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Neurochemical studies with the novel anticonvulsant levetiracetam in mouse brain

Graeme J. Sills [a,*], John Paul Leach [a], Caroline M. Fraser [a], Gerard Forrest [a], Philip N. Patsalos [b], Martin J. Brodie [a]

[a] *Epilepsy Unit, University Department of Medicine and Therapeutics, Western Infirmary, Glasgow G11 6NT, UK*
[b] *Institute of Neurology, National Hospital, Queen Square, London, UK*

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Abstract

Levetiracetam is a novel antiepileptic agent with a wide spectrum of activity against experimental and clinical seizures. The mechanism of its anticonvulsant action remains to be determined. We have investigated the effects of levetiracetam on several γ-aminobutyric acid (GABA)-related neurochemical parameters in mouse brain. Adult male mice were randomised into two groups and administered levetiracetam (0–300 mg/kg) intraperitoneally either as a single dose or twice daily for 5 days. Four hours after the final dose, animals were killed and their brains removed. Brain tissues were analysed for concentrations of GABA, glutamate and glutamine and for the activities of GABA-transaminase and glutamic acid decarboxylase. Single dose and repeated levetiracetam treatments were without effect on all of the parameters investigated. The anticonvulsant action of levetiracetam is unlikely to be mediated via an action on the GABAergic system. © 1997 Elsevier Science B.V.

Keywords: Levetiracetam; GABA (γ-aminobutyric acid); Anti-epileptic drug; Epilepsy

1. Introduction

The novel antiepileptic drug, levetiracetam, is the *S* enantiomer of the ethyl analogue of piracetam, a widely used nootropic agent (Löscher and Schmidt, 1993). It is currently undergoing clinical trial for the treatment of epilepsy and initial reports suggest that it has efficacy against a wide range of seizure types (Stables et al., 1995). Whereas piracetam and other cognitive enhancers have only limited anticonvulsant effects (Schmidt, 1990), levetiracetam has demonstrated a broad spectrum of activity in experimental seizure models. It is effective against both threshold (Löscher and Hönack, 1993) and maximal (Gower et al., 1992) convulsions induced by pentylenetetrazol and electroshock. It also protects against seizures induced by amygdaloid kindling and those precipitated by bicuculline, picrotoxin and *N*-methyl-D-aspartate (Gower et al., 1992; Löscher and Hönack, 1993). The drug has also demonstrated efficacy against audiogenic seizures in the genetically epilepsy-prone rat and against spike-wave discharges in the genetic absence epilepsy rat Strasbourg (GAERS) model of absence epilepsy (Gower et al., 1995).

The precise mechanism by which levetiracetam exerts its antiepileptic effects remains to be determined. It fails to interact with classical receptor and ion channel sites in the brain (Noyer et al., 1995). The drug has, however, been proposed to bind in a stereoselective manner to a specific site in central nervous system (CNS) membranes where it is weakly displaced by several other antiepileptic drugs and γ-aminobutyric acid (GABA)-related substances (Noyer et al., 1995). As a result, we have investigated the effects of single and repeated administration of levetiracetam on several GABA-related neurochemical parameters in mouse brain.

2. Materials and methods

2.1. Materials

Adult male mice (ICR strain; 25–30 g) were obtained from Harlan Olac (Bicester, UK) and were housed in a

* Corresponding author. Tel.: (44-141) 211-2770; Fax: (44-141) 334-9329; e-mail: g.j.sills@clinmed.gla.ac.uk

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controlled temperature and humidity environment with day/night cycle conditions and access to food and water ad libitum. All experimental work was governed by the Animals (Scientific Procedures) Act, 1986 (UK). Levetiracetam was obtained from UCB Pharmaceutical Sector (Chemin du Foriest, Belgium) and was prepared daily for intraperitoneal (i.p.) injection in 0.9% saline to varying concentrations for uniformity of injection volume. Radiolabelled GABA (γ-[^{14}C(U)]aminobutyric acid) was obtained from NEN Research Products (Stevenage, UK). All chemicals (reagent grade) were obtained from Sigma (Poole, UK) and solvents (high-performance liquid chromatography (HPLC) grade) were purchased from Rathburn (Walkerburn, UK).

2.2. Protein concentrations

Protein concentrations were determined by the sensitive BIORAD method which relies on the colour change of a dye (Coomassie Brilliant Blue G-250). Standards were prepared over the range 5–20 μg/ml bovine serum albumin. Samples of unknown protein concentration were diluted into this range. Bio-Rad dye reagent was diluted 1:1 with water and added to standards and samples alike. Tubes were mixed and incubated at room temperature for 5 min and then read at 595 nm in a spectrophotometer (MR5000, Dynatech, Guernsey, Channel Islands, UK). Results were corrected for dilution and expressed in mg/ml.

2.3. Amino acid concentrations

Amino acid (GABA, glutamate, glutamine) concentrations were analysed by a modification of the method of Durkin et al. (1988). Stock amino acid solutions (1 mg/ml in distilled water) were prepared monthly and diluted to working standard solutions daily as required. The derivatisation reagent mixture *o*-phthalaldehyde-3-mercaptopropionic acid was prepared weekly by dissolving 50 mg *o*-phthalaldehyde in a solution of 4.5 ml methanol, 0.5 ml borate buffer and 50 μl 3-mercaptopropionic acid. The borate buffer was prepared weekly by adjusting 0.5 M boric acid to pH 9.5 with 1 M NaOH.

All HPLC analyses were performed at room temperature on a Beckman Ultrasphere 5μ reversed phase column with a system which consisted of a Waters 6000 A pump (Waters/Millipore, Harrow, UK), a Shimadzu SIL-9A auto-injector (Dyson Instruments, Houghton-le-Spring, UK) and a Perkin-Elmer LS5 fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, UK). The excitation and emission wavelengths were 330 and 440 nm respectively with slitwidths set at 15 and 20 nm respectively. Chromatograms were recorded and integrated on a Jones Chromatography JCL6000 chromatography data system (Crawford Scientific, Strathaven, UK).

GABA concentrations were determined in mobile phase consisting of 60:40 (v/v) 0.2 M acetate buffer (pH 3.8, containing 100 mg/l ethylene diaminetetra-acetic acid (EDTA))/acetonitrile. Acetate buffer (0.2 M) was prepared by diluting 11.5 ml glacial acetic acid to 1 litre with water, adding 100 mg EDTA and adjusting the pH to 3.8 with 3 M NaOH. Glutamate and glutamine concentrations were determined simultaneously in mobile phase consisting of 80:20 (v/v) 0.57 M acetate buffer (pH 3.4, containing 100 mg/l EDTA)/acetonitrile. Acetate buffer (0.57 M) was prepared by diluting 32.8 ml glacial acetic acid to 1 litre with water, adding 100 mg EDTA and adjusting the pH to 3.4 with 3 M NaOH. Flow rates were 1.0 ml/min throughout.

Calibration curves were constructed for GABA (2–20 μg/ml), glutamate (15–150 μg/ml) and glutamine (5–50 μg/ml) and were seen to be linear ($r \geq 0.970$) in all cases. The limits of detection in a 50 μl sample were found to be 5 ng/ml, 25 ng/ml and 12.5 ng/ml for GABA, glutamate and glutamine respectively. Intra- and inter-assay variations were calculated at 2.8% and 7.9% for GABA (5 μg/ml), 4.7% and 9.2% for glutamate (30 μg/ml), and 4.1% and 8.5% for glutamine (10 μg/ml), respectively.

Mouse brains were homogenised in 10 volumes (v/w) of 1% perchloric acid. Samples were centrifuged at $600 \times g$ for 5 min at room temperature, the supernatant decanted and diluted 1/10 (v/v) with water prior to derivatisation. A 50 μl aliquot of the diluted supernatant was reacted with 200 μl methanol, 200 μl borate buffer, and 50 μl *o*-phthalaldehyde-3-mercaptopropionic acid solution. D,L-Norvaline (50 μl of 1 μg/ml stock) was added as an internal standard. Reaction mixtures were vortexed and allowed to stand at room temperature for 4 min prior to injection of 10 μl onto the column. Amino acid concentrations were calculated by comparison of peak height ratios of analyte to internal standard, quantified in relation to the wet weight of tissue and expressed as μg/g.

2.4. GABA transaminase activity

This method was devised from modifications of the methods of White and Sato (1978) and Larsson et al. (1986). An EDTA buffer, prepared weekly, consisted of 0.1 mM EDTA, 0.5 mM dithiothreitol, and 0.1 mM KH_2PO_4. Pyridoxal phosphate (PLP) was added daily as required (final concentration = 0.2 mM) and the buffer adjusted to pH 8.0 with 1 M NaOH. A [^{14}C]GABA incubation medium, prepared every 2–3 months, consisted of 2.70 mM GABA (specific activity 0.37 mCi/mmol), 1.8 mM EDTA, and 200 mM KH_2PO_4 with the pH adjusted to 8.0 with 1 M NaOH.

Mouse brains were homogenised in 4 volumes (v/w) of EDTA buffer. Samples were centrifuged at $600 \times g$ for 20 min at 4°C. The resultant supernatant was decanted and its protein content determined by the Bio-Rad method. The

remaining supernatant was adjusted to a protein concentration of 1 mg/ml with EDTA buffer. A 50 μl volume of the adjusted supernatant was added to 25 μl of 0.68 mM α-ketoglutaric acid and 25 μl of the [^{14}C]GABA incubation medium. Assays were performed in duplicate with a blank assay included for each sample by replacing the α-ketoglutaric acid with 25 μl water. Samples were mixed and incubated for 60 min at 37°C. The reaction was terminated by the addition of 10 μl 2 M HCl. The incubation mixtures were transferred to the surface of a resin in small disposable ion-exchange columns (Dowex AG50Wx8, pre-washed with deionised water, 0.5×3.0 cm, in 9 inch glass Pasteur pipettes plugged with a glass bead). Radioactive products were eluted directly into glass scintillation vials using 3 portions of 0.5 ml water. Twelve ml of Picofluor 40 (Canberra Packard, Pangbourne, UK) scintillation fluid was added to each vial and the disintegrations per minute (dpm) were counted in a liquid scintillation counter (2000CA TRI-CARB, Canberra Packard).

The radioactive content of samples was analysed in comparison to the dpm obtained from standard solutions containing known amounts of radioligand. Results were corrected for background and blank sample counts and quantified in relation to protein content and reaction time. Enzyme activities were expressed as nmol/min per mg protein.

2.5. Glutamic acid decarboxylase activity

This method was devised from modifications of the methods of Kochhar et al. (1989) and Wolf and Klemisch (1991). A sodium phosphate-2-aminoethylisothiouronium bromide (AET) buffer was prepared weekly and consisted of 0.1 M Na_2HPO_4 and 1 mM AET. The buffer was adjusted to pH 7.0 with 0.1 M NaH_2PO_4. An incubation medium was prepared daily and consisted of 50 mM L-glutamic acid, 250 μM PLP, 0.4% 2-mercaptoethanol and 57 μM gabaculline.

Mouse brains were homogenised in 10 volumes (v/w) sodium phosphate-AET buffer. Samples were centrifuged at $600 \times g$ for 10 min at room temperature. The supernatant was decanted and an aliquot taken for determination of protein content by the BIORAD method. Incubation medium (100 μl) was added to each of two 100 μl aliquots of supernatant per sample. The reaction in one aliquot (blank) was terminated immediately while the other (test) was allowed to continue for a period of 60 min at 37°C. Termination was performed in both cases by the addition of 100 μl 1% perchloric acid. Terminated blank and test reaction mixtures were diluted 1:10 with water and assayed for GABA content by the HPLC method described above.

Enzyme activity was calculated by subtraction of the blank GABA concentration from the test GABA concentration to give a value for GABA production during the reaction period. Results were quantified in relation to both reaction time and protein concentration and were expressed as nmol/min per mg protein.

2.6. Single dose studies

Mice were randomised into six treatment groups ($n = 12$/group) and levetiracetam was administered (i.p.) in doses of 1, 3, 10, 30, 100 and 300 mg/kg. A seventh group (control) received vehicle (0.9% saline) alone. At 4 h post-administration, the animals were killed and their brains removed. Brains were divided into two hemispheres by a sagittal incision and stored at −70°C until required.

2.7. Multiple dose studies

Mice were randomised into six treatment groups ($n = 12$/group) and levetiracetam was administered (i.p.) in doses of 1, 3, 10, 30, 100 and 300 mg/kg. A seventh group (control) received vehicle (0.9% saline) alone. Treatment was continued twice daily (8 a.m. and 4 p.m.) for 5 days. At 4 h after the final dose, the animals were killed and their brains removed. Brains were divided into two hemispheres by a sagittal incision and stored at −70°C until required.

2.8. Neurochemical assays

Six left hemispheres from each group (control and drug treatments) in each phase (single and multiple dose) were assayed for GABA concentrations. Six right hemispheres from each group in each phase were employed for the simultaneous determination of glutamate and glutamine concentrations. The remaining six left hemispheres were assayed for GABA-transaminase activity and the remaining six right hemispheres were employed for determination of glutamic acid decarboxylase activity.

2.9. Statistical methods

Statistical analysis was performed using MINITAB for Windows statistical package (Version 10.1) on a Viglen 4DX266 microcomputer. All results were calculated as the percentage of mean control values. Group results were then expressed as mean percentages ± the standard error of the mean (S.E.M.). Statistical differences from control were determined using one-way analysis of variance with a Dunnett correction for multiple comparisons.

3. Results

3.1. Amino-acid concentrations

Single dose and repeated treatments with levetiracetam were without effect on the concentrations of GABA (Fig. 1), glutamate (Fig. 2) and glutamine (Fig. 3) in mouse brain at 4 h post-administration.

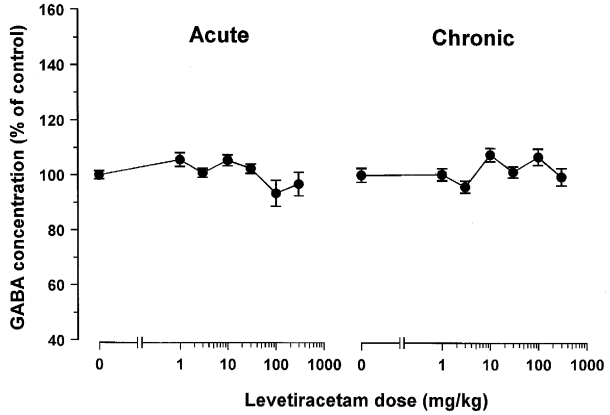

Fig. 1. Effect of levetiracetam (0–300 mg/kg) on mouse brain GABA concentration at 4 h after acute (single dose; left graph) and chronic (twice daily for 5 days; right graph) administration. Results ($n = 6$) are expressed as the percentage of individual control values and error bars denote the standard error of the mean.

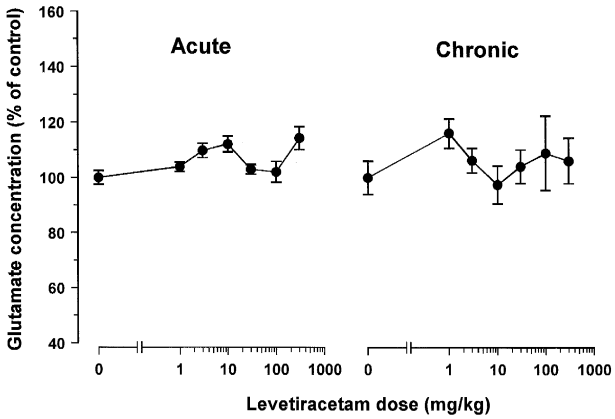

Fig. 2. Effect of levetiracetam (0–300 mg/kg) on mouse brain glutamate concentration at 4 h after acute (single dose; left graph) and chronic (twice daily for 5 days; right graph) administration. Results ($n = 6$) are expressed as the percentage of individual control values and error bars denote the standard error of the mean.

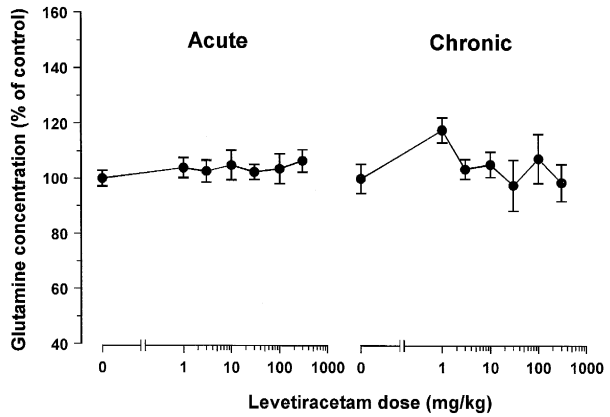

Fig. 3. Effect of levetiracetam (0–300 mg/kg) on mouse brain glutamine concentration at 4 h after acute (single dose; left graph) and chronic (twice daily for 5 days; right graph) administration. Results ($n = 6$) are expressed as the percentage of individual control values and error bars denote the standard error of the mean.

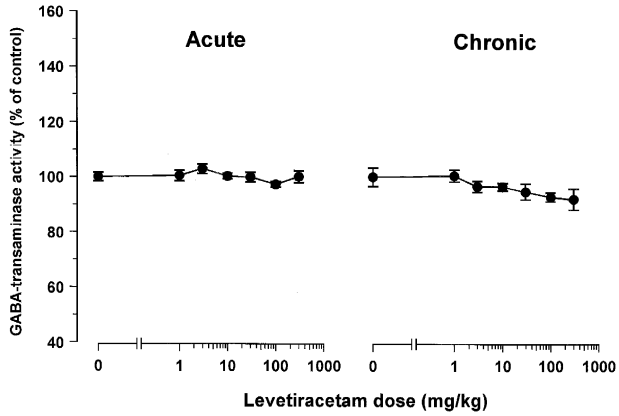

Fig. 4. Effect of levetiracetam (0–300 mg/kg) on mouse brain GABA-transaminase activity at 4 h after acute (single dose; left graph) and chronic (twice daily for 5 days; right graph) administration. Results ($n = 6$) are expressed as the percentage of individual control values and error bars denote the standard error of the mean.

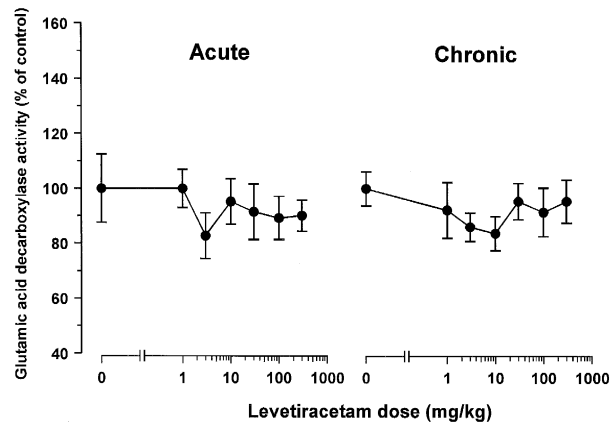

Fig. 5. Effect of levetiracetam (0–300 mg/kg) on mouse brain glutamic acid decarboxylase activity at 4 h after acute (single dose; left graph) and chronic (twice daily for 5 days; right graph) administration. Results ($n = 6$) are expressed as the percentage of individual control values and error bars denote the standard error of the mean.

3.2. Enzyme activities

Single dose and repeated treatments with levetiracetam were without effect on the activities of GABA-transaminase (Fig. 4) and glutamic acid decarboxylase (Fig. 5) in mouse brain at 4 h post-administration.

4. Discussion

Levetiracetam is a new antiepileptic agent with a novel spectrum of activity in both experimental seizure models and in man (Gower et al., 1992; Löscher and Hönack, 1993; Sharief et al., 1996). The mechanism by which it exerts these effects remains unknown. The drug has been shown to bind to a specific site in CNS membranes where it is weakly displaced by several other anticonvulsant

drugs and GABA-related compounds (Noyer et al., 1995). In an attempt to investigate its mechanism of action, we have made a preliminary study of the neurochemical effects of levetiracetam in mouse brain following both single and repeated administration. This approach, arguably applicable to the investigation of the mechanism of action of all antiepileptic drugs, has been employed in our laboratory to highlight previously unreported neurochemical actions of gabapentin (Leach et al., 1997) and remacemide (Leach et al., data not shown).

Levetiracetam failed to influence the concentrations of GABA, glutamate and glutamine in mouse brain following both single and repeated treatment. It was similarly without effect on the activities of GABA-transaminase and glutamic acid decarboxylase. These results imply that the GABAergic system is not involved in the mechanism of action of levetiracetam. However, it remains possible that subtle changes in amino-acid concentrations and/or enzyme activities were masked by the tissue volume employed. The use of techniques such as microdissection and microdialysis, which afford greater regional resolution, might clarify this issue. Nevertheless, Margineanu and Wülfert (1995) proposed that the reduction in bicuculline-induced hyperexcitability in rat hippocampal CA3 observed with levetiracetam was manifested via a non-GABAergic mechanism. In addition, the efficacy of the drug in the GAERS rat, a genetic animal model of absence seizures (Gower et al., 1995) lends further weight to the non-GABAergic theory of levetiracetam action. It has previously been proposed that antiepileptic drugs which exert their effects via a GABAergic mechanism exacerbate the spontaneous discharges observed in this model (Vergnes et al., 1984). Thus, it is possible that levetiracetam may be effective against generalised absence seizures in man.

Drawing a similar parallel, the efficacy of levetiracetam against maximal electroshock seizures (Gower et al., 1992), audiogenic seizures in genetically epilepsy-prone rats (Gower et al., 1995) and amygdaloid-kindled seizures (Löscher and Hönack, 1993) might suggest clinical activity in partial and secondary generalised epilepsy. This theory is supported by preliminary evidence in refractory patients (Sharief et al., 1996). Traditionally, drugs active in these experimental seizure models have inhibitory actions at voltage-sensitive sodium channels in the brain (Löscher and Schmidt, 1988; Rogawski and Porter, 1990). However, there is, at present, no evidence to suggest that levetiracetam has any activity at this site (Saccan and Lloyd, 1994).

The theoretical evidence discussed above, derived from studies with experimental seizure models, might suggest a multi-factorial mechanism of action for levetiracetam, such as that proposed for sodium valproate (Davies, 1995) and the new antiepileptic drug topiramate (Coulter et al., 1995; White et al., 1995). This proposal is supported by the similarity in experimental anticonvulsant profiles of levetiracetam and sodium valproate (Rogawski and Porter, 1990; Gower et al., 1992; Löscher and Hönack, 1993).

In conclusion, the new antiepileptic drug levetiracetam failed to influence GABA-related neurochemistry in mouse brain following both single and repeated administration. Although these results suggest that the drug is unlikely to exert its anticonvulsant effects by an action on the GABA system, they are far from conclusive. There is evidence to suggest that levetiracetam has a wide spectrum of anticonvulsant activity and thus perhaps multiple mechanisms of action. Further studies are required to investigate this suggestion.

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