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Synergistic interaction between valproate and lamotrigine against seizures induced by 4-aminopyridine and pentylenetetrazole in mice

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Abstract

We compared the effects of adding a non-protective dose of valproate to increasing doses of lamotrigine with those of monotherapy and vice versa in CD1 mice. Anticonvulsant effects were evaluated against seizures induced by both 4-aminopyridine and pentylenetetrazole, and neurotoxic effects were evaluated by the rotarod test. Changes in anticonvulsants, γ-aminobutyric acid (GABA) and glutamate concentrations in the whole brain were also assessed. Lamotrigine increased the potency ratio of valproate against 4-aminopyridine and pentylenetetrazole but not on rotarod, the protective index being increased from 1.1 to 2.4 against 4-aminopyridine and from 1.9 to 3.8 against pentylenetetrazole, without changes in brain valproate, and with a significant increase in brain GABA. Valproate increased the potency ratio of lamotrigine against 4-aminopyridine but not on rotarod, the protective index being increased from 4.4 to 7.3; valproate also increased brain lamotrigine (but only at low doses), brain GABA and brain glutamate. In conclusion, non-protective doses of lamotrigine increased the therapeutic index of valproate and vice versa, and these effects appeared to be pharmacodynamic.

Keywords: Valproate; Lamotrigine; Pentylenetetrazole; 4-Aminopyridine; Anticonvulsant; Drug interactions

1. Introduction

The clinical benefits of the association between lamotrigine and valproate against refractory seizures in humans have been highlighted in several reports and the existence of a pharmacodynamic interaction underlying this synergy has been proposed (Panayiotopoulos et al., 1993; Pisani et al., 1993, 1999; Ferrie et al., 1995; Leach, 1997; McCabe et al., 1998; Privitera and Welty, 1998; Michel et al., 1998). This association has shown favorable results against absences (Panayiotopoulos et al., 1993; Pisani et al., 1993; Ferrie et al., 1995) and refractory seizures (Pisani et al., 1993, 1999; Ferrie and Panayiotopoulus, 1994; Brodie et al., 1997; McCabe et al., 1998, 2001; Privitera and Welty, 1998; Arzimanoglou et al., 2001). Furthermore, some side effects observed when valproate and lamotrigine are used concomitantly have also been attributed to a possible synergism (Reutens et al., 1993; Ferrie and Panayiotopoulus, 1994; Brodie et al., 1997; Pisani et al., 1999). The hypothetical pharmacodynamic interaction between both anticonvulsants that appears to underlie this synergism is supported by the following points: (i) this synergy is not explained by the well-known pharmacokinetic influence of valproate on lamotrigine concentration in those studies in which plasma lamotrigine concentrations were available; (ii) both anticonvulsants act through different mechanisms since valproate mainly inhibits voltage-dependent Na^+ channels, it blocks T Ca^{++} channels and enhances γ-aminobutyric acid (GABA)ergic tone, whereas lamotrigine inhibits voltage-dependent Na^+ channels, stabilizing neuronal membranes and prolonging depolarization, leading to a reduction in glutamate release (Pisani et al., 1993; Davis et al., 1994; Meldrum and Leach, 1994; Fariello et al., 1995; Leach, 1997; Macdonald, 1999). However, a true pharmacodynamic interaction is difficult to demonstrate in the human setting due to ethical and methodological limitations (i.e., low possibility of discarding a real pharmacokinetic interaction).

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Data supporting a synergistic interaction between lamotrigine and valproate in animals are scarce. An increase has been described in the anticonvulsant effects of valproate against audiogenic seizures when a non-protective dose of lamotrigine is associated to valproate in DBA/2 mice (De Sarro et al., 1996). However, the possibility of a pharmacokinetic interaction was not totally discarded in this study since brain concentrations were not measured. Furthermore, we consider it worth investigating whether a non-protective dose of valproate increases the anticonvulsant effects of lamotrigine and if these interactions are observed in other models of convulsions such as those induced by 4-aminopyridine and pentylenetetrazole.

The aim of this study was to analyze the influence that adding a non-protective dose of valproate to increasing single doses of lamotrigine, and conversely, adding a non-protective dose of lamotrigine to increasing single doses of valproate has on the anticonvulsant effects against hind limb extension induced by 4-aminopyridine and against clonus induced by pentylenetetrazole, and on the neurotoxic effects evaluated on rotarod in mice taking into account the possibility of pharmacokinetic interactions and changes in GABA and glutamate in the whole brain.

2. Materials and methods

2.1. Animals

Adult male albino mice (CD No. 1 strain, Charles River) weighing 20–28 g were housed for at least 7 days before experiments at controlled temperature (19 to 24 °C) and under natural light/dark conditions; food and water were available ad libitum. All procedures were performed from 9:00 to 14:00 h. Protocol complies with the European Community guidelines for the use of experimental animals and was approved by the Physiology and Pharmacology Department of our University.

2.2. Anticonvulsant and neurotoxic models

Anticonvulsant efficacy was tested against seizures induced by the 97%-convulsant dose (CD_{97}) of subcutaneous 4-aminopyridine or pentylenetetrazole, both purchased from Sigma (St. Louis, MO, USA). The CD_{97} established in our Laboratory for these mice was 14 mg/kg for 4-aminopyridine and 110 mg/kg for pentylenetetrazole. 4-Aminopiridine and pentylenetetrazole were dissolved in 0.9% Na^+ chloride and administered into a loose fold of skin in the midline of the neck in a volume of 0.01 ml/g of body weight. Fresh drug solutions were prepared each day. The main end-points were the abolition of the hind limb extension induced by 4-aminopyridine or the abolition of the clonus lasting at least 5 s induced by pentylenetetrazole. The periods of observation were 30 min for both the 4-aminopyridine and the pentylenetetrazole model. Periods of latency until hind limb extension or clonus were evaluated as secondary end-points.

Minimal neurotoxicity was assessed by the rotarod test (Dunham and Miya, 1957). Before the experiment, mice were placed on a 3-cm rod rotating (Rota-rod Treadmill 7600, Ugo-Basile) at 6 rpm in two training sessions lasting 10 and 5 min, respectively. After anticonvulsant administration, mice were tested again on the rotarod. The end-point to evaluate the minimal neurotoxicity was the inability of the mouse to maintain its equilibrium for 1 min in each of three trials.

Valproate (Sanofi-Winthrop) and lamotrigine (Glaxo-Wellcome) were administered in a volume of 0.01 ml/g of body weight. Valproate was administered intraperitoneally in 0.9% Na^+ chloride solution, whereas lamotrigine was given by transesophageal gavage in 1% methylcellulose. Valproate and lamotrigine were administered 20 and 60 min prior to testing, respectively.

2.3. Groups of study and experimental design

Anticonvulsant efficacy and toxicity were evaluated on groups of at least 8 mice for each dose of each drug in monotherapy and in association. A minimum of 3 percentage points between the limits of 0% and 100% was required. Control groups were included in each experiment.

The anticonvulsant effects of valproate in monotherapy were studied at doses of 175, 225, 275, 300, 350, 400 and 450 mg/kg against the hind limb extension induced by 4-aminopyridine, and at doses of 50, 63, 75, 150, 200, 250 and 350 mg/kg against clonus induced by pentylenetetrazole. The anticonvulsant effects of lamotrigine in monotherapy were studied at doses of 3, 5, 12, 20, 30, 40, 60 and 80 mg/kg against the hind limb extension induced by 4-aminopyridine, and at doses of 0.75, 1.5, 3.5, 5, 8, 16, 32, 50 and 65 mg/kg against the clonus induced by pentylenetetrazole.

For each anticonvulsant in monotherapy, the highest non-protective dose was established in our laboratory with the following criteria: (i) it was the highest dose that did not produce a statistically significant effect, and (ii) it was the highest dose unable to protect 10% of mice against 4-aminopyridine or pentylenetetrazole seizures. The highest non-protective dose of lamotrigine in the 4-aminopyridine model was 5 mg/kg (7% of mice protected from hind limb extension, ns). In the pentylenetetrazole model it was 1.5 mg/kg (6% of mice protected from clonus, ns, data not shown). The highest non-protective dose of valproate in the 4-aminopyridine model was 225 mg/kg (0% of mice protected from hind limb extension, ns). The neurotoxic effects of valproate in monotherapy were studied at doses of 225, 400, 450, 500 and 600 mg/kg and the neurotoxic effects of lamotrigine in monotherapy at doses of 20, 40, 50, 60 and 80 mg/kg.

The anticonvulsant interaction between valproate and lamotrigine was assessed bidirectionally: (i) the influence

of a non-protective dose of lamotrigine (5 mg/kg) on the effects of 175, 225, 275 and 350 mg/kg of valproate against 4-aminopyridine and the influence of a non-protective dose of lamotrigine (1.5 mg/kg) on the effects of 75, 150 and 200 mg/kg of valproate against pentylenetetrazole, and (ii) the influence of a non-protective dose of valproate (225 mg/kg) on the effects of 3, 5, 12 and 20 mg/kg of lamotrigine against 4-aminopyridine. The influence of valproate on the anticonvulsant effects of lamotrigine in the pentylenetetrazole model could not be analyzed because lamotrigine in monotherapy was only partly effective in this model.

The interaction between valproate and lamotrigine on neurotoxic effects was also assessed bidirectionally: (i) the influence of 5 mg/kg of lamotrigine (the highest dose associated to valproate in the anticonvulsant studies) on the neurotoxic effects of 225, 400 and 450 mg/kg of valproate, and (ii) the influence of 225 mg/kg of valproate on the neurotoxic effects of 40, 50 and 60 mg/kg of lamotrigine.

Pharmacokinetic interactions and neurochemical changes were studied in groups of at least five mice. These groups were different from those used in the anticonvulsant and neurotoxic experiments. Again the study was bidirectional: (i) the influence of 5 mg/kg of lamotrigine on whole brain concentrations of valproate, GABA and glutamate achieved 20 min after the administration of 100, 225 and 350 mg/kg of valproate, and (ii) the influence of 225 mg/kg of valproate on whole brain concentrations of lamotrigine, GABA and glutamate achieved 60 min after the administration of 5, 12 and 20 mg/kg of lamotrigine. Mice were killed by cervical dislocation, and the whole brain was immediately removed, weighed and homogenized in 2 ml of methanol. Homogenates were centrifuged at 2500 × *g* for 10 min at 4 °C and the upper phase stored at −20 °C until assay.

2.4. Assays

Brain valproate concentration was assayed in duplicate by fluorescence polarization immunoassay (FPIA), using the TDx analyzer and the kits supplied by Abbott (Abbott Laboratories, North Chicago, IL, USA). Coefficients of variation within assays and between assays were lower than 10% (usually lower than 5%).

Brain lamotrigine concentration was assayed by the modified liquid chromatographic method used by Fraser et al. (1995). Briefly: 200 μl of brain homogenate supernatant or lamotrigine calibrators (0.5, 1, 2.5, 5, 10 and 25 mg/l), 100 μl of BW725C78 (10 mg/l) as internal standard (Glaxo-Wellcome Lab.), and 1 ml of 2 mol/l NaOH were added to 5 ml of high-performance liquid cromatographic (HPLC)-grade ethyl acetate. After mixing for 10 min on a horizontal mixer and centrifuging at 2000 rpm for 5 min, 3 ml of the upper organic layer were transferred into a 10-ml pyrex tube and evaporated to dryness under a stream of nitrogen in a heating block at 60 °C. The dry residue was dissolved in 200 μl of methanol and 50 μl injected in the liquid chromatograph. Isocratic separation at room temperature was performed using a Kontron modular HPLC system equipped with a 250 × 4.6 mm i.d. column packed with 5 μ Hypersil ODS and a Waters ultraviolet variable wavelength detector at 210 nm. The mobile phase consisted of 30% acetonitrile and 70% of a mixture of 12.5 ml of 0.5 M Na^+ phosphate buffer (pH 5.71) and 987.5 ml of Mili Q water at a flow rate of 1.5 ml/min. Within-assay coefficients of variation in two control samples with 0.75 and 3.0 mg/l of lamotrigine were 6.0% (S.D. = 5.6) and 7.5% (S.D. = 8.8), respectively, and between-assay coefficients of variation were 9.4% and 10.1%, respectively.

Brain GABA concentration was assayed by the liquid chromatographic method of Turnell and Cooper (1982) modified as described by Valdizán and Armijo (1992). Mean brain GABA concentration in control groups was 1.99 (S.D. = 0.1) μmol/g of tissue. Within-assay and between-assay coefficients of variation in a control sample with 0.75 mmol/l of GABA were 4.3% (S.D. = 3.2) and 17%, respectively.

Brain glutamate concentration was assayed by the modified liquid chromatographic method used by Löscher et al. (1993). Briefly: 25 μl of brain homogenate supernatant or glutamate calibrators (1, 2, 2.5, 3 and 4 mmol/l), 100 μl of 1 mmol/l L-α-aminoadipic acid as internal standard (Sigma) and 200 μl of 1% 5-sulfosalicylic acid as deproteinizing reagent were vortex-mixed and centrifuged at 10,500 × *g* at room temperature for 2 min and maintained in an ice bath. Just before injection, 25 μl of the supernatant were vortex-mixed with 100 μl of daily prepared *O*-phthalaldehyde-2-mercaptoethanol derivatizing reagent and 50 μl immediately injected in the liquid chromatograph. Isocratic separation was performed at room temperature using a Kontron modular HPLC system equipped with a 100 μ-Bondapak C_{18} guard column (Waters), a 125 × 4 mm i.d. LiChroCart column packed with 5 μm LiChrosphere 100 RP-18 (Merk), and a Waters 420 AC fluorescence detector with 338 nm of excitation and 425 nm of emission wavelength filters. The mobile phase consisted of 8% methanol and 92% of a mixture of 0.1 mmol/l Na^+ acetate buffer (at pH 7.2)–methanol–tetrahydrofurane (90:9.5:0.5%, v/v/v) at a flow rate of 1.5 ml/min. Mean brain glutamate concentration in control groups was 6.73 (S.D. = 0.53) μmol/g of tissue. Within-assay coefficients of variation in two control samples with 1.25 and 2.75 mmol/l of glutamate were 3.3% (S.D. = 3.4) and 4.8% (S.D. = 5.4), respectively, and between-assay coefficients of variation were 1.6% and 5.7%, respectively.

2.5. Statistical analysis

Median effective doses (ED_{50}), median neurotoxic doses (TD_{50}), relative potencies between monotherapy and association (i.e., ED_{50} monotherapy/ED_{50} association or TD_{50} monotherapy/TD_{50} association) and 95%-confidence interval (CI 95%) were calculated by Litchfield and Wilcoxon's

(1949) log-probit method using the Pharm/PCS v. 4.0 software. The protective index (TD_{50}/ED_{50}) for valproate and lamotrigine in monotherapy and in association was also calculated. Qualitative variables were compared by the chi-square test or Fisher's exact test, and quantitative variables by means of one-way analysis of variance (followed by the Newman–Keuls test) or by the non-parametric Mann–Whitney *U*-test using SPSS for Windows V 6.0 software. A two-sided $P<0.05$ was considered significant throughout. Data are expressed as mean and standard deviation (S.D.) in text and tables and as mean and standard error of the mean (S.E.M.) in figures.

3. Results

3.1. Influence of lamotrigine on valproate

3.1.1. Influence on the anticonvulsant and neurotoxic effects

The effects of single increasing doses of valproate in monotherapy and those in association with a non-protective dose of lamotrigine on the incidence and latency of hind limb extension induced by 4-aminopyridine and clonus induced by pentylenetetrazole are shown in Table 1. Lamotrigine shifted the dose–response curve of valproate against the incidence of hind limb extension induced by 4-aminopyridine to the left, this effect being more pronounced at low than at high doses of valproate (Fig. 1A). The ED_{50} of valproate decreased from 349.5 mg/kg in monotherapy to 173.9 mg/kg in association with lamotrigine, the relative anticonvulsant potency of valproate being 2.01 ($P<0.05$). Lamotrigine also shifted the dose–response curve of valproate against the incidence of clonus induced by pentylenetetrazole to the left (Fig. 1B). The ED_{50} of valproate decreased from 204.6 mg/kg in monotherapy to 109.7 mg/kg in association with lamotrigine, the relative anticonvulsant potency of valproate being 1.87 ($P<0.05$) (Table 2). Furthermore, lamotrigine prolonged the periods of latency until hind limb extension induced by 4-aminopyridine and until clonus induced by pentylenetetrazole observed with valproate (Table 1). In contrast, lamotrigine did not significantly modify the

Table 1
Influence of a non-protective dose of lamotrigine on the effects that increasing single doses of valproate have on the incidence and latency of hind limb extension induced by 4-aminopyridine and clonus induced by pentylenetetrazole in CD1 mice

VPA dose (mg/kg)	VPA monotherapy			VPA + LTG			$P'<$	$P''<$
	N	Protection (%)	Latency (min) Mean (S.D.)	*N*	Protection (%)	Latency (min) Mean (S.D.)		
(A) Effects on hind limb extension induced by 14 mg/kg of subcutaneous 4-aminopyridine								
0	29	0	10.7 (4.8)	27	7	12.9 (5.7)	NS	NS
175	12	0	12.8 (3.7)[a,b]	12	50[c,d]	25.9 (5.0)[c,d]	0.05	0.001
225	25	0	17.6 (5.1)[b,c,e]	12	67[c,d]	27.6 (4.5)[c,d]	0.001	0.001
275	12	17	23.2 (5.5)[c,e]	12	67[c,d]	27.2 (5.8)[c,d]	0.05	0.05
300	18	6[b]	19.3 (5.1)[b,c,e]	–	–	–	–	–
350	12	58[c,e]	27.8 (3.6)[c,e]	12	83[c,d]	28.6 (4.1)[c,d]	NS	NS
400	12	67[c,e]	27.3 (4.5)[c,e]	–	–	–	–	–
450	12	100[c,e]	30.0 (0.0)[c,e]	–	–	–	–	–
P	–	<0.001	<0.001	–	NS	NS	–	–
(B) Effects on clonus induced by 110 mg/kg of subcutaneous pentylenetetrazole								
0	20	0	7.0 (5.0)	18	6	13.7 (8.5)	NS	0.01
50	18	0	7.8 (7.1)	–	–	–	–	–
63	12	0	4.3 (3.6)	–	–	–	–	–
75	24	8	10.8 (8.1)	12	25[a,b]	16.2 (9.6)[b,f]	NS	NS
150	18	17	10.4 (9.7)[b]	12	83[c,d,e]	28.8 (3.7)[c,d,e]	0.01	0.001
200	24	46[e,f]	19.7 (10.9)[c,e]	12	67[c,d]	26.5 (7.7)[c,d,e]	NS	NS
250	19	63[c,e]	23.8 (9.5)[b,c,e]	–	–	–	–	–
350	20	90[c,e]	29.3 (4.7)[c,e]	–	–	–	–	–
P	–	<0.001	<0.001	–	<0.05	<0.001	–	–

LTG = lamotrigine. NS = non-significant. Protection: percentage of mice protected from convulsions. *P*: significance between doses of valproate (excluding control). *P'*: significance vs. the effect on protection of the same dose in monotherapy. *P''*: significance vs. the effect on latencies of the same dose in monotherapy. VPA = valproate.

[a] $P<0.05$ vs. control (untreated mice).

[b] Statistically significant vs. the following higher dose.

[c] $P<0.001$ vs. control (untreated mice).

[d] Statistically significant vs. the effect of the non-protective dose of lamotrigine (5 mg/kg in the 4-aminopyridine and 1.5 mg/kg in the pentylenetetrazole model).

[e] Statistically significant vs. lower doses.

[f] $P<0.01$ vs. control (untreated mice).

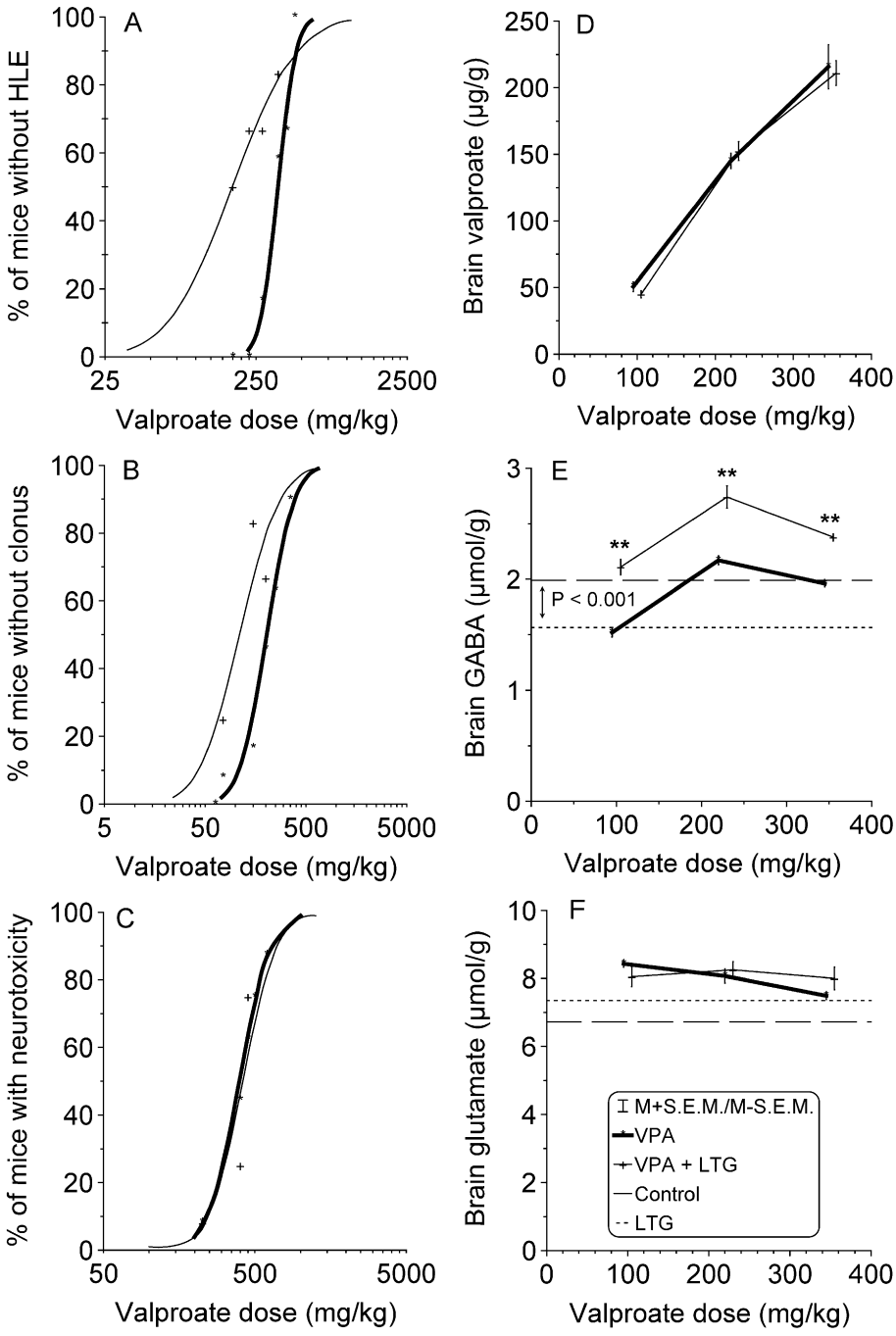

Fig. 1. Valproate dose–response curves against hind limb extension (HLE) induced by 4-aminopyridine (A) or against clonus induced by pentylenetetrazole (B), and dose–neurotoxic response evaluated by rotarod (C) after single increasing doses of valproate both in monotherapy (VPA) and in association with a non-protective dose of lamotrigine of 5 mg/kg in the 4-aminopyridine and of 1.5 mg/kg in the pentylenetetrazole model (VPA + LTG). Influence of adding 5 mg/kg of lamotrigine to increasing single doses of valproate on whole brain valproate concentration (D), and on whole brain GABA (E) and glutamate (F) concentrations in CD1 mice. $^{**}P < 0.01$ vs. the same dose in monotherapy. Data are mean (M) ± standard error of the mean (S.E.M.). *P*: significance between control and the non-protective dose of lamotrigine.

neurotoxic effects of valproate (Fig. 1C). Consequently, the protective index of valproate increased from 1.1 in monotherapy to 2.4 in association with lamotrigine in the 4-aminopyridine model and from 1.9 to 3.8 in the pentylenetetrazole model (Table 2).

3.1.2. Influence on brain valproate and on brain GABA and glutamate concentrations

Lamotrigine did not statistically modify valproate concentration in the whole brain (Fig. 1D). However, lamotrigine significantly increased the effects of valproate on brain

Table 2
Influence of a non-protective dose of lamotrigine on anticonvulsant and neurotoxic effects of increasing single doses of valproate and vice versa in CD1 mice

Groups	ED_{50} (mg/kg)		TD_{50} (mg/kg)	PI (TD_{50}/ED_{50})	
	4-AP	PTZ		4-AP	PTZ
(A) Influence of a non-protective dose of lamotrigine on increasing doses of valproate					
VPA	349.5 (318.3–383.7)	204.6 (171.3–244.2)	394.5 (328.6–473.7)	1.1	1.9
VPA + LTG	173.9 (127.7–236.9)	109.7 (78.2–153.9)	415.7 (329.3–524.7)	2.4	3.8
Relative potency VPA/(VPA + LTG)	2.01 (1.45–2.77)[a]	1.87 (1.27–2.73)[a]	0.95 (0.71–1.28)	–	–
(B) Influence of a non-protective dose of valproate on increasing doses of lamotrigine					
LTG	12.3 (9.5–15.8)	–	54.1 (43.3–67.7)	4.4	–
LTG + VPA	6.0 (3.9–9.2)	–	43.7 (39.8–48.1)	7.3	–
Relative potency LTG/(LTG + VPA)	2.06 (1.25–3.38)[a]	–	1.23 (0.97–1.57)	–	–

4-AP = 4-aminopyridine. LTG = lamotrigine. PI = protective index. PTZ = pentylenetetrazole. VPA = valproate. Data are median and 95%-confidence interval in brackets.

[a] $P < 0.05$ by the Litchfield and Wilcoxon's method (1949).

GABA at doses of 100 mg/kg (from 1.52 to 2.11 μmol/g, $P < 0.01$), 225 mg/kg (from 2.17 to 2.74 μmol/g, $P < 0.01$), and 350 mg/kg of valproate (from 1.96 to 2.38 μmol/g, $P < 0.01$). This synergistic effect on brain GABA was unexpected because 5 mg/kg of lamotrigine in monotherapy significantly reduced brain GABA in relation to control (from 1.99 to 1.56 μmol/g, $P < 0.01$) (Fig. 1E). In contrast, lamotrigine did not statistically modify the increase in brain glutamate produced by valproate alone (Fig. 1F).

3.2. Influence of valproate on lamotrigine

3.2.1. Influence on the anticonvulsant and neurotoxic effects

The effects of single increasing doses of lamotrigine in monotherapy and in association with a non-protective dose of valproate on incidence and latency of hind limb extension induced by 4-aminopyridine are shown in Table 3. Valproate shifted the dose–response curve of lamotrigine against the incidence of hind limb extension induced by 4-aminopyridine to the left, this effect being more pronounced at low than at high doses of lamotrigine (Fig. 2A). The ED_{50} of lamotrigine decreased from 12.3 mg/kg in monotherapy to 6.0 mg/kg in association with valproate, the relative anticonvulsant potency of lamotrigine being 2.06 ($P < 0.05$) (Table 2). Furthermore, valproate significantly increased the periods of latency in relation to those observed with lamotrigine alone (Table 3). Valproate increased the slope of the dose–neurotoxic response curve of lamotrigine (Fig. 2B) but it did not significantly modify the relative neurotoxic potency. Consequently, the protective index of lamotrigine increased from 4.4 in monotherapy to 7.3 in association with valproate (Table 2).

Table 3
Influence of a non-protective dose of valproate on the effects that increasing single doses of lamotrigine have on the incidence and latency of hind limb extension induced by 14 mg/kg of subcutaneous 4-aminopyridine in CD1 mice

LTG dose (mg/kg)	LTG monotherapy			LTG + VPA			P' <	P'' <
	N	Protection (%)	Latency (min) Mean (S.D.)	N	Protection (%)	Latency (min) Mean (S.D.)		
0	29	0	10.7 (4.8)	25	0	17.6 (5.1)	NS	0.001
3	12	0	11.4 (2.4)	12	0[a]	18.4 (4.5)[a,b]	NS	0.001
5	27	7	12.9 (5.7)[a]	12	67[b,c,d]	27.6 (4.5)[b,c,d]	0.001	0.001
12	12	33[a,e]	22.0 (6.9)[a,b,c]	12	83[b,c,d]	28.2 (4.3)[b,c,d]	0.05	0.05
20	24	79[b,c]	27.4 (5.6)[b,c]	12	83[b,c,d]	28.9 (2.9)[b,c,d]	NS	NS
30	12	100[b,c]	30.0 (0.0)[b,c]	–	–	–	–	–
40	12	100[b,c]	30.0 (0.0)[b,c]	–	–	–	–	–
60	12	100[b,c]	30.0 (0.0)[b,c]	–	–	–	–	–
80	12	100[b,c]	30.0 (0.0)[b,c]	–	–	–	–	–
P'	–	< 0.001	< 0.001	–	< 0.001	< 0.001	–	–

LTG = lamotrigine. NS = non-significant. Protection: percentage of mice protected from convulsions. P: significance between doses of lamotrigine (excluding control). P': significance vs. the effect on protection of the same dose in monotherapy. P'': significance vs. the effect on latencies of the same dose in monotherapy. VPA = valproate.

[a] Statistically significant vs. the following higher dose.

[b] $P < 0.001$ vs. control (untreated mice).

[c] Statistically significant vs. lower doses.

[d] Statistically significant vs. the effect of the non-protective dose of valproate (225 mg/kg).

[e] $P < 0.01$ vs. control (untreated mice).

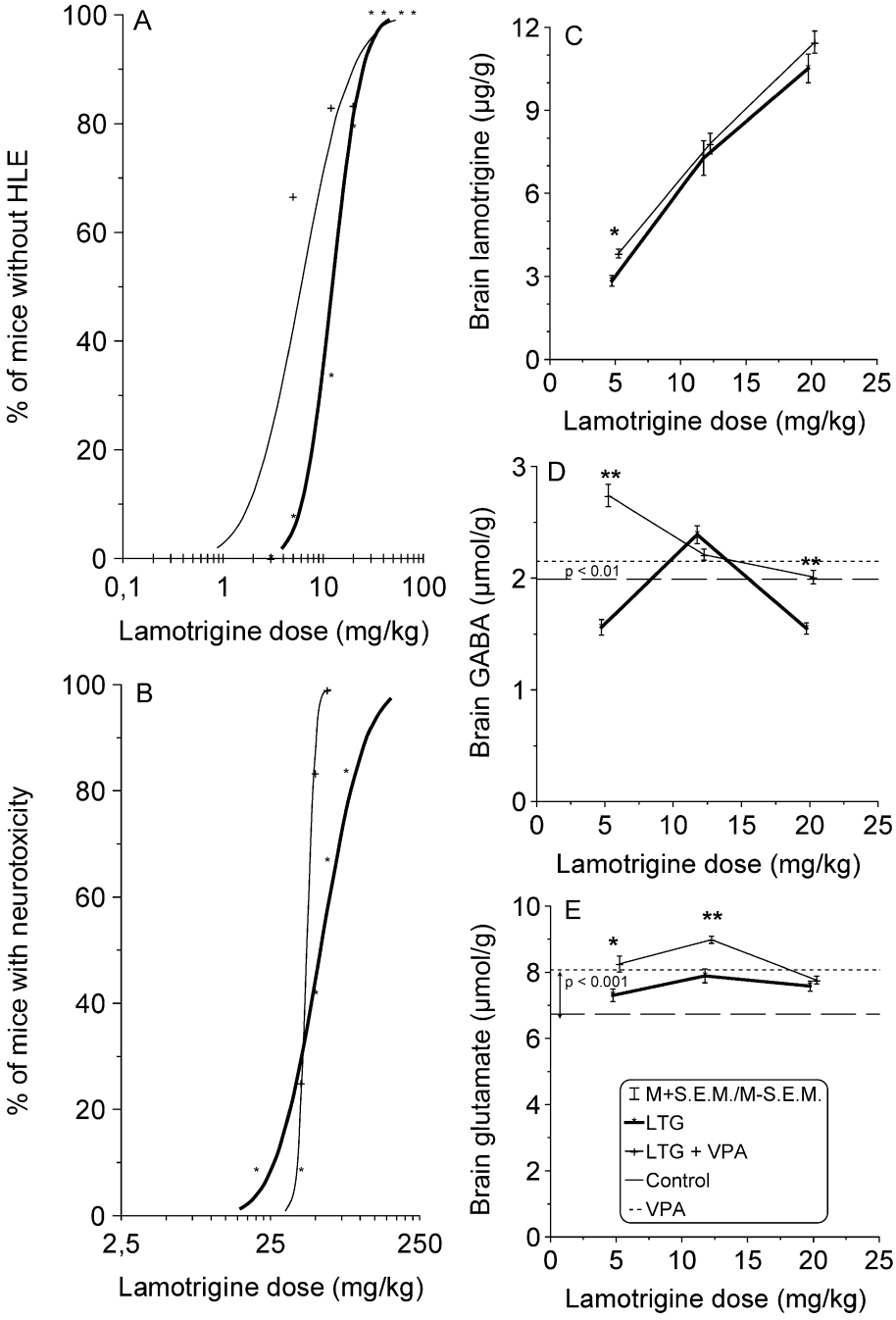

Fig. 2. Lamotrigine dose–response curves against hind limb extension (HLE) induced by 4-aminopyridine (A) and dose–neurotoxic response evaluated by rotarod (B) after single increasing doses of lamotrigine both in monotherapy (LTG) and in association with a non-protective dose of valproate of 225 mg/kg (LTG + VPA). Influence of adding 225 mg/kg of valproate to increasing single doses of lamotrigine on whole brain lamotrigine concentration (C), and on whole brain GABA (D) and glutamate (E) concentrations in CD1 mice. *$P<0.05$ and **$P<0.01$ vs. the same dose in monotherapy. Data are mean (M) ± standard error of the mean (S.E.M.). *P*: significance between control and the non-protective dose of valproate.

3.2.2. Influence on brain lamotrigine and on brain GABA and glutamate concentrations

The non-protective dose of 225 mg/kg of valproate increased lamotrigine concentrations achieved in the whole brain by 5 mg/kg of lamotrigine from 2.84 to 3.83 µg/g ($P<0.05$) but not those achieved by 12 and 20 mg/kg (Fig. 2C). Valproate also increased brain GABA in relation to the effects of lamotrigine alone at doses of 5 mg/kg (from 1.56 to 2.74 µmol/g, $P<0.01$) and 20 mg/kg (from 1.55 to 2.01 µmol/g, $P<0.01$) but not with 12 mg/kg of lamotrigine (Fig. 2D). Some increase in brain GABA was expected because 225 mg/kg of valproate in monotherapy increased brain GABA in relation to control from 1.99 to 2.17 µmol/g ($P<0.01$), but the increase in brain GABA observed in the

association was higher than that expected from this effect (Fig. 2D). Valproate also increased brain glutamate with regard to the effects of lamotrigine alone at doses of 5 mg/kg (from 7.30 to 8.25 μmol/g, $P<0.05$) and 12 mg/kg (from 7.89 to 8.98 μmol/g, $P<0,01$) but not with 20 mg/kg of lamotrigine (Fig. 2E). This greater effect on brain glutamate was expected because 225 mg/kg of valproate in monotherapy increased glutamate in relation to control from 6.73 to 8.07 μmol/g ($P<0.001$).

4. Discussion

This study shows that a low and ineffective dose of lamotrigine increases the protective index of valproate against seizures induced by both 4-aminopyridine and pentylenetetrazole by enhancing valproate anticonvulsant effects without a significant increase in its neurotoxicity. Conversely, a low and ineffective dose of valproate increases lamotrigine protective index against seizures induced by 4-aminopyridine by enhancing lamotrigine anticonvulsant effect but not its neurotoxicity. The absence of a relevant pharmacokinetic interaction between both anticonvulsants suggests that a pharmacodynamic interaction underlies these effects.

A part of our results coincide with those of De Sarro et al. (1996), which found an increase in the therapeutic index of valproate after adding a non-effective dose of lamotrigine in another convulsions model. These investigators showed an augmentation of valproate anticonvulsant activity against audiogenic seizures without a concomitant increase in toxicity after adding a non-effective dose of lamotrigine to increasing doses of valproate in DBA/2 mice. No pharmacokinetic interaction was observed when they studied the influence of lamotrigine on total plasma levels of valproate. However, as the investigators recognized, by measuring total plasma levels of the anticonvulsants they could not definitely exclude the possibility that lamotrigine could alter protein binding and the relative free vs. protein bound ratio. Following a similar design, our study adds new evidence of a favorable pharmacodynamic increase of valproate effects by lamotrigine in mice: (i) we have confirmed an increase in the therapeutic index of valproate against two other different convulsions models (i.e., 4-aminopyridine and pentylenetetrazole) and (ii) the influence of lamotrigine on valproate described in our study cannot be explained by any influence of lamotrigine on whole brain valproate concentrations, which is the gold standard by which a possible pharmacokinetic interaction in animals is assessed (Levy and Bourgeois, 1997).

Furthermore, our study shows for the first time that a low and ineffective dose of valproate also enhances the therapeutic index of lamotrigine without a consistent and homogeneous influence of valproate on brain lamotrigine concentrations. In fact, there was a statistically significant 857% enhancement in protection against hind limb extension after adding a non-protective dose of valproate to 5 mg/kg of lamotrigine with only a significant but minor increase of 35% in brain lamotrigine concentration. Furthermore, the significant 152% increase in protection against hind limb extension observed when valproate was added to 12 mg/kg of lamotrigine was associated to only a slight and non-significant 7% increase in brain lamotrigine concentration.

Our results suggest that the association of low doses of valproate to lamotrigine may be as worthwhile as the converse, showing an interesting bidirectional synergy. Finally, our results and those of De Sarro et al. (1996) support the hypothesis of a beneficial pharmacodynamic interaction between valproate and lamotrigine emerging from clinical studies (Panayiotopoulos et al., 1993; Pisani et al., 1993, 1999; Ferrie et al., 1995; Leach, 1997).

We have tried for the first time to relate the anticonvulsant interaction between valproate and lamotrigine to changes in whole brain GABA and glutamate concentrations. The most interesting result was that lamotrigine, an inhibitor of excitatory neurotransmission that even reduced brain GABA concentrations in monotherapy, significantly and homogeneously increased GABA concentrations in relation to valproate alone at the same doses that produced an anticonvulsant synergy. In contrast, this potentiation could not be explained by an antiglutamatergic effect since an increase in brain glutamate was observed both with valproate in monotherapy and in association with lamotrigine. Certainly, GABA or glutamate changes in the whole brain are not sensitive enough to explain subtle drug effects and it is more suitable to study neurochemical interactions in discrete brain areas and even better at the synaptosomal level. However, the neurochemical analysis in the whole brain performed in our study may be a valid approach and an acceptable source of new hypothesis to investigate (Leach et al., 1997).

In this study, the anticonvulsant action has been tested in two chemical models of acute induced convulsions: 4-aminopyridine and pentylenetetrazole. The 4-aminopyridine model has been used in our study instead of the maximal electroshock test. Maximal electroshock test has been widely used to study anticonvulsant synergy between antiepileptic drugs, because it is a well-known model and one of the reference tests used in the screening of new anticonvulsants; it is fast (with a short observation time), and is easy to perform and reproduce (Gordon et al., 1993; Chez et al., 1994; Sofia, 1995; White et al., 1998). The tonic extension of the hind limbs observed in both models is considered representative of seizure spread (Yamaguchi and Rogawski, 1992; Cramer et al., 1994). 4-Aminopyridine is a potassium channel antagonist; its mechanism of action is partially unknown but it seems to enhance the release of excitatory neurotransmitters. 4-Aminopyridine has been proposed as a substitute for the maximal electroshock test because the overall profile of compounds active against 4-aminopyridine convulsions resembles that against maximal electroshock, and the resultant convulsion resembles that of maximal

electroshock. 4-Aminopyridine test is also fast and is easier to perform than maximal electroshock test. Our results show that 4-aminopyridine is suitable to study the anticonvulsant interaction between lamotrigine and valproate in mice.

Pentylenetetrazole is a well-known agent extensively used for identifying new anticonvulsant drugs and also to assess the anticonvulsant efficacy of different antiepileptic combinations (Bourgeois, 1988a,b; White et al., 1998). Its mechanism of action is only partially understood, although an inhibition of the chloride conductance by binding to picrotoxin sites of $GABA_A$ receptor complex and a benzodiazepine receptor antagonism have been reported (Sayin et al., 1993). The efficacy against the clonus induced by pentylenetetrazole is considered predictive of the efficacy against generalized absences. However, in spite of the extensive use of the pentylenetetrazole model for screening and research, the validity of the results can hardly be generalized to spontaneous seizures in epileptic patients. In fact, the lack of efficacy of lamotrigine in the pentylenetetrazole model found in our study confirms the results of other investigators (Miller et al., 1986), and it contrasts with the anti-absence effects of lamotrigine observed in humans. Therefore, the influence of valproate on the anticonvulsant effects of lamotrigine should be analyzed in other models in which lamotrigine is active such as photically evoked afterdischarges and photoconvulsive responses (Lamb and Miller, 1985), electrically induced EEG afterdischarges (Wheatley and Miller, 1989) or the genetic epilepsy-prone rat (Smith et al., 1993).

In summary, we have observed that both the addition of a non-protective dose of valproate to increasing single doses of lamotrigine and, conversely, the addition of a non-protective dose of lamotrigine to increasing single doses of valproate produce a significant increase of the anticonvulsant effect achieved in monotherapy against the hind limb extension produced by 4-aminopyridine in mice without increasing neurotoxicity. Similarly, the addition of a non-protective dose of lamotrigine to increasing single doses of valproate produces a significant increase of the anticonvulsant effect of valproate against the clonus induced by pentylenetetrazole. These effects result in an increase in the therapeutic index of the association in relation to respective monotherapies. Furthermore, the anticonvulsant potentiation was not explained by a pharmacokinetic interaction. In addition, the anticonvulsant synergy was accompanied by an increase of GABA concentration in whole brain increase after adding a non-protective dose of lamotrigine to valproate, which was unexpected because lamotrigine in monotherapy significantly reduced brain GABA and warrants a further and detailed investigation. Our results add a new evidence of a synergistic pharmacodynamic interaction between valproate and lamotrigine and support the need for further experimental studies (in animals and humans) that definitely provide the basis for development of adjunctive therapy between them when monotherapy is ineffective.

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References

Arzimanoglou, A., Kulak, I., Bidaut-Mazel, C., Baldy-Moulinier, M., 2001. Optimal use of lamotrigine in clinical practice: results of an open multicenter trial in refractory epilepsy. Rev. Neurol. 157, 525–536.

Bourgeois, B.F.D., 1988a. Anticonvulsant potency and neurotoxicity of valproate alone and in combination with carbamazepine or phenobarbital. Clin. Neuropharmacol. 11, 348–359.

Bourgeois, B.F.D., 1988b. Combination of valproate and ethosuximide: antiepileptic and neurotoxic interaction. J. Pharmacol. Exp. Ther. 247, 1128–1132.

Brodie, M.J., Yuen, A.W.C.105 Study Group, 1997. Lamotrigine substitution study: evidence for synergism with sodium valproate? Epilepsy Res. 26, 423–432.

Chez, M.G., Bourgeois, B.F.D., Pippenger, C.E., Knowles, W.D., 1994. Pharmacodynamic interactions between phenytoin and valproate: individual and combined antiepileptic and neurotoxic actions in mice. Clin. Neuropharmacol. 17, 32–37.

Cramer, C.L., Stagnitto, M.L., Knowles, M.A., Palmer, G.C., 1994. Kainic acid and 4-aminopyridine seizure models in mice: evaluation of efficacy of anti-epileptic agents and calcium antagonists. Life Sci. 54, 271–275.

Davis, R., Peters, D.H., McTavish, D., 1994. Valproic acid: a reappraisal of its pharmacological properties and clinical efficacy in epilepsy. Drugs 47, 332–372.

De Sarro, G., Nava, F., Aguglia, U., De Sarro, A., 1996. Lamotrigine potentiates the antiseizure activity of some anticonvulsants in DBA/2 mice. Neuropharmacology 35, 153–158.

Dunham, N.W., Miya, T.S., 1957. A note on a simple apparatus for detecting neurological deficit in rats and mice. J. Am. Pharm. Assoc. 46, 208–209.

Fariello, R.G., Varasi, M., Smith, M.C., 1995. Valproic acid: mechanisms of action. In: Levy, R.H., Mattson, R.H., Meldrum, B.S. (Eds.), Antiepileptic Drugs, 4th ed. Raven Press, New York, NY, pp. 581–588.

Ferrie, C.D., Panayiotopoulus, C.P., 1994. Therapeutic interactions of lamotrigine and sodium valproate in intractable myoclonic epilepsy. Seizure 3, 157–159.

Ferrie, C.D., Robinson, R.O., Knott, C., Panayiotopoulos, C.P., 1995. Lamotrigine as an add-on drug in typical absence seizures. Acta Neurol. Scand. 91, 200–202.

Fraser, A.D., MacNeil, W., Isner, A.F., Camfield, P.R., 1995. Lamotrigine analysis in serum by high-performance liquid chromatography. Ther. Drug Monit. 17, 174–178.

Gordon, R., Gels, M., Wichmann, J.K., Diamantis, W., Sofia, R.D., 1993. Interaction of felbamate with several other antiepileptic drugs against seizures induced by maximal electroshock in mice. Epilepsia 34, 367–371.

Lamb, R.J., Miller, A.A., 1985. Effect of lamotrigine and some known anticonvulsant drugs on visually-evoked after-discharge in the conscious rat. Br. J. Pharmacol. 86, 765P (abstract).

Leach, J.P., 1997. Polypharmacy with anticonvulsants: focus on synergism. CNS Drugs 8, 366–375.

Leach, J.P., Sills, G.J., Butler, E., Forrest, G., Thompson, G.G., Brodie, M.J.,

1997. Neurochemical actions of gabapentin in mouse brain. Epilepsy Res. 27, 175–180.

Levy, R.H., Bourgeois, B.F.D., 1997. Drug–drug interactions. In: Engel, J., Pedley, T.A. (Eds.), Epilepsy: A Comprehensive Textbook. Lippincott-Raven, Philadelphia, pp. 1175–1179.

Litchfield, J.T., Wilcoxon, F., 1949. A simplified method of evaluating dose–effect experiments. J. Pharmacol. Exp. Ther. 96, 99–113.

Löscher, W., Fassbender, C.P., Gram, L., Gramer, M., Hörstermann, D., Zahner, B., Stefan, H., 1993. Determination of GABA and vigabatrin in human plasma by a rapid and simple HPLC method: correlation between clinical response to vigabatrin and increase in plasma GABA. Epilepsy Res. 14, 245–255.

Macdonald, R.L., 1999. Cellular actions of antiepileptic drugs. In: Eadie, F.J.E., Vajda, F.J.E. (Eds.), Antiepileptic Drugs. Pharmacology and Therapeutics. Springer-Verlag, Berlin, pp. 123–149.

McCabe, P.H., Michel, N.C., McNew, C.D., 1998. Clinical efficacy of combination therapy with valproic acid and lamotrigine in intractable epilepsy: high success rate with low incidence of rash. Epilepsia 39, 49 (abstract).

McCabe, P.H., McNew, C.D., Michel, N.C., 2001. Effect of divalproex–lamotrigine combination therapy in frontal lobe seizures. Arch. Neurol. 58, 1264–1268.

Michel, N.C., McCabe, P.H., McNew, C.D., 1998. Intractable epilepsy in mentally handicapped patients: valproic acid and lamotrigine combination improves seizure control and quality life. Epilepsia 39, 127.

Meldrum, B.S., Leach, M., 1994. The mechanism of action of lamotrigine. Rev. Contemp. Pharmacother. 5, 107–114.

Miller, A.A., Wheatley, P., Sawyer, D.A., Baxter, M.G., Roth, B., 1986. Pharmacological studies on lamotrigine, a novel potential antiepileptic drug: I. Anticonvulsant profile in mice and rats. Epilepsia 27, 483–489.

Panayiotopoulos, G.P., Ferrie, C.D., Knott, C., Robinson, R.O., 1993. Interaction of lamotrigine with sodium valproate. Lancet 341, 445 (letter).

Pisani, F., Di Perri, R., Perucca, E., Richens, A., 1993. Interaction of lamotrigine with sodium valproate. Lancet 341, 1224 (letter).

Pisani, F., Oteri, G., Russo, M.F., Di Perri, R., Perucca, E., Richens, A., 1999. The efficacy of valproate–lamotrigine comedication in refractory complex partial seizures: evidence for a pharmacodynamic interaction. Epilepsia 40, 1141–1146.

Privitera, M., Welty, T.E., 1998. Lamotrigine combined with valproate for idiopathic generalized epilepsy. Epilepsia 39, 52 (abstract).

Reutens, D.C., Duncan, J.S., Patsalos, P.N., 1993. Disabling tremor after lamotrigine with sodium valproate. Lancet 342, 185–186 (letter).

Turnell, D.C., Cooper, J.D.H., 1982. Rapid assay for amino acids in serum or urine by pre-column derivatization and reversed-phase liquid chromatography. Clin. Chem. 28, 527–531.

Sayin, Ü., Cengiz, S., Altug, T., 1993. Vigabatrin as an anticonvulsant against pentylenetetrazole seizures. Pharmacol. Res. 28, 325–331.

Smith, S.E., al-Zubaidy, Z.A., Chapman, A.G., Meldrum, B.S., 1993. Excitatory aminoacids, lamotrigine and BW 1003C87 as anticonvulsants in genetically epilepsy-prone rat. Epilepsy Res. 15, 101–111.

Sofia, R.D., 1995. Felbamate: mechanisms of action. In: Levy, R.H., Mattson, R.H., Meldrum, B.S. (Eds.), Antiepileptic Drugs, 4th ed. Raven Press, New York, NY, pp. 791–797.

Valdizán, E.M., Armijo, J.A., 1992. Effects of single and multiple increasing doses of vigabatrin on brain GABA metabolism and correlation with vigabatrin plasma concentration. Biochem. Pharmacol. 43, 2143–2150.

Wheatley, P.L., Miller, A.A., 1989. Effects of lamotrigine on electrically induced afterdischarge duration in anaesthetised rat, dog, and marmoset. Epilepsia 30, 34–40.

White, H.S., Wolf, H.H., Woodhead, J.H., Kupferberg, H.J., 1998. The national institutes of health anticonvulsant drug development program: screening for efficacy. In: French, J., Leppik, I., Dichter, M.A. (Eds.), Antiepileptic Drug Development. Advances in Neurology, vol. 76. Lippincott-Raven, Philadelphia, pp. 29–39.

Yamaguchi, S., Rogawski, M.A., 1992. Effects of anticonvulsant drugs on 4-aminopyridine-induced seizures in mice. Epilepsy Res. 11, 9–16.