulator of the glycine receptor were recorded for increasing LEV concentrations. Results are expressed as in Figure 3 (mean$\pm$s.e.mean; $n=4-20$ for B, 5–33 for D, 5–33 for F and 5–44 for H). The curve fitting procedure yielded a half-maximal effective LEV concentration of 0.7 ± 0.1 μM in spinal cord neurons and 0.04 ± 0.02 μM in hippocampal neurons for zinc-induced inhibitions of glycine currents. ***$P<0.001$ and *$P<0.05$ using ANOVA followed by Dunnett's multiple comparisons post-tests.

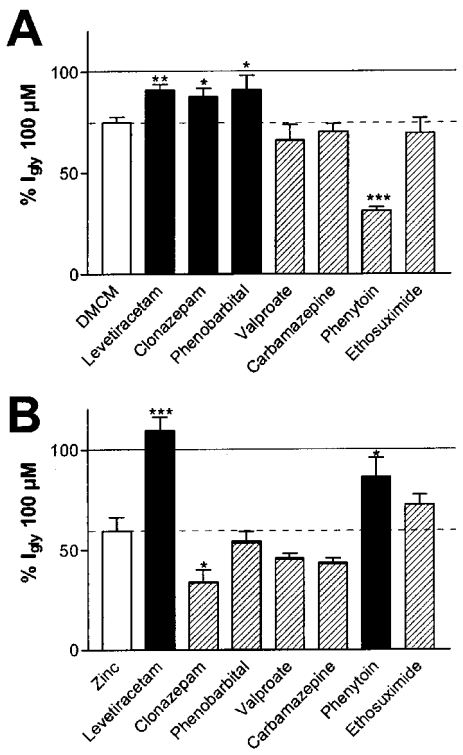

Figure 7 Comparison of the potencies of reference AEDs in reversing the inhibitory effects of DMCM (A) and zinc (B) on glycine-induced currents in 3–5 DIV cultured spinal cord neurons. Currents evoked by 100 μM glycine in the presence of 15 μM DMCM or 15 μM $ZnCl_2$ were recorded with the different AEDs (same concentrations as in Figure 4). Results are expressed and represented as described in Figure 4 (mean ± s.e.mean; $n = 5–19$).

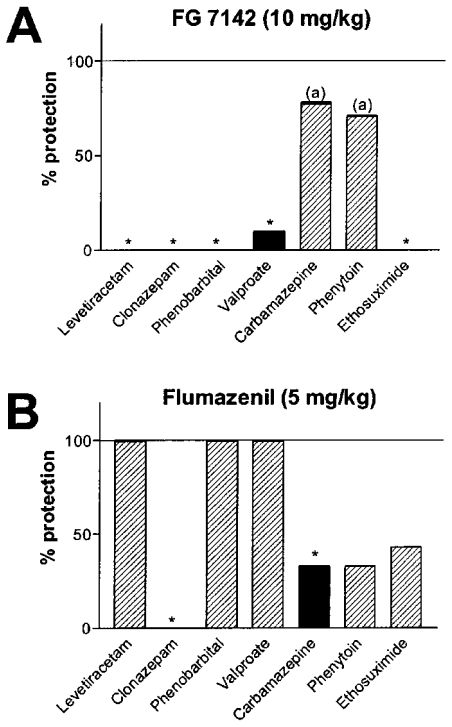

Figure 8 Effect of FG 7142 (A) and flumazenil (B) on the protection afforded by LEV and reference AEDs against clonic convulsions in sound-sensitive mice. LEV (17 mg kg^{-1}), carbamazepine (23.6 mg kg^{-1}), clonazepam (0.032 mg kg^{-1}), phenobarbital (14.2 mg kg^{-1}), phenytoin (45.4 mg kg^{-1}), valproate (230 mg kg^{-1}) and ethosuximide (254 mg kg^{-1}) were all administered i.p. 30 min before testing. The effect of FG 7142 (10 mg kg^{-1} i.p., 15 min before testing) and flumazenil (5 mg kg^{-1} p.o., 15 min before testing) was determined on the seizure protection afforded by LEV and the reference AEDs against clonic convulsions in audiogenic-susceptible mice. The protection given by LEV and reference AEDs administered alone was (%): (A) LEV (90), carbamazepine (90), clonazepam (80), phenobarbital (100), phenytoin (70), valproate (100) and ethosuximide (80); (B) LEV (90), carbamazepine (90), clonazepam (90), phenobarbital (100), phenytoin (60), valproate (100) and ethosuximide (70). To allow a direct comparison between the different drugs, the results from the interaction studies were expressed as percentage of a maximal protective effect (100%) obtained with each AEDs and indicated by the horizontal line. *$P < 0.05$ using a Fisher test *versus* AED administered alone. (a) a higher dose FG 7142 (50 mg kg^{-1}) was used for carbamazepine and phenytoin.

the same brains also reveal significant alterations in the subunit composition of the $GABA_A$ receptors from DGCs, when compared to controls. This appears to render these 'epileptic' $GABA_A$ receptors markedly more sensitive to the inhibitory action of zinc. Thus, it was postulated that epileptogenesis may involve the presence of zinc-containing sprouted mossy fibre terminals innervating DGCs containing zinc-sensitive $GABA_A$ receptors. When repetitive stimulation results in release of zinc this cation may in turn diffuse to and block these 'epileptic' $GABA_A$ receptors on DGCs. This would induce a vicious circle of disinhibition, which may result in aberrant activity triggering epileptiform discharges in the epileptic hippocampus. In that respect, it was interesting that LEV revealed a potent ability to suppress the inhibition of zinc on GABA-gated currents in hippocampal neurons.

Only few studies have addressed the implication of the glycine receptor in epilepsy. This may partly reflect the difficult design of specific glycine agonists (Bohme & Luddens, 2001) and thereby a scarcity in relevant probes for pharmacological studies of the function of the glycine receptor. Another reason probably arises from the classical separation among inhibitory neurotransmitters: GABA for the upper part of the neuraxis and glycine for the lower part. However, glycine receptors are also expressed in rostral brain regions like the adult striatum (Sergeeva & Haas, 2001), the amygdala (McCool & Farroni, 2001), the motor cortex (Furukawa *et al*., 1994) and the hippocampus where they seem to participate in neurotransmission (McMahon & Chattipakorn, 2000). Among the studies that addressed the consequences of glycine receptor subunits mutations, the well-documented concerns hyperekplexia (startle disease) that results from a mutation in the α_1 subunit (Celesia, 2001; Pierce *et al*., 2001; Moorhouse *et al*., 1999). Until now, only one work screened for gene mutations in glycine receptors in some forms of genetic epilepsies in humans (coding for the α_3 and the β subunits), but no significant results were reported (Sobetzko *et al*., 2001).

Our finding that LEV, an atypical but potent AED, acts with an even larger efficacy on glycine receptors than on $GABA_A$ receptors in reversing the effects of some of their inhibitors, could support a role for glycine receptors in the pathophysiology of epilepsy. This agrees with previous reports on the experimental drug milacemide, ascribing a

major part of its anti-epileptic activity to its metabolism into glycine (Roba *et al*., 1986). However, the electrophysiological techniques used in the present study only allowed the recording of whole-cell currents, i.e. those carried by both synaptic and extrasynaptic receptors. Hence, additional experiments have to be performed in order to demonstrate the effect of LEV on synaptic neurotransmission, which may differ from its effect on ligand-gated currents as assessed by whole-cell patch-clamp. Further studies are also needed to unravel the implication of glycinergic neurotransmission in epilepsy.

At the cellular and molecular levels, the reversal by LEV of the inhibition induced by β-carbolines and zinc on both GABA- and glycine-gated currents, together with its weak modulatory effect on GABA responses, at high concentrations, suggest an interaction of LEV with both of these receptors. However, LEV has never been shown to displace known ligands of the $GABA_A$ or the glycine receptors (Noyer *et al*., 1995). For example, antagonism of the β-carbolines effect would suggest interaction with the benzodiazepine site of the $GABA_A$ receptor. However, LEV has no effect on the binding of flunitrazepam or flumazenil (Noyer *et al*., 1995). Moreover, the lack of antagonism by flumazenil against LEV's seizure protection reported in the present study together with an absence of activity in the pentylenetetrazole seizure model previously reported (Klitgaard *et al*., 1998) argues against an interaction of LEV with the benzodiazepine site. To explain these discrepancies, only speculations can currently be made. We favour the hypothesis of an allosteric or at least functional coupling between two distinct binding sites. In that context, the existence on the $GABA_A$ receptor of a second site of action for benzodiazepines has recently been reported with an affinity in the micromolar range (compared to nanomolar for the 'classic' one) which is associated with residues in the TM2 region and which is less subunit specific than the nanomolar site (Walters *et al*., 2000). The same could be true for β-carbolines, classically described as benzodiazepine inverse agonists, which we used in the micromolar range to block not only GABA- but also glycine-evoked currents, suggesting a site of action that differs from the classical benzodiazepine site. Finally, LEV also reverses the inhibition of GABA responses by Ro 5-4864, a benzodiazepine which binds with nanomolar affinity to peripheral benzodiazepine receptors and with micromolar affinity to an unknown, highly controversial, site on the $GABA_A$ receptor (Gee *et al*., 1988; Lan *et al*., 1995; Puia *et al*., 1989). Taken together, these data could suggest that LEV interacts with a common site on both these inhibitory receptor channels.

In conclusion, it appears that the ability of LEV to reverse the inhibitory action of zinc and other negative allosteric modulators on both GABA- and glycine-gated currents could explain at least part of its anti-seizure effect as well as its potential anti-epileptogenic properties as demonstrated in the kindling model of chronic epilepsy.

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