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Tiagabine prevents seizures, neuronal damage and memory impairment in experimental status epilepticus

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Abstract

A novel antiepileptic drug, tiagabine ((*R*)-*N*-[4,4-di-(3-methylthien-2-yl)but-3-enyl] nipecotic acid hydrochloride), was studied in rats in order to determine its efficacy in preventing seizures, seizure-induced neuronal damage and impairment of spatial memory in the perforant pathway stimulation model of status epilepticus. In pilot experiments, administration of tiagabine (50, 100 or 200 mg/kg/day) with subcutaneously implanted Alzet osmotic pumps led to a dose-dependent increase in tiagabine concentrations in the serum and brain. Two days of tiagabine treatment at a dose range of 50–200 mg/kg/day did not change the levels of γ-aminobutyric acid (GABA), glutamate or aspartate in cisternal cerebrospinal fluid (CSF) compared to the controls. In the pentylenetetrazol test, the maximal anticonvulsive effect of tiagabine administered via osmotic pumps was achieved already with a dose of 50 mg/kg/day. In the perforant pathway model of status epilepticus, subchronic treatment with tiagabine (Alzet pumps, 50 mg/kg/day) completely prevented the appearance of generalized clonic seizures during stimulation ($P < 0.001$). In the same rats, tiagabine treatment reduced the loss of pyramidal cells in the CA3c and CA1 fields of the hippocampus ($P < 0.05$) but not the loss of somatostatin immunoreactive neurons in the hilus. Two weeks after perforant pathway stimulation, the tiagabine-treated rats performed better in the Morris water-maze test than the vehicle-treated rats did ($P < 0.001$). Our results show that tiagabine treatment reduces the severity of seizures in the perforant pathway stimulation model of status epilepticus. Possibly associated with the reduction in seizure number and severity, tiagabine treatment also reduced seizure-induced damage to pyramidal cells in the hippocampus as well as the impairment of the spatial memory associated with hippocampal damage.

Keywords: GABA (γ-aminobutyric acid); Epilepsy; Hippocampus; Neuroprotection; Perforant pathway stimulation

1. Introduction

Hippocampal damage is found in 60–70% of patients undergoing temporal lobe surgery for drug-refractory temporal lobe epilepsy (Babb and Pretorius, 1993). The damage includes a loss of hippocampal pyramidal neurons and hilar interneurons as well as a reduction in the number of hilar mossy cells (Babb and Pretorius, 1993; Gloor, 1991). Data obtained from the kindling model of temporal lobe epilepsy show that the severity of neuronal damage as well as the amount of accompanying mossy-fiber sprouting correlate with the number of seizures (Cavazos et al., 1991, 1994). Some data suggest that also in human epilepsy the seizures may cause progressive neuronal damage in the hippocampus (Mathern et al., 1995; Mourizen-Dam, 1980; DeGiorgio et al., 1992; Saukkonen et al., 1994). Interestingly, it was recently suggested that this kind of selective and progressive neuronal damage and the reorganization of the synaptic connections of the surviving neurons change the properties of synaptic circuitries, making them more favorable for the generation of seizures both in animals and human (Sloviter, 1994).

We recently found that pretreatment of rats with vigabatrin, a γ-aminobutyric acid (GABA) transaminase inhibitor, prevents seizure-induced neuronal damage in the hippocampus as well as impairment of spatial memory in the perforant pathway stimulation model of status epilepticus (Ylinen et al., 1991; Pitkänen and Halonen, 1994). We also found that carbamazepine treatment prevents seizures as effectively as vigabatrin does (Pitkänen and Halonen,

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1994). However, carbamazepine treatment did not prevent neuronal damage. Sutula et al. (1992) found that in seizures induced with kainic acid, phenobarbital treatment prevents seizures, mossy fiber sprouting and damage to the hilar neurons. However, phenobarbital did not prevent damage to CA3 and CA1 neurons. Thus, the data available indicate that both novel and conventional anticonvulsants are effective in preventing seizures but may differ in terms of neuroprotective efficacy. This may be significant in evaluating the final position of the drug in the treatment of epilepsy.

Tiagabine ((*R*)-*N*-[4,4-di-(3-methylthien-2-yl)but-3-enyl] nipecotic acid hydrochloride, previously called NO-328) is a new antiepileptic drug which inhibits GABA uptake into neurons and glia (Braestrup et al., 1990; Nielsen et al., 1991). Tiagabine readily crosses the blood-brain barrier and in vivo increases the extracellular levels of GABA in the brain (Fink-Jensen et al., 1992). Tiagabine has anticonvulsant efficacy in several animal models of epilepsy (Faingold et al., 1994; Nielsen et al., 1991; Walton et al., 1994; Suzdak and Jansen, 1995). According to first Phase II studies, 25–50% of patients with uncontrolled complex partial seizures experienced a 50% or greater reduction in seizure frequency during the open-label phase of the study compared to the baseline (Chadwick et al., 1991; Richens et al., 1992). Little is known, however, about the effects of tiagabine on neuronal damage and the associated impairment of memory.

The present study was designed to answer three questions: (1) Does tiagabine treatment prevent partial and generalized seizures in the perforant pathway model of status epilepticus? (2) Does it prevent seizure-induced neuronal damage in the temporal lobe structures, in particular, in the hippocampus? (3) Does it prevent the impairment of spatial memory caused by prolonged seizures?

2. Materials and methods

2.1. Animals

Male Han:Wistar rats ($n = 71$) (300–350 g) were used in this study. After surgery the rats were housed in individual cages at a temperature of 20 ± 1°C with humidity maintained at 50–60% and lights on from 07:00 to 19:00 h. Standard food pellets and water were freely available. The rats were weighed at the beginning and end of the experiments.

2.2. Pilot studies aimed at investigating the usefulness of Alzet osmotic pumps for subchronic administration of tiagabine

The biological half-life of tiagabine in the blood of the rat is only 2–3 h following chronic administration of tiagabine (Suzdak and Jansen, 1995). Therefore, to avoid fluctuations in the tiagabine level in the serum and brain, we did a pilot study in which we investigated the usefulness of subcutaneously implanted osmotic minipumps for subchronic delivery of tiagabine. We also determined whether tiagabine administration resulted in a dose-dependent increase of tiagabine in serum and brain tissue. In addition, we investigated whether the tiagabine administered via minipumps had any effects (1) on the levels of neurotransmitter amino acids (GABA, glutamate and aspartate) in the cisternal cerebrospinal fluid (CSF) and (2) on behavioral seizures induced with pentylenetetrazol.

2.2.1. Determination of the in vitro pumping rate

Tiagabine (Novo Nordisk, Malov, Denmark) was dissolved in propylene glycol at concentrations of 70, 140 and 280 mg/ml (the concentrations were adjusted to correspond to in vivo doses of 50, 100 and 200 mg/kg/day, respectively). Thereafter, Alzet 2ML1 osmotic pumps (volume 2 ml) (Alza Corp., Palo Alto, CA, USA) were filled with the tiagabine solution (two pumps per concentration), immersed in a 50-ml test tube prefilled with 30 ml of isotonic saline, and incubated at 37°C for 4 h. The pumps were transfered to new tubes filled with saline every 24 h for 7 days. For determination of tiagabine concentrations in order to calculate the pumping rate of tiagabine, a 2 ml aliquot was taken from each tube daily. The samples were stored at −70°C until analyzed. At the end of the in vitro test, the tiagabine solution remaining in the pumps was removed for determination of the amount of tiagabine left in the pump.

2.2.2. Placement of cannulas into cisterna magna for CSF collection

The animals ($n = 27$) were anesthetized with an intraperitoneal injection of an anesthetic cocktail (60 mg/kg sodium pentobarbital and 100 mg/kg chloral hydrate) and placed in a David Kopf stereotaxic frame. Permanent polyethylene cannulas were implanted into the cisterna magna as described by Jolkkonen et al. (1986).

2.2.3. Administration of different doses of tiagabine via Alzet osmotic pumps

Two days after implantation of the cannulas, Alzet 2ML1 osmotic pumps were filled with different doses of tiagabine dissolved in propylene glycol. The tiagabine concentrations were calculated individually for each rat to obtain daily administration of 50 ($n = 7$), 100 ($n = 7$), or 200 mg/kg/day ($n = 6$), at a pumping rate of 9.56 μl/h (this value is based on information provided by the manufacturer). Thus, the tiagabine concentrations in the pumps were adjusted to 68.4 ± 0.6, 140.7 ± 2.9 or 277.6 ± 13.8 mg/ml, respectively.

The animals were anesthetized and the pumps were implanted subcutaneously under the skin on the back slightly posterior to the scapulae, according to the protocol of the manufacturer.

2.2.4. CSF collection

CSF was collected from freely moving rats 2 days after implantation of the Alzet osmotic pump. The plug was removed, and cisternal CSF (about 100 μl) was slowly withdrawn with a syringe (CSF was obtained from 19 rats). The samples were centrifuged immediately, and the supernatant was frozen in liquid nitrogen and stored at −70°C until assayed.

2.2.5. Anticonvulsant efficacy of subchronic administration of tiagabine via Alzet osmotic pumps in the pentylenetetrazol test

Seven days after implantation of the osmotic pump (tiagabine doses 50, 100 or 200 mg/kg/day), the rats were injected with 70 mg/kg (i.p.) of pentylenetetrazol. The appearance of behavioral seizures was observed for 1 h. Behavioral motor seizures were classified as follows: (1) partial motor seizure (jaw clonus, head nodding, myoclonic jerks or partial clonus of a forelimb), (2) generalized clonic seizure (bilateral forelimb clonus, rearing, rearing and falling), and (3) generalized tonic-clonic seizure.

Two different control groups were studied: (1) rats with vehicle in the Alzet pump ($n = 7$), and (2) rats without pumps ($n = 14$).

2.2.6. Collection of blood and brain tissue samples

Immediately after the pentylenetetrazol test, the rats were deeply anesthetized and blood samples were collected by cardiac puncture. Thereafter, the brains were removed from the skull and frozen in liquid nitrogen. Serum and brain samples were kept at −70°C until analyzed.

2.2.7. Determination of GABA, glutamate and aspartate levels in cisternal CSF

The concentrations of GABA, glutamate and aspartate in the cisternal CSF were analyzed by reverse-phase high performance liquid chromatography (HPLC) by using precolumn derivatization of amino acids with *o*-phthaldialdehyde (Valtonen et al., 1995). Briefly, amino acids were separated on a Nova-Pak C18 (3.9 × 150 mm, 4 μm, 60 Å) column with detection by a Merck Hitachi F1000 fluorescence spectrophotometer (ex 330 nm/em 450 nm). Mobile phase A was 0.10 M Na_2HPO_4 (pH 6.4) and 10% methanol, and B was 62.5% methanol (linear gradient, 35 min) at a rate of 1.2 ml/min. The limit of detection for GABA, glutamate and aspartate was < 30 fmol.

2.2.8. Determination of tiagabine levels in the in vitro test, serum and brain samples

Samples from in vitro tests were diluted in the mobile phase used for HPLC, to which internal standard had been added, and assayed by the HPLC assay used for serum. Calibrators were also prepared in the mobile phase. The concentration of tiagabine base (ng/ml = 0.87 × tiagabine HCl concentration) was determined in serum samples (200 μl), using solid-phase extraction for clean up and HPLC with electrochemical detection (Gustavson and Chu, 1992).

Brains were homogenized for 30 s in demineralized water (3.0 ml/g tissue) using an Ultra Turrax T25 homogenizer. After centrifugation at 2500 × *g* for 20 min, 1.0 ml samples of the supernatant were assayed in the same way as serum. For preparation of the calibration curve, the initial stock solution of tiagabine was serially diluted in the drug-free homogenate of the control rats.

2.3. Effects of tiagabine on seizures, neuronal damage and spatial memory impairment induced by perforant pathway stimulation

2.3.1. Electrode placement

The animals were anesthetized and placed in a Kopf stereotaxic frame (lambda and bregma at the same horizontal level). For recording population spikes, a pair of insulated stainless steel electrodes (electrode tips 0.8 mm apart) were implanted bilaterally into the hippocampus (4.1 mm posterior, 2.6 mm lateral, and 3.5 mm ventral to the bregma according to the atlas of the rat brain compiled by Paxinos and Watson (1986)). The lower tip of the electrode was aimed at the hilus of the dentate gyrus and the upper tip at the pyramidal cell layer of CA1. For stimulation of the perforant pathway, a similar pair of electrodes (tip separation 0.5 mm) was implanted bilaterally into the angular bundle (atlas coordinates 7.0 mm posterior, 4.5 mm lateral, and 4.1 mm ventral to the bregma). Two stainless steel screws inserted bilaterally into the skull above the cerebellum served as indifferent and ground electrodes. The electrodes were fixed with dental acrylate. The location of the electrodes was later verified histologically, and only the rats with correct electrode placements were included in the analysis.

2.3.2. Electrical stimulation of perforant pathway

Rats were allowed to recover from the surgery for 6–8 days. Thereafter, the location of the stimulation electrodes was tested just before pump implantation by stimulating the angular bundle and recording the evoked potentials in the hilus. Both sides were tested separately. Rats in which the evoked potentials in the hilus could be elicited at a current level of 1.5 mA or less were used in the experiments. The side on which the population spikes could be evoked with a lower current was chosen for the experiment. Three days after the beginning of drug administration (see section 2.3.4), the perforant pathway was stimulated by using a modification of the method of Sloviter (1983, 1987). In the present experiments, the perforant pathway was stimulated in unanesthetized, freely moving rats with 2 mA current (20 Hz, 0.1 ms pulse duration) for 60 min (Grass 88 stimulator, Grass PSIU 6 stimulus isolation units).

2.3.3. Electrophysiological and behavioral characterization of seizures

During the stimulation session, the appearance of population spikes in the hilus and the duration of total spiking time were measured. A modification of Racine's scoring scale (Racine, 1972) was used to characterize the severity of behavioral seizures: Class 1: jaw clonus, head nodding; Class 2: clonic jerks of the contralateral forelimb; Class 3: bilateral forelimb clonus; Class 4: forelimb clonus and rearing with back fully extended; Class 5: forelimb clonus with rearing and falling. The number and duration of different behavioral seizures during perforant pathway stimulation were measured.

2.3.4. Tiagabine administration

Three groups of rats were included in the study: (1) sham-operated, vehicle-treated controls, which had electrodes implanted in the angular bundle and hippocampus but which were not stimulated ($n = 10$); (2) vehicle-treated, stimulated rats ($n = 10$); (3) tiagabine-treated, stimulated rats ($n = 10$).

Tiagabine was given via Alzet osmotic pumps, which allowed administration of tiagabine at a constant rate (see the results obtained in the pilot study). The dose of 50 mg/kg/day was chosen based on data obtained in our pilot studies, which showed that 50 mg/kg/day of tiagabine was enough to prevent tonic-clonic seizures in the pentylenetetrazol test. This dose also resulted in tiagabine concentrations in the serum and brain that had previously been shown to be anticonvulsant in other models of epilepsy (Walton et al., 1994; Suzdak and Jansen, 1995). Tiagabine and vehicle treatments were started immediately after the evoked potentials were recorded. Tiagabine was dissolved in propylene glycol (61.76 ± 4.18 mg/ml). The Alzet 2ML1 osmotic pumps (pumping rate 10.61 μl/h, information provided by the manufacturer) were filled with either the tiagabine or the vehicle solution. The pumps were implanted subcutaneously according to the protocol of the manufacturer 3 days before stimulation of the perforant pathway. The pumps were removed 4 days after perforant pathway stimulation. All surgical operations were done under anesthesia and aseptic conditions.

2.3.5. Morris water maze

The spatial learning and memory of rats was tested in the Morris water maze for 5 days (Morris et al., 1982) (for a detailed description of the equipment used, see Riekkinen et al., 1990). The first trial was started 5 days after discontinuation of the drug. The daily tests consisted of ten 60-s trials. The location of the platform was changed daily so that on days 1, 3, and 5 the platform was in quadrant 1, and on days 2 and 4 in quandrant 3 (the opposite site of the maze pool). The escape latency, i.e. the time taken by the rats to find the platform, the swimming speed and the total swim path length (distance) were measured.

2.3.6. Histological processing of brain tissue

2.3.6.1. Fixation. Two weeks after stimulation of the perforant pathway (immediately after finishing the water maze test), the rats were perfused intracardially according to the following fixation protocol: 0.9% NaCl (30 ml/min) for 2 min followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (30 ml/min), +4°C, for 30 min. The brains were postfixed in the same fixative for 2 h and then placed in a cryoprotection solution containing 20% glycerol in 0.02 M potassium phosphate buffered saline (KPBS), pH 7.4, for 24–36 h. The brains were then cut into blocks, frozen in dry ice and stored at −70°C until cut. They were sectioned in the coronal plane (1-in-5 series) at a thickness of 30 μm with a sliding microtome. The sections were stored in a cryoprotectant tissue-collecting solution (30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer) at −20°C until processed.

2.3.6.2. Immunohistological staining of somatostatin. A 1-in-5 series of free-floating sections was collected from tissue-collecting solution and stained by the avidin-biotin technique. The sections were washed 3 times (10 min each time) in 0.02 M KPBS, pH 7.4. To block nonspecific binding, the sections were incubated in a solution containing 10% normal horse serum and 0.5% Triton X-100 in 0.02 M KPBS for 2 h at room temperature. The primary incubation was performed with a 1 : 1000 dilution of monoclonal mouse anti-somatostatin antibody (No. K121, Biomeda, CA, USA), 0.5% Triton X-100, 1% NHS in 0.02 M KPBS, pH 7.4, at +4°C for 3 days. Then the sections were washed 3 times (10 min each time) in 0.02 M KPBS containing 2% NHS. Thereafter, they were incubated in a solution containing horse biotinylated antimouse IgG (Vector, BA-2000; Burlingame, CA, USA), 1% NHS and 0.3% Triton X-100 in 0.02 M KPBS, pH 7.4 for 1 h at room temperature. The sections were washed twice as described above and incubated for 45 min at room temperature in avidin-biotin solution (11001-1, Biomeda, Super ABC kit) in 0.02 M KPBS, pH 7.4. The sections were washed 2 times (10 min each time) with 2% NHS in KPBS. They were then incubated with the second antibody solution for 45 min. After two 10-min washes in KPBS, they were incubated with avidin-biotin complex for 30 min, and again washed in KPBS 3 times (10 min each time). The sections were then incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (0.05% DAB and 0.04% H_2O_2 in 0.02 M KPBS, pH 7.4) and washed 3 times (10 min each time) in KPBS. The sections were then mounted on gelatin-coated slides, dried overnight at 37°C and the stain was intensified with OsO_4 and thiocarbohydrazide according to the method of Lewis et al. (1986).

2.3.6.3. Silver impregnation. Degeneration of neurons was determined by silver impregnation using the procedure described by Gallyas et al. (1980). Briefly, an adjacent

series of sections was incubated for 10 min in a pretreatment solution containing 2% NaOH and 2.5% NH_4OH. Thereafter, the sections were incubated in an impregnating solution containing 0–0.8% NaOH, 2.5% NH_4OH and 0.5% $AgNO_3$. Sections were washed 3 times (5 min each time) in a solution containing 0.5% Na_2CO_3 and 0.01% NH_4NO_3 in 30% ethanol. Thereafter, they were developed in a solution containing 0.4–0.6% formaldehyde and 0.01% citric acid in 10% ethanol (pH 5.0–5.5) for 1 min and washed 3 times (10 min each time) in 0.5% acetic acid. All steps were done at room temperature. Sections were mounted on gelatin-coated slides, dehydrated and covered with coverslips and Depex.

2.3.6.4. Other stainings. To identify the cytoarchitectonic boundaries of different regions of the brain, an adjacent series of sections was stained for thionin. In addition, thionin-stained sections were analyzed for detection of damaged neurons and gliosis.

2.3.7. Analysis of sections

The sections were analyzed with a Nikon Optiphot-2 microscope with both brightfield and darkfield optics. All analyses were performed blindly.

2.3.7.1. Silver impregnation. The scale described by Freund et al. (1991) was used for semiquantitative analysis of cell death in the hippocampus. Briefly, a score of (0) no damage, (1) 1–10% of the neurons damaged, (2) 10–50% of the neurons damaged, and (3) more than 50% of the neurons damaged. Only shrunken argyrophilic neurons were considered irreversibly degenerated. The silver score given for each animal was the mean score calculated for 12 successive sections (1-in-5 series, 30 μm) from the septal end of the hippocampus. In other areas of the temporal lobe (piriform cortex, entorhinal cortex, perirhinal cortex, subiculum, presubiculum, parasubiculum and the amygdaloid complex) the presence of damage was analyzed but its severity was not scored. Neuronal damage and gliosis in adjacent Nissl-stained sections were analyzed side-by-side with silver-stained sections.

2.3.7.2. Calculation of the number of somatostatin immunoreactive neurons. The number of somatostatin-immunoreactive neurons in the hippocampus (section thickness 30 μm, 1-in-5 series) was calculated manually at a magnification of 200 × with the aid of an ocular grid. The area of the hilus in the same sections was then measured using a Quantimet 570 Image Analysis System (Leica Cambridge, Cambridge, UK). Statistical analysis of the mean areas of the hilus did not indicate any shrinkage of these areas compared to sham-operated controls. Thus, the somatostatin-immunoreactive cell counts are expressed as somatostatin-immunoreactive neurons/mm^2 instead of mean numbers of cells. For each rat, the density of somatostatin-immunoreactive neurons/mm^2 in the septal end of the hippocampus was obtained by calculating the mean density of somatostatin-immunoreactive neurons from 12 successive sections adjacent to the silver-stained sections.

Photomicrographs were taken with the Nikon Optiphot-2 system.

2.4. Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the dose-response effect of tiagabine. For statistical evaluation of the anticonvulsive efficacy of tiagabine, the Pearson χ^2-test was used. For evaluation of the severity of neuronal damage in the hippocampus, the Mann-Whitney U-test was used to compare the different treatment groups. Wilcoxon's test for paired samples was used to compare the differences between the ipsilateral and contralateral sides. The Morris water maze data were evaluated by using an analysis of variance (ANOVA) between groups with training days as covariate. The level of significance was $P < 0.05$.

3. Results

3.1. Dose-response study

3.1.1. In vitro release of tiagabine

In in vitro conditions the rate of release of tiagabine from osmotic pumps loaded with 70, 140, or 280 mg/ml of tiagabine stabilized by the second day. For the different loadings the mean amounts of tiagabine released from pumps during days 2–7 were 14.1 ± 3.6, 25.2 ± 4.2, and 48.8 ± 5.6 mg/day, respectively. This represents 78.9–105.0% of the expected release (Table 1). When the concentration of tiagabine left in the pumps on the 7th day of the experiment was verified by determining its concentration in the solution remaining in the osmotic pumps, it was found to be 84.6–101.6% of the expected concentrations (Table 1).

3.1.2. GABA, glutamate and aspartate concentrations in CSF during tiagabine treatment

The concentrations of GABA, glutamate and aspartate in the cisternal CSF were measured 2 days after implantation of the Alzet osmotic pumps. Compared to the controls, the GABA levels were elevated by 27%, 6%, and 27% in rats receiving 50 ($n = 5$), 100 ($n = 5$) or 200 ($n = 4$) mg/kg of tiagabine, respectively, per day (statistically nonsignificant) (Fig. 1A). During the tiagabine treatment, the levels of glutamate and aspartate in the cisternal CSF were also unaltered (Fig. 1B and C).

3.1.3. Tiagabine concentrations in serum and brain tissue

Tiagabine concentrations increased dose dependently in serum with increasing doses of tiagabine administered ($P < 0.001$, one-way ANOVA) (Fig. 2A). We found no

Table 1
Release of tiagabine from Alzet osmotic pumps.

Pump No.	Target concentration in the pumps (mg/ml)	Actual concentration in the pumps (mg/ml)	Percentage of target concentration	Target release (mg) (mean of days 2–7)	Actual release (mg/day)	Percentage of target release
1	70.0	59.3	84.7	15.15	15.91	105.0
2	70.0	62.9	89.8	15.15	12.26	81.0
3	140.0	122.1	87.2	30.27	26.54	87.7
4	140.0	127.3	90.9	30.27	23.88	78.9
5	280.0	266.0	95.0	60.54	48.72	80.5
6	280.0	284.4	101.6	60.54	48.76	80.5

Tiagabine was dissolved in 100% propylene glycol. The in vitro recovery study was done according to the manufacturer's suggestions (see Materials and methods).

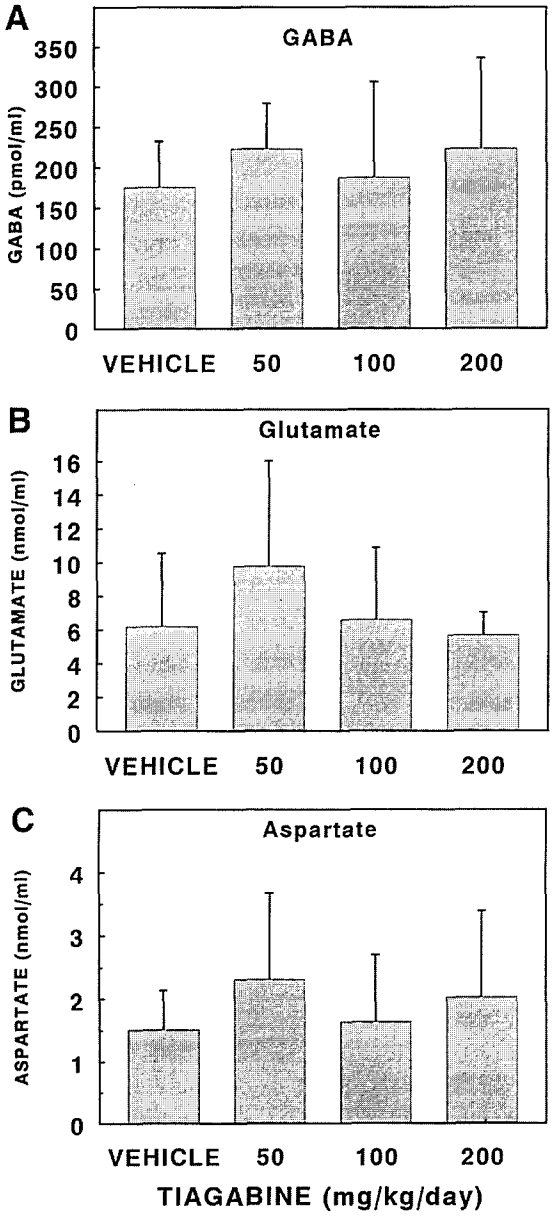

Fig. 1. Concentrations of (A) GABA (pmol/ml ± S.D.), (B) glutamate (nmol ± S.D.), and (C) aspartate (nmol/ml ± S.D.) in the cisternal cerebrospinal fluid of rats receiving vehicle or different doses of tiagabine (50, 100, or 200 mg/kg/day) via Alzet 2ML1 osmotic pumps. The differences were not statistically significant.

correlation between the serum concentration of tiagabine and the GABA levels in the CSF ($r = 0.055$, $P > 0.05$).

With increasing doses of tiagabine, tiagabine concentrations in brain tissue also increased dose dependently ($P < 0.001$, one-way ANOVA) (Fig. 2B). Tiagabine concentrations in the brain correlated well with those in the serum ($r = 0.946$, $P < 0.001$). We found no correlation between the tiagabine concentration in the brain and the GABA concentration in the CSF ($r = 0.020$, $P > 0.05$).

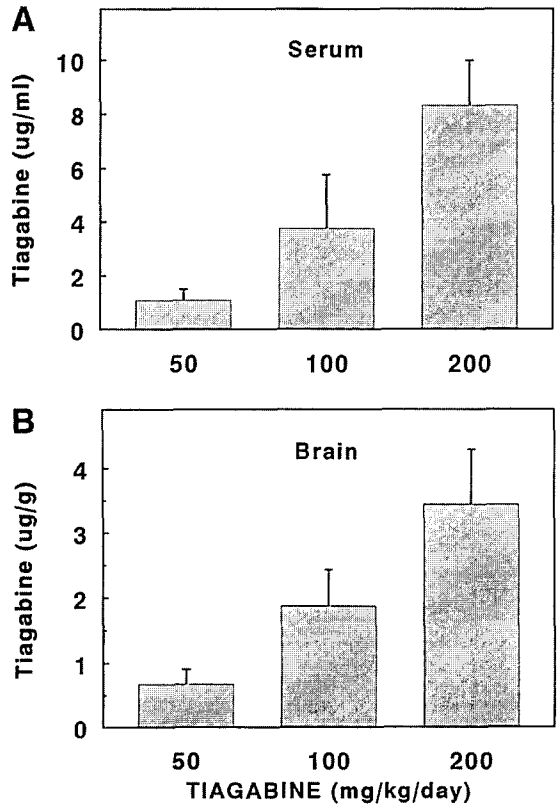

Fig. 2. Tiagabine concentrations in (A) serum and (B) brain tissue of rats receiving vehicle or different doses of tiagabine (50, 100, or 200 mg/kg/day) via Alzet 2ML1 osmotic pumps. Tiagabine concentrations (μg/ml ± S.D. and μg/g brain tissue ± S.D., respectively) increased dose dependently in both serum and brain tissue ($P < 0.001$, one-way analysis of variance).

3.1.4. Effect of tiagabine treatment of seizures induced with pentylenetetrazol

Pentylenetetrazol (70 mg/kg, i.p.) induced tonic-clonic seizures in 13 of 14 (92.3%) unoperated control rats (no minipump placement). Five of seven (71.4%) vehicle-treated rats had pentylenetetrazol-induced tonic-clonic seizures. None of the tiagabine-treated rats (three dose groups, $n = 20$) had tonic-clonic seizures ($P < 0.001$ combined tiagabine groups versus vehicle controls, χ^2-test). Tiagabine did not, however, prevent the appearance of partial or generalized clonic seizures induced by pentylenetetrazol (Table 2).

3.2. Perforant pathway stimulation

3.2.1. Appearance of seizures induced by perforant pathway stimulation

During stimulation of the perforant pathway, the duration of spiking was similar in the different groups of rats, which suggests that the granule cells were equally activated in all treatment groups (Fig. 3A).

The mean number of partial (Class 1–2) and generalized (Class 3–5) seizures in the vehicle and tiagabine groups is presented in Fig. 3B and C. During stimulation tiagabine effectively decreased the number and duration of partial seizures ($P < 0.05$ compared to vehicle-treated rats) and completely prevented the appearance of generalized seizures ($P < 0.001$ compared to vehicle-treated rats).

3.2.2. Morris water maze task

Perforant pathway-stimulated rats (vehicle and tiagabine groups) were impaired in finding the platform compared to the sham-operated, unstimulated controls (increased escape latency, $P < 0.001$). For both the vehicle and tiagabine groups the swim path length was also increased compared to that of the sham-operated controls ($P < 0.001$). However, the tiagabine group had a shorter escape latency and swim path length compared to the vehicle group ($P < 0.001$) (Fig. 4A and B).

The vehicle group had an increased swimming speed and the tiagabine group a decreased swimming speed compared to the sham-operated controls ($P < 0.05$ and $P < 0.01$, respectively). In addition, the tiagabine group

Table 2
Effect of tiagabine on seizures induced by pentylenetetrazol (70 mg/kg, i.p.)

Treatment	Most severe seizure type [c]			
	Partial clonic	Generalized clonic	Tonic-clonic	n
Controls	0	1	13	14
Vehicle	0	2	5	7
Tiagabine (mg/kg/day)				
50	1	6	0 [b]	7
100	0	7	0 [b]	7
200	2	4	0 [b]	6
all	3	17	0 [a]	20

The most severe type of behavioral seizure seen in an animal after injection of pentylenetetrazol was used to classify the rat into a certain seizure type (partial clonic, generalized clonic, tonic-clonic). Tiagabine protected against tonic-clonic seizures induced by pentylenetetrazol ([a] $P < 0.001$, tiagabine-treated rats [rats receiving different doses were combined] compared with the vehicle group; [b] $P < 0.01$, rat groups receiving different doses of tiagabine compared with the vehicle group, χ^2-test). Abbreviations: i.p., intraperitoneal, n, total number of rats in each group.
[c] See details of the seizure classification in Materials and methods.

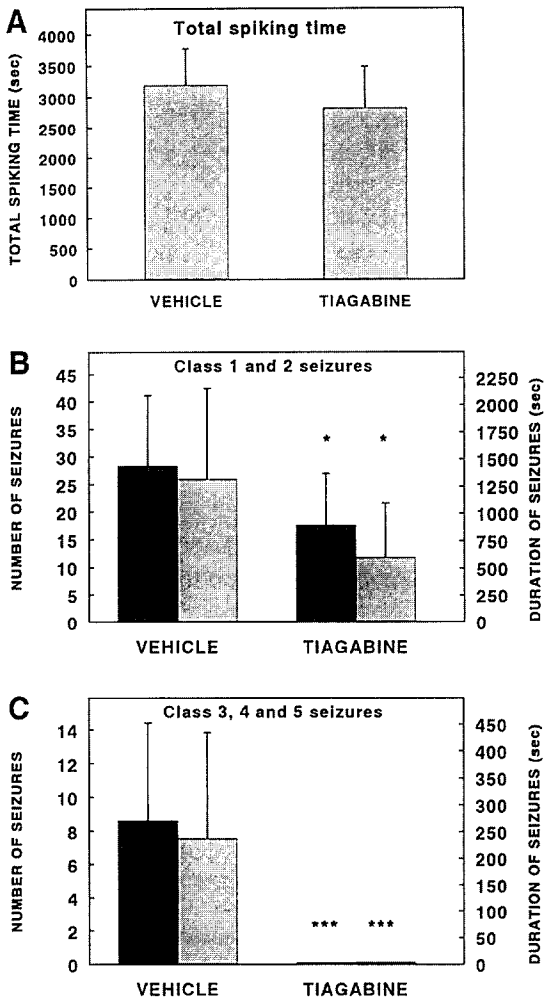

Fig. 3. Panel A: Total time of appearance of population spikes in the dentate gyrus during perforant pathway stimulation (mean ± standard deviation). In each group, the mean spiking time was > 50% of the total stimulation time (60 min). No difference was found between the vehicle-treated and tiagabine-treated groups. Panels B and C show the efficacy of tiagabine treatment in preventing behavioral seizures during stimulation of the perforant pathway. Different treatment groups are shown on the x-axis. Seizure number (darker bar) is shown on the left y-axis and seizure duration (lighter bar) on the right y-axis. Panel B: Partial seizures (Class 1 and 2). The number and duration of partial seizures decreased in the tiagabine group compared to the vehicle-treated group ($P < 0.05$, Mann-Whitney U-test). Panel C: Generalized clonic seizures (Class 3–5) in vehicle and tiagabine-treated groups. Tiagabine completely prevented the appearance of generalized seizures ($P < 0.001$, Mann-Whitney U-test).

had a decreased swimming speed compared to the vehicle-treated animals ($P < 0.001$) (Fig. 4C).

3.2.3. Severity of neuronal damage

The number of somatostatin-immunoreactive neurons in the hilus and the severity of neuronal damage in the pyramidal cells of the hippocampus proper in the different treatment groups are summarized in Tables 3 and 4. Representative sections from each treatment group demonstrating the extent and severity of neuronal damage in the hippocampus are presented in Fig. 5.

3.2.3.1. Hippocampal formation. All stimulated, vehicle-treated rats had neuronal damage in the hippocampus (Fig. 5C and D). The neuronal damage in vehicle-treated animals was more severe in the septal end of the hippocampus than in the temporal end (data not shown).

Somatostatin immunoreactive neurons in the hilus: The number of somatostatin-immunoreactive neurons in the hilus of the septal end of the dentate gyrus in different treatment groups is summarized in Table 3.

Septal end ipsilaterally. In vehicle-treated stimulated rats, 62% of the somatostatin-immunoreactive neurons were left compared to the 100% of sham-operated, unstimulated controls ($P < 0.05$). In the tiagabine group, 74% of the somatostatin-immunoreactive neurons were preserved, which did not differ from the sham-operated controls or from the vehicle-treated rats.

Septal end contralaterally. In vehicle-treated rats, 76% of the somatostatin-immunoreactive neurons were left. In the tiagabine group, 71% of the somatostatin-immunoreactive neurons remained, which did not differ from the percentage in sham-operated controls or vehicle-treated rats.

Pyramidal cells in CA3: According to the nomenclature of Lorente de No (1934), the transverse axis of the CA3 can be divided into three parts (Fig. 5A): the proximal portion of CA3 or CA3c, the mid-portion or CA3b, and the distal portion or CA3a. The severity of neuronal damage, as determined from silver staining in different portions of CA3, varied and is summarized in Table 3.

Septal end ipsilaterally. In vehicle-treated rats, the damage was more severe in the CA3c region than in the CA3b or CA3a regions (Table 3) (Fig. 5C). In the tiagabine group, the pyramidal cell damage in CA3c was less severe than in the vehicle group ($P < 0.05$) and did not differ from that in sham-operated controls (Table 3) (Fig. 5A and E). In the CA3b and CA3a regions, pyramidal cell damage in the vehicle and tiagabine groups did not differ from that in the sham-operated controls.

Septal end contralaterally. In the CA3c, CA3b and CA3a regions, pyramidal cell damage in the vehicle and tiagabine groups did not differ from that in the sham-operated controls.

Pyramidal cells in CA1: In the vehicle-treated rats, the hippocampal pyramidal cell damage in CA1 was more severe in the septal end than in the temporal end (data not shown). The CA1 pyramidal cell damage was equally severe in both hemispheres.

Septal end ipsilaterally. In the tiagabine group, the pyramidal cell damage in the CA1 region was more severe ($P < 0.001$) than that in the sham-operated controls (Table 3) but less severe than that in the vehicle group ($P < 0.05$) (Table 3). In this same tiagabine group, the CA1 damage was more severe on the stimulation side than on the contralateral side ($P < 0.05$).

Septal end contralaterally. In the tiagabine group, the pyramidal cell damage in the CA1 region was more severe than in the sham-operated controls ($P < 0.01$) (Table 3), but less severe than in the vehicle group ($P < 0.05$).

3.2.3.2. Other temporal lobe areas. Our initial analysis of silver-impregnated sections from vehicle-treated rats indicated that the dentate gyrus and the hippocampus proper were not the only areas of the temporal lobe that were damaged. Damage was also found in the subiculum, the

Table 3
Neuronal cell damage in different regions of the septal end of the hippocampus

Group	SOM-ir in hilus	Silver score			
	(neurons/mm^2)	CA3c	CA3b	CA3a	CA1
Ipsilateral to stimulation					
Sham (10)	92.8 ± 33.5	0	0	0	0
Vehicle (10)	57.7 ± 16.5 [a]	1.20 ± 1.23 [b]	0.40 ± 0.84	0.50 ± 1.08	1.80 ± 0.92 [c]
Tiagabine (10)	69.4 ± 24.3	0.20 ± 0.63 [d]	0	0	1.00 ± 0.47 [c,d,e]
Contralateral to stimulation					
Sham (10)	97.8 ± 31.9	0	0	0	0
Vehicle (10)	74.2 ± 34.9	0.80 ± 1.32	0.50 ± 0.85	0.60 ± 1.08	1.50 ± 1.18 [c]
Tiagabine (10)	69.3 ± 34.8	0	0	0	0.50 ± 0.71 [a,d]

Statistical significances: [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ compared to unstimulated, sham-operated controls (Mann-Whitney U-test); [d] $P < 0.05$ compared to the vehicle group (Mann-Whitney U-test); [e] $P < 0.05$ compared to the contralateral side (Wilcoxon's test). The number of rats in each group is shown in parentheses. Sham-operated rats were not stimulated, vehicle- and tiagabine-treated rats were stimulated as described in Materials and methods. SOM-ir = somatostatin-immunoreactive neurons.

parasubiculum and the amygdaloid complex as well as in the entorhinal, perirhinal and piriform cortices (Table 4). In the tiagabine group, the damage in the extrahippocampal areas was less extensive than in the vehicle group

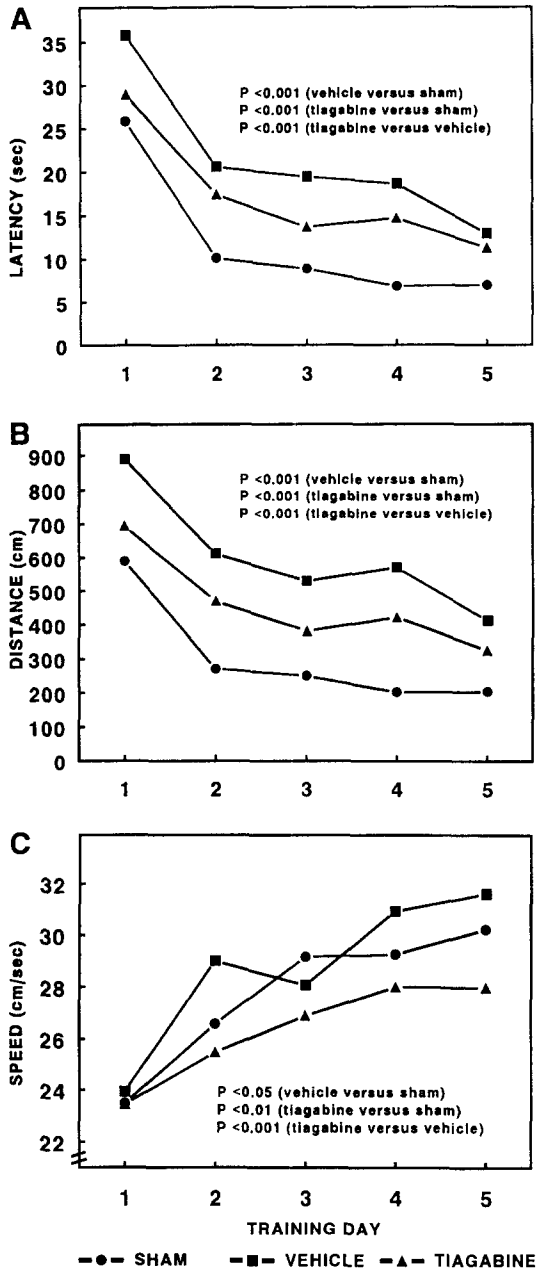

Fig. 4. Performance of different rat groups in the Morris water maze task. (A) Escape latency (s), (B) swim path length (distance, cm), and (C) swimming speed (cm/s) to submerged platform in different groups. Stimulated rats (the vehicle and tiagabine groups) had an increased escape latency ($P < 0.001$) and distance swum ($P < 0.001$) compared to unstimulated, sham-operated controls (analysis of variance). The tiagabine group had shorter escape latency and distance swum compared to the vehicle group ($P < 0.001$). The vehicle group had an increased and the tiagabine group decreased swimming speed compared to the sham-operated controls ($P < 0.05$ and $P < 0.01$, respectively). In addition, the tiagabine group had a decreased swimming speed compared to the vehicle-treated animals ($P < 0.001$).

Table 4
Appearance of neuronal damage in the extrahippocampal temporal lobe regions in different treatment groups

	Number of rats with ipsilateral damage/contralateral damage		
	Sham (10)	Vehicle (10)	Tiagabine (10)
Subiculum	0/0	2/2	0/0
Presubiculum	0/0	0/0	0/0
Parasubiculum	0/0	4/3	4/4
Amygdala	0/0	5/5	2/1
Piriform cortex	0/0	3/3	0/0
Perirhinal cortex	0/0	6/5	5/0
Entorhinal cortex	0/0	6/6	3/3

The number of rats in each group is shown in parentheses. Vehicle- and tiagabine-treated rats were stimulated as described in Materials and Methods. Sham-operated rats underwent the same surgical operation as the rats in the vehicle and tiagabine groups but were not stimulated. In vehicle and tiagabine groups the neuronal damage in the extrahippocampal regions differed on the stimulation side and on the contralateral side from that of sham-operated controls ($P < 0.001$). Tiagabine-treated rats had less damage on the stimulation and contralateral sides than did the vehicle-treated animals ($P < 0.05$ and $P < 0.01$, respectively, χ^2-test).

(ipsilateral side: $P < 0.05$; contralateral side: $P < 0.01$, χ^2-test) and tended to be limited to the side of stimulation (Table 4).

4. Discussion

In the present study, we evaluated the efficacy of tiagabine in preventing epileptic seizures, seizure-induced neuronal damage and memory impairment. The major findings of this study were as follows. First, the use of osmotic pumps provided a convenient way to deliver tiagabine at a constant rate for several days. Second, tiagabine completely prevented generalized seizures during electrical stimulation of the perforant pathway. The number of partial seizures was also reduced. Third, the decrease in neuronal damage produced by tiagabine treatment was clearer in the pyramidal cell layer of the hippocampus than in the hilus of the dentate gyrus or in the extrahippocampal areas. The neuroprotective effect was clearest in the hippocampus contralateral to the side of stimulation. Fourth, tiagabine treatment did not change the CSF levels of GABA, glutamate and aspartate. Fifth, tiagabine pretreatment reduced the seizure-induced impairment of memory.

We found that high concentrations (> 400 mg/ml) of tiagabine can be dissolved in propyleneglycol. Its high solubility makes it possible to administer tiagabine via osmotic pumps at anticonvulsant doses. The in vitro test showed that tiagabine was released from the osmotic pumps at a relatively constant rate and that a constant pumping rate was achieved by the second day. Owing to the short half-life of tiagabine, this is a clear advantage in experimental designs in which subchronic treatment with

tiagabine is desired. This is also favored by the fact that tiagabine is a relatively stable compound and does not decompose at body temperature during a one-week study (Novo Nordisk, unpublished observation).

By administering tiagabine via osmotic pumps, the concentrations of tiagabine in the serum and brain increased in a dose-dependent manner. In our study, the tiagabine dose of 50 mg/kg/day resulted in a serum concentration of

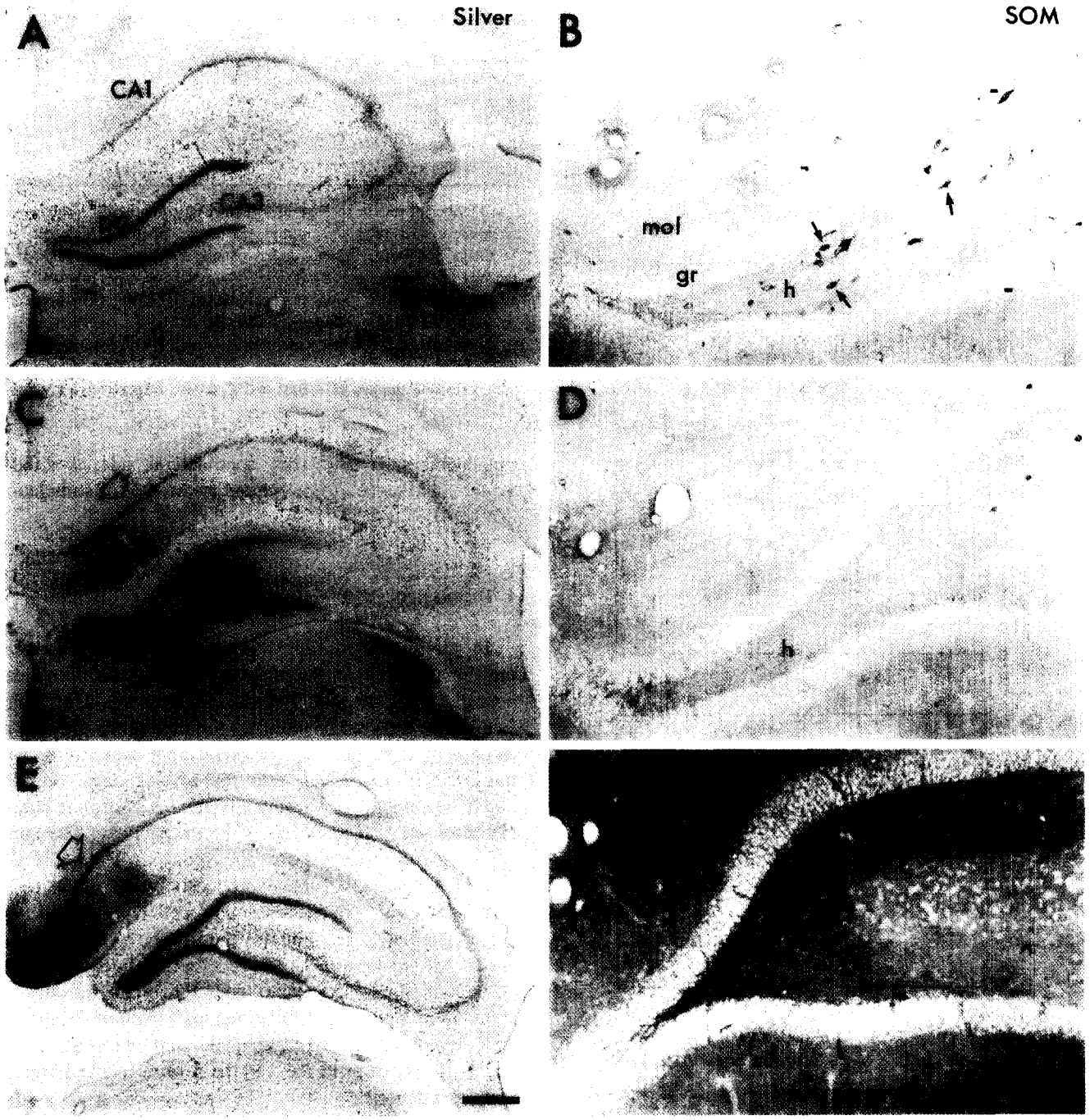

Fig. 5. Appearance of neuronal damage in the septal end of the hippocampus in representative sections from each treatment group: panels A and B, unstimulated, sham-operated control rats; panels C and D, stimulated, vehicle-treated rats; panels E and F, stimulated, tiagabine-treated rats (50 mg/kg/day via Alzet pumps). Photomicrographs on the left (panels A, C and E) demonstrate the appearance of damage in silver-stained sections. Note the argyrophilic damaged neurons in the CA3c (open arrowhead) and CA1 (closed arrow) regions of the hippocampus proper in a vehicle-treated rat (panel C). In the tiagabine-treated animal (panel E), the extent of damage was less severe than in the vehicle-treated animal (compare panels C and E; closed arrow indicates damage in the CA1 region in panel E). Photomicrographs on the right (panels B, D and F) demonstrate the severity of loss of somatostatin-immunoreactive neurons in the hilus of the dentate gyrus in different treatment groups (sections are adjacent to the silver-stained sections). Some immunopositive neurons are indicated with arrowheads in panel B. The vehicle-treated rat (panel D) had 56% and the tiagabine-treated rat (panel F) 65% of the somatostatin-immunoreactive neurons left compared to the controls. Abbreviations: DG, dendate gyrus; gr, granule cell layer; h, hilus; mol, molecular layer; SOM, somatostatin-immunoreactive neurons. Scalebars A, C, E equal 200 μm; B, D, F equal 100 μm.

1091 ng/ml, which corresponds to the mean C_{max} (1202 ng/ml) obtained with the administration of a single oral dose of 40 mg/kg of tiagabine (Novo Nordisk, unpublished observation). Similar serum concentrations of tiagabine (1000 ng/ml) were also obtained with the 8.3 mg/kg (i.p.) dose of tiagabine required to control (ED_{50}) generalized tonic-clonic seizures in status epilepticus induced by injection of homocysteine thiolactone into cobalt-lesioned rats (Walton et al., 1994). Our results show that the serum concentration obtained by administering 50 mg/kg/day of tiagabine via minipumps is high enough to prevent the appearance of tonic-clonic seizures in the pentyleneterazole model as well as to prevent generalized seizures in the perforant pathway stimulation model.

GABA levels in the CSF reflect the concentration of GABA in the brain (Grove et al., 1983). In previous microdialysis studies, systemic administration of tiagabine (11.5 or 21.0 mg/kg i.p as a single dose) increased the extracellular concentrations of GABA by 100–250% in the striatum and pars reticulata portion of the substantia nigra (Fink-Jensen et al., 1992). In the present study, we found that with anticonvulsant doses of tiagabine of 50–200 mg/kg/day, the GABA levels in the cisternal CSF were elevated only 6–27%. This statistically nonsignificant increase did not correlate with the tiagabine dose or the tiagabine concentration in the serum and brain. This finding was not unexpected because tiagabine increases the GABA concentrations mainly locally in synaptic clefts but leaves the metabolism of GABA intact. In this respect, tiagabine differs from another anticonvulsant, vigabatrin, which increases GABA levels by 5- to 10-fold in brain tissue by irreversibly inhibiting the GABA-metabolizing enzyme, GABA transaminase, both in neurons and in glia (see Grant and Heel, 1991). In vigabatrin-treated rats, this dose-dependent increase in the tissue level of GABA results in a dose-dependent increase in the CSF level of GABA (Halonen et al., 1991).

We found that tiagabine treatment completely prevented the appearance of tonic-clonic seizures in the pentylenetetrazol test (model of primary generalized seizures) as well as generalized seizures during perforant pathway stimulation (model of status epilepticus with focal onset) but only partially prevented the appearance of generalized clonic seizures after pentylenetetrazol injection or partial seizures during perforant pathway stimulation. These findings agree with earlier studies which showed that tiagabine is particularly efficient in controlling tonic seizures induced with pentylenetetrazol in rats and mice (Nielsen et al., 1991) or by homocysteine thiolactone injection in cobalt-lesioned rats (Walton et al., 1994). Tiagabine also effectively controls audiogenic seizures in mice (Nielsen et al., 1991) and rats (Faingold et al., 1994; Smith et al., 1995) that are genetically prone to epilepsy. Furthermore, tiagabine suppresses the generalized clonic seizures induced by DMCM in mice (Nielsen et al., 1991) or by electrical stimulation of the amygdaloid complex in kindled rats (Suzdak and Jansen, 1995). Thus, tiagabine treatment seems to prevent the generalization of seizures, probably by suppressing the spread of seizures in the extrahippocampal regions. This suggestion agrees with our finding that there was less extrahippocampal neuronal damage in the tiagabine group than in the vehicle group. In addition, the contralateral damage in the hippocampus was less severe than the ipsilateral damage.

In this study, the neuronal damage in the hippocampus was less severe in tiagabine-treated rats than in vehicle-treated rats. The tiagabine treatment most effectively prevented damage to the pyramidal cells in the CA3c and CA1 fields of the hippocampus proper. The somatostatin-immunoreactive cells in the hilus, in contrast, were not protected. It is difficult to conclude whether the neuroprotective effect of tiagabine treatment was associated with its anticonvulsant properties during stimulation or with its potency to prevent delayed neuronal damage after stimulation. Interestingly, we found that the duration of population spiking in the dentate gyrus during the perforant pathway stimulation did not differ between the vehicle-treated and tiagabine-treated animals. This indicates that the discharge rate of dentate granule cells was similar in both groups and that the tiagabine treatment did not reduce the activation of granule cells during perforant pathway stimulation. It is interesting that in our earlier study with the same seizure model, the duration of population spiking in the vigabatrin group was similar to that of the tiagabine-treated rats in the present study. Nevertheless, vigabatrin treatment failed to prevent the appearance of generalized seizures (Pitkänen and Halonen, 1994), whereas in the present study generalized seizures were completely prevented. However, the neuronal damage, particularly the loss of somatostatin-immunoreactive neurons in the hilus, was prevented more effectively by vigabatrin treatment (92% left) than by tiagabine treatment (75% left) (Pitkänen and Halonen, 1994). These results suggest that different anticonvulsant drugs are not equally potent in their neuroprotective efficacy. Previously, Mikati et al. (1994) found that chronic phenobarbital treatment actually exacerbated the neuronal damage in the CA1 pyramidal cells of rats previously subjected to kainate-induced seizures and status epilepticus. In another study, Sutula et al. (1992) reported that phenobarbital treatment prevented damage to hilar neurons but not to the CA3 and CA1 pyramidal neurons in the hippocampus of rats treated with kainic acid. Thus, the results of these studies suggest that the neuroprotective efficacy of an anticonvulsant may differ in different areas of the brain as well as in different populations of neurons. Furthermore, our study indicates that partial seizures may cause neuronal damage also even in the extrahippocampal areas.

Neuronal damage in the hippocampus and the impairment of declarative memory are not uncommon findings in patients with chronic drug-refractory epilepsy of temporal lobe origin (Bornstein et al., 1988; Lencz et al., 1992). The

animal data indicate even more clearly the association between hippocampal neuronal loss and memory impairment (Cavazos et al., 1994). Several studies have shown that the extent of neuronal damage in the hippocampus is associated with the impairment of spatial memory in the Morris water maze (Morris et al., 1982; Sutherland et al., 1983; Ylinen et al., 1991). Accordingly, in the present study the tiagabine-treated rats had less severe damage in the CA3 and CA1 areas of the hippocampus and were less impaired in the water-maze task than the vehicle-treated animals were.

In conclusion, we have shown that tiagabine administered at a dose of 50 mg/kg/day via osmotic pumps is anticonvulsive in the perforant pathway stimulation model of status epilepticus. Tiagabine treatment also prevented seizure-induced pyramidal cell damage in the hippocampus and alleviated impairment of spatial learning and memory, which may partly be explained by the prevention of generalized seizures and hippocampal damage. The data obtained in the present experimental studies suggest that tiagabine is a promising drug for the treatment of human epilepsy.

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