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# NMDA receptor-mediated pilocarpine-induced seizures: characterization in freely moving rats by microdialysis

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**1** Pilocarpine administration has been used as an animal model for temporal lobe epilepsy since it produces several morphological and synaptic features in common with human complex partial seizures. Little is known about changes in extracellular neurotransmitter concentrations during the seizures provoked by pilocarpine, a non-selective muscarinic agonist.

**2** Focally evoked pilocarpine-induced seizures in freely moving rats were provoked by intrahippocampal pilocarpine (10 mM for 40 min at a flow rate of 2 $\mu$l min$^{-1}$) administration via a microdialysis probe. Concomitant changes in extracellular hippocampal glutamate, $\gamma$-aminobutyric acid (GABA) and dopamine levels were monitored and simultaneous electrocorticography was performed. The animal model was characterized by intrahippocampal perfusion with the muscarinic receptor antagonist atropine (20 mM), the sodium channel blocker tetrodotoxin (1 $\mu$M) and the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (dizocilpine maleate, 100 $\mu$M). The effectiveness of locally (600 $\mu$M) or systemically (10 mg kg$^{-1}$ day$^{-1}$) applied lamotrigine against the pilocarpine-induced convulsions was evaluated.

**3** Pilocarpine initially decreased extracellular hippocampal glutamate and GABA levels. During the subsequent pilocarpine-induced limbic convulsions extracellular glutamate, GABA and dopamine concentrations in hippocampus were significantly increased. Atropine blocked all changes in extracellular transmitter levels during and after co-administration of pilocarpine. All pilocarpine-induced increases were completely prevented by simultaneous tetrodotoxin perfusion. Intrahippocampal administration of MK-801 and lamotrigine resulted in an elevation of hippocampal dopamine levels and protected the rats from the pilocarpine-induced seizures. Pilocarpine-induced convulsions developed in the rats which received lamotrigine perorally.

**4** Pilocarpine-induced seizures are initiated via muscarinic receptors and further mediated via NMDA receptors. Sustained increases in extracellular glutamate levels after pilocarpine perfusion are related to the limbic seizures. These are arguments in favour of earlier described NMDA receptor-mediated excitotoxicity. Hippocampal dopamine release may be functionally important in epileptogenesis and may participate in the anticonvulsant effects of MK-801 and lamotrigine. The pilocarpine-stimulated hippocampal GABA, glutamate and dopamine levels reflect neuronal vesicular release.

**Keywords:** Microdialysis; electrocorticography; glutamate; GABA; dopamine; NMDA; pilocarpine; lamotrigine; hippocampus

## Introduction

In epilepsy research one of the challenges is to develop an animal model that resembles as close as possible the neuropharmacological, neurophysiological and morphological sequelae of human epilepsy. Elucidation of the basic mechanisms of action of new and even marketed antiepileptic drugs in such an animal model, may contribute to the optimization of pharmacotherapy in epilepsy. Since Olney *et al*., (1983) showed that cholinoceptor agonists induce seizure-related brain damage, a variety of histological, behavioural, receptor binding and electrophysiological studies in which cholinomimetics – such as the non-selective muscarinic agonist pilocarpine – were applied, have been published (review Turski *et al*., 1989). However, little is known about alterations in extracellular neurotransmitter levels during such seizures.

Hippocampus is an area of interest to investigate the pilocarpine-induced seizures, because it is one of the most vulnerable brain areas for epilepsy-related brain damage and plays a main role in the development and maintenance of limbic seizures. The projection from the medial septal area to the hippocampus is cholinergic (Moor *et al*., 1994). Muscarinic receptors (Rotter, 1984) and NMDA receptors (Cotman *et al*., 1987) are widely distributed in the hippocampal region. The hippocampal formation contains a rich glutamatergic and $\gamma$-aminobutyric acid (GABA)-ergic input, GABA-ergic interneurones containing peptide co-transmitters and the glutamatergic perforant pathway which interconnects the entorhinal cortex, subiculum, CA1–CA3 fields and dentate gyrus (Ottersen & Storm-Mathisen, 1984 Kupferman, 1991).

Systemic administration of pilocarpine has been used as an animal model for temporal lobe epilepsy and has several features in common with the human complex partial seizures. The most striking similarity was probably that pilocarpine produced marked changes in morphology, membrane properties and synaptic responses of hippocampal rat neurones, comparable to those observed in human epileptic hippocampal neurones (Isokawa & Mello, 1991). The N-methyl-D-aspartate (NMDA) receptor was involved in the altered synaptic responses of pilocarpine-treated dentate granule cells. An NMDA receptor-mediated component of excitatory synaptic input to gyrus dentatus was also found in 70% of hippocampi surgically removed from human brain for the treatment of medically intractable complex partial seizures (Urban *et al*., 1990). Paradoxically, pilocarpine-induced seizures in mice were aggravated after systemic administration of the selective non-competitive NMDA receptor antagonist MK-801 (Starr & Starr, 1993).

'Focally evoked' pilocarpine-induced seizures mimic the generation of complex partial seizures closer than limbic epilepsy induced by systemic administration of a drug. Millan *et al*., (1993) were the first to evoke such seizures by administering pilocarpine via a microdialysis probe in the hip-

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pocampus of anaesthetized rats. In this microdialysis study, we used this experimental approach in freely moving rats. Seizures were provoked by intrahippocampal pilocarpine perfusion, concomitant changes in hippocampal GABA, glutamate and dopamine efflux were monitored and simultaneous electrocorticography (ECoG) recordings were registered. The animal model was characterized by use of the muscarinic receptor antagonist atropine, the sodium channel blocker tetrodotoxin and the NMDA receptor antagonist MK-801. Since it is presumed that pilocarpine-induced seizures are primarily mediated via muscarinic receptor stimulation, we must provide evidence that the effects can be abolished by muscarinic receptor blockade. Blood-brain barrier permeability changes during seizures could interfere with possible changes in extracellular amino acid levels (Pont *et al*., 1995). Thus, for investigating the neuronal origin of hippocampal amino acid and dopamine release, tetrodotoxin was administered. Due to contradictory results on a NMDA receptor-mediated mechanism, the effect of intrahippocampal perfusion with MK-801 was tested. Furthermore, the effectiveness of locally or systemically applied lamotrigine against pilocarpine-induced seizures was evaluated. Lamotrigine is a quite novel antiepileptic drug which inhibits voltage-sensitive sodium currents through a preferential interaction with slow inactivated sodium channels, thereby suggesting that it may act selectively against high frequency epileptiform discharges (Fitton & Goa, 1995).

## Methods

### *Chromatographic assays*

Chromatographic conditions and precolumn derivatization procedures for the amino acids have been described previously in detail (Smolders *et al*., 1995). For GABA reversed-phase microbore liquid chromatography with isocratic elution and electrochemical detection was used. Precolumn derivatization was performed with *o*-phtalaldehyde/*tert*-butylthiol and iodoacetamide. Glutamate analysis was carried out after precolumn derivatization with *o*-phtalaldehyde/$\beta$-mercaptoethanol, by reversed-phase microbore liquid chromatography with gradient elution and fluorescence detection. The chromatographic assay for dopamine was an isocratic reversed-phase ion-pair microbore liquid chromatography method with electrochemical detection and has also been described previously (Smolders *et al*., 1996a).

### *Microdialysis*

The protocols for the animal experiments described in this study were performed according to national rules on animal experiments and institutional guidelines of the Faculty of Medicine of our University.

Male albino Wistar rats, weighing 270–300 g, were anaesthetized with a mixture of ketamine:diazepam (50:5 mg $kg^{-1}$) and mounted on a stereotaxic frame. Intracranial guides with inner cannulae (CMA Microdialysis, Stockholm, Sweden) were implanted in the dorsal hippocampus. The coordinates towards bregma were L +4.6, A −5.6 and V +4.6 (Paxinos & Watson, 1986). Immediately after surgery the inner cannulae were replaced by CMA 10 microdialysis probes (CMA Microdialysis, Stockholm, Sweden) with a 3 mm membrane length. Probes were continuously perfused with modified Ringer solution at a flow rate of 2 $\mu$l $min^{-1}$ (CMA 100 microdialysis pump, CMA Microdialysis, Stockholm, Sweden). The animals were placed in the plexiglass microdialysis cages in which the experiments were to be carried out, and were allowed to recover from surgery overnight. During the experiments, dialysates were collected every 20 min, yielding 40 $\mu$l samples, from freely moving animals. All drugs were administered at the same flow rate of 2 $\mu$l $min^{-1}$ when applied intrahippocampally. Collection vials contained 10 $\mu$l of the below described antioxidant mixture. At the end of the experiments, the rats were killed with an overdose of pentobarbitone.

### *Group 1 (control group): intrahippocampal perfusion with pilocarpine*

The hippocampus was perfused with modified Ringer solution and 8 dialysates were collected in basal conditions. Then, 10 mM pilocarpine (s.f. pilocarpine hydrochloride) was administered into the hippocampus via the microdialysis probe for 40 min (2 dialysates), after which the perfusion fluid was switched back to modified Ringer solution until the end of the experiment.

### *Group 2: intrahippocampal perfusion with atropine followed by co-administration of pilocarpine*

After 6 basal dialysates, 20 mM atropine (s.f. atropine sulphate) dissolved in modified Ringer solution was administered via the microdialysis probe into the hippocampus for 180 min (9 dialysates). Then, the perfusion fluid was switched to a combined 20 mM atropine/10 mM pilocarpine solution for 2 collections, after which the perfusion fluid was switched back to the atropine solution.

### *Group 3: intrahippocampal perfusion with tetrodotoxin followed by co-administration of pilocarpine*

The same protocol as described for group 2 was followed, but 1 $\mu$M tetrodotoxin was administered instead of 20 mM atropine.

### *Group 4: intrahippocampal perfusion with MK-801 followed by co-administration of pilocarpine*

The same protocol as described for group 2 was followed, but 100 $\mu$M MK-801 (s.f. MK-801 hydrogen maleate) was administered instead of 20 mM atropine.

### *Group 5: intrahippocampal perfusion with lamotrigine followed by co-administration of pilocarpine*

The same protocol as described for group 2 was followed, but 600 $\mu$M lamotrigine was administered instead of 20 mM atropine.

### *Group 6: peroral pretreated animals with lamotrigine followed by intrahippocampal pilocarpine administration*

During the 7 days of pretreatment, the rats received every day a peroral administration of 10 mg $kg^{-1}$ lamotrigine suspended in sesame oil. On the day of the microdialysis experiment, the same protocol was followed as for the control group.

### *Electrocorticography (ECoG)*

A parasagital groove at each side of the brain was drilled in the skull of the anaesthetized animal (ketamine:diazepam, 50:5 mg $kg^{-1}$). Electrodes for the ECoG recordings were implanted as shown in Figure 1 and fixed with dental cement, so that the electrode tips touch the dura mater. Monopolar ECoG towards a prefrontal reference electrode were polygraphically amplified and recorded with a time constant of 0.15 s, a high cut-off filter at 70 Hz and a sensitivity of 500 $\mu$V $cm^{-1}$. The signals were further sampled at a frequency of 256 Hz by use of the Nicolet Brainlab System.

### *Chemicals and reagents*

GABA ($\gamma$-aminobutyric acid), L-glutamate, dopamine, pilocarpine hydrochoride, atropine sulphate and tetrodotoxin were supplied by Sigma (St. Louis, MO, U.S.A.). (+)-MK-801

hydrogen maleate (dizocilpine maleate) was purchased from RBI (Natick, MA, U.S.A.). Lamotrigine was obtained from the Wellcome Research Laboratories (Beckenham, Kent, U.K.). All other chemicals were analytical reagent grade or better and supplied by Merck (Darmstadt, Germany). Aqueous solutions were made in fresh water purified by a Seralpur pro 90 CN (Belgolabo, Overijse, Belgium) and filtered through a 0.2 $\mu$m membrane filter. As perfusion fluid for the microdialysis experiments, a modified Ringer solution was used containing in mM: NaCl147, $CaCl_2$2.3 and KCl4, pH 7.3. The antioxidant mixture to prevent degradation of the sampled dopamine consisted of 0.02 M HCl, 0.2% sodium metabisulphite and 0.02% $Na_2$ EDTA. All drugs were dissolved in modified Ringer solution when applied via the microdialysis probe. Lamotrigine was suspended in sesame oil when administered perorally.

### *Statistical analysis*

All results are expressed as mean amino acid or dopamine dialysate concentrations in $\mu$M or nM, respectively, with s.e.mean. The basal values in the figures (i.e. 100% baseline values) are the mean of at least 5 or 6 stable neurotransmitter dialysate levels obtained in the conditions before drug administration via the microdialysis probe. The dialysate levels were not corrected for the recovery across the dialysis membrane. Statistical analysis of the changes of GABA, glutamate or dopamine dialysate concentrations in time was performed by one-way analysis of variance (ANOVA) for repeated measures and Fisher's protected least significant difference (Fisher's PLSD) *post hoc* test ($\alpha = 0.05$). The significance of differences between peak dialysate concentrations was determined by Mann Whitney's test ($\alpha = 0.05$).

## Results

### *Basal values of GABA, glutamate and dopamine in the hippocampus*

The basal hippocampal dialysate concentrations (mean $\pm$ s.e.mean) ($n = 30$) were, respectively, $0.041 \pm 0.005$ $\mu$M for GABA, $0.439 \pm 0.069$ $\mu$M for glutamate and $0.289 \pm 0.027$ nM for dopamine.

### *Group 1 (n = 6) (control group): intrahippocampal pilocarpine administration*

We observed limbic seizures, characterized by tremor, scratching and wet dog shakes starting about 1 h after the start of perfusion with pilocarpine, followed by tonic-clonic limb movements, salivation, intense masticatory jaw movements and rearing. The ECoG recordings showed during the pilocarpine administration a slowing of the rhythmic activity, resulting in theta and delta waves. About 1 h after the start of perfusion with pilocarpine, we observed on the ECoG recordings clear patterns of tonic and tonic-clonic epileptic seizure activity, which was sustained until the end of the experiment (Figure 2). The focally evoked seizures became secondarily generalized: we noticed seizure activity at the ipsi- and contralateral side of the focus. Pilocarpine administration (40 min) in the hippocampus (Figure 3), resulted in significant decreases of the basal hippocampal overflow of glutamate ($P = 0.0003$) and GABA ($P = 0.0082$). For both glutamate and GABA, ceasing the administration of pilocarpine, resulted in a significant increase to about 200% ($P = 0.0001$) (Figure 3), with a significant elevation of both amino acid transmitters remaining until the end of the experiment. Intrahippocampal pilocarpine administration resulted in a significant increase of the basal extracellular hippocampal dopamine release (Figure 3) to about 500% ($P = 0.0001$). The extracellular dopamine levels remained significantly elevated for 140 min.

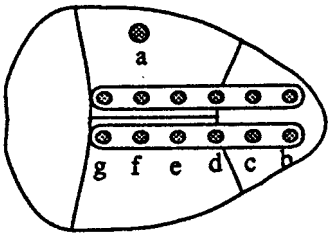


**Figure 1** View of the skull of the animal and implantation sites for the microdialysis probe (a) and the electrodes for ECoG recordings (b–g). (b) Reference electrode; (c) ground and (d–g) monitoring electrodes.

### *Group 2 (n = 6): intrahippocampal perfusion with atropine followed by co-administration of pilocarpine*

Atropine (20 mM) administration produced an increase in the exploratory and locomotor activity of the rats. However, none of the rats pretreated with atropine displayed the typical sequence of convulsive behaviour observed in the control group after the simultaneous pilocarpine perfusion. Intrahippocampal administration of atropine (Figure 4a)

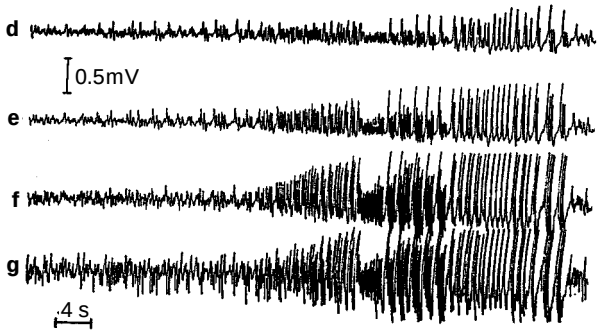


**Figure 2** ECoG recording obtained from a control rat after 10 mM pilocarpine administration and during the limbic convulsions. Monopolar ECoG recordings are determined between monitoring electrodes (d–g) and the prefrontal reference electrode (b).

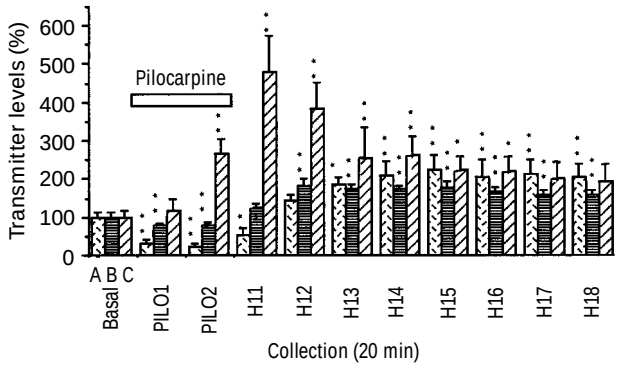


**Figure 3** Hippocampal (H) microdialysate concentrations (as % of the baseline level) (mean $\pm$ s.e.mean) ($n = 6$) of glutamate (columns A), GABA (columns B) and dopamine (columns C), before (basal), during (PILO1–PILO2) and after (H11–H18), intrahippocampal administration of 10 mM pilocarpine via the microdialysis probe. Each column represents a 20 min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD *post hoc* test. Asterisks denote the values significantly different from corresponding baseline values; $^*P < 0.05$, $^{**}P < 0.01$.

resulted in a complete disappearance of glutamate dialysate levels during the whole experiment ($P=0.0001$), exerted no significant changes on extracellular GABA overflow and increased dopamine release ($P=0.0267$). During and after the simultaneous perfusion with 10 mM pilocarpine and 20 mM atropine (Figure 4b), there were no further significant changes in extracellular GABA or dopamine concentrations. Glutamate concentrations stayed below the level of detection.

### *Group 3 (n=6): intrahippocampal perfusion with tetrodotoxin followed by co-administration of pilocarpine*

No specific behavioural changes were observed during the intrahippocampal application of tetrodotoxin 1 $\mu$M. Continuous administration of tetrodotoxin into the hippocampus (Figure 5a) resulted in a sustained decrease of hippocampal dopamine release to about 25% ($P=0.0001$), but exerted no significant changes on extracellular glutamate or GABA concentrations. During the simultaneous tetrodotoxin/pilocarpine perfusion (Figure 5b) the extracellular glutamate and GABA concentrations decreased to about 10% ($P=0.0001$) and 50% ($P=0.0001$), respectively. After the pilocarpine perfusion had ceased no further significant changes in extracellular glutamate, GABA or dopamine concentrations were noticed (Figure 5b), nor did the animals develop the typical limbic convulsions.

### *Group 4 (n=6): intrahippocampal perfusion with MK-801 followed by co-administration of pilocarpine*

No behavioural changes were noticed during local MK-801 and simultaneous MK-801/pilocarpine administration. The rats were completely protected from limbic seizures after the pilocarpine perfusion had ceased. The intrahippocampal MK-801 administration (100 $\mu$M) (Figure 6a) increased hippocampal dopamine release to about 250% ($P=0.0014$), but exerted no changes on extracellular hippocampal glutamate or GABA overflow. During the simultaneous MK-801/pilocarpine perfusion (Figure 6b) the extracellular glutamate and GABA levels decreased to about 12% ($P=0.0007$) and 60% ($P=0.0345$), respectively. After the pilocarpine perfusion had ceased (Figure 6b) the extracellular hippocampal MK-801-induced dopamine concentration

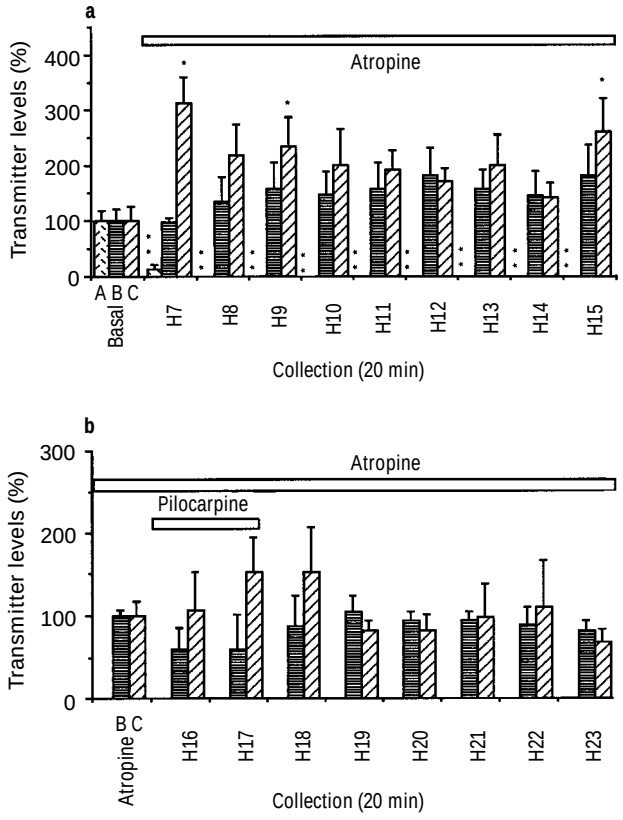


**Figure 4** (a) Hippocampal (H) microdialysate concentrations (as % of the baseline level) (mean ± s.e.mean) ($n=6$) of glutamate (columns A), GABA (columns B) and dopamine (columns C) during intrahippocampal administration of 20 mM atropine via the microdialysis probe. Each column represents a 20 min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc test. Asterisks denote the values significantly different from corresponding baseline values; $*P<0.05$, $**P<0.01$. (b) Hippocampal (H) microdialysate concentrations (mean ± s.e.mean) $n=6$) of GABA (columns B) and dopamine (columns C) during (H16–H17) and after (H18–H23) simultaneous intrahippocampal administration of 20 mM atropine and 10 mM pilocarpine via the microdialysis probe. The 100% baseline level (atropine) was taken as the mean ± s.e.mean of the 8 preceding dialysate concentrations under atropine perfusion presented in Figure 4a (H8–H15). Each column represents a 20 min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD *post hoc* test, not significant at the 5% level of significance.

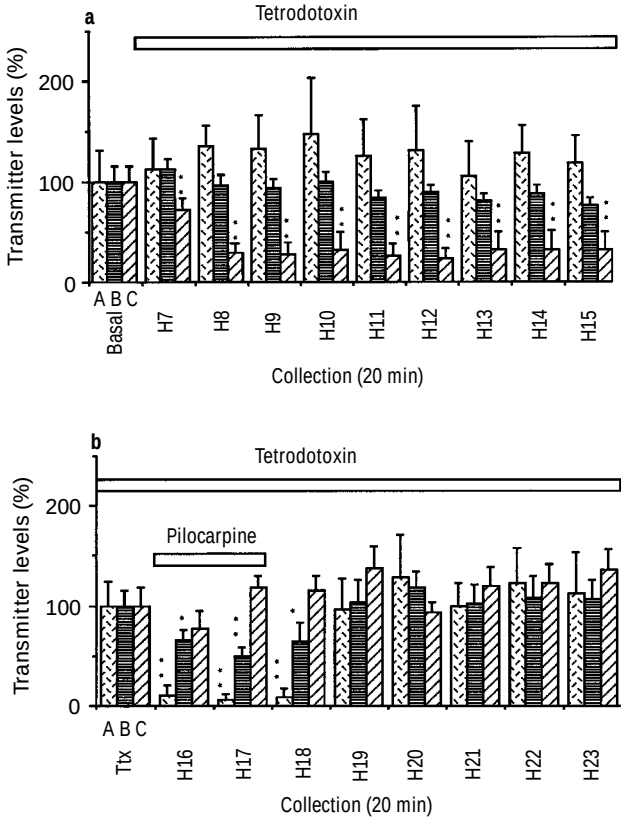


**Figure 5** (a) Hippocampal (H) microdialysate concentrations (as % of the baseline level) (mean ± s.e.mean) ($n=6$) of glutamate (columns A), GABA (columns B) and dopamine (columns C) during intrahippocampal administration of 1 $\mu$M tetrodotoxin via the microdialysis probe. Each column represents a 20 min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD *post hoc* test. Asterisks denote the values significantly different from corresponding baseline values; $**P<0.01$. (b) Hippocampal (H) microdialysate concentrations (mean ± s.e.mean) ($n=6$) of glutamate (A), GABA (B) and dopamine (C) during (H16–H17) and after (H18–H23) simultaneous intrahippocampal administration of 1 $\mu$M tetrodotoxin and 10 mM pilocarpine via the microdialysis probe. The 100% baseline level (Ttx) was taken as mean ± s.e.mean of the 8 preceding dialysate concentrations under tetrodotoxin perfusion presented in (a) (H8–H15). Each column represents a 20 min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD *post hoc* test. Asterisks denote the values significantly different from corresponding baseline values; $*P<0.05$, $**P<0.01$.

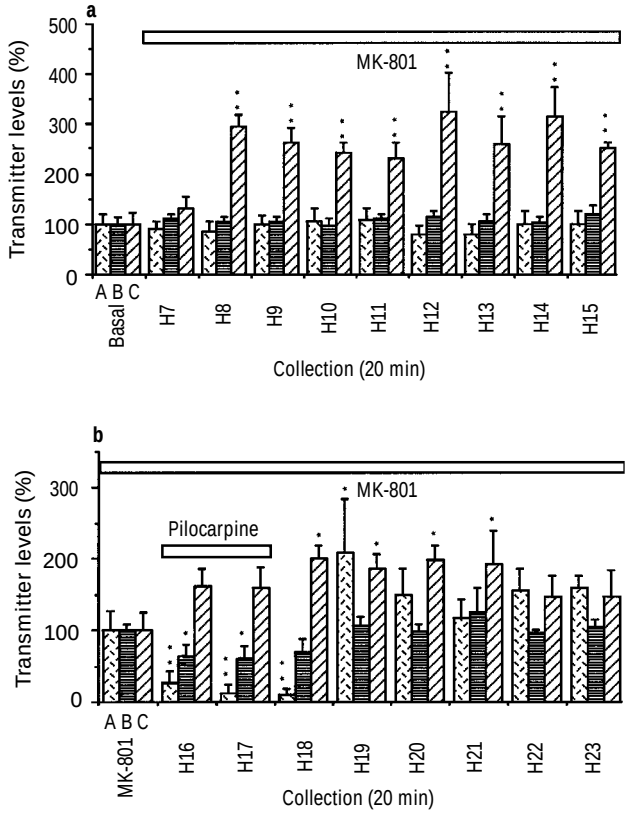


**Figure 6** (a) Hippocampal (H) microdialysate concentrations (as % of the baseline level) (mean ± s.e.mean) ($n=6$) of glutamate (columns A), GABA (columns B) and dopamine (columns C) during intrahippocampal administration of 100 $\mu$M MK-801 via the microdialysis probe. Each column represents a 20 min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD *post hoc* test. Asterisks denote the values significantly different from corresponding baseline values; $^{**}P<0.01$. (b) Hippocampal (H) microdialysate concentrations (mean ± s.e.mean) ($n=6$) of glutamate, GABA and dopamine during (H16–H17) and after (H18–H23) simultaneous intrahippocampal administration of 100 $\mu$M MK-801 and 10 mM pilocarpine via the microdialysis probe. The 100% baseline level (MK-801) was taken as the mean ± s.e.mean of the 8 preceding dialysate concentrations under MK-801 perfusion presented in (a) (H8–H15). Each column represents a 20 min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD *post hoc* test. Asterisks denote the values significantly different from corresponding baseline values; $^{*}P<0.05$, $^{**}P<0.01$.

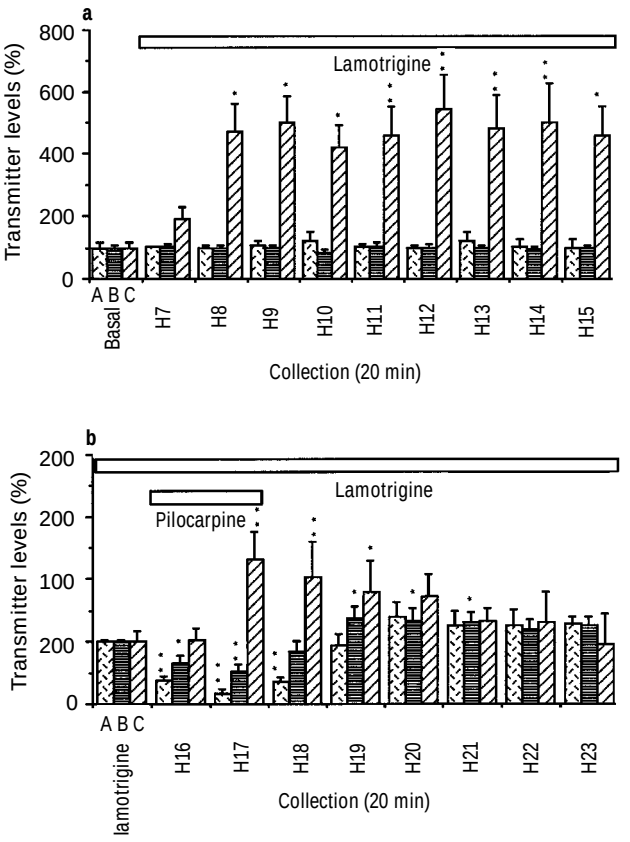


**Figure 7** (a) Hippocampal (H) microdialysate concentrations (as % of the baseline level) (mean ± s.e.mean) ($n=6$) of glutamate (columns A), GABA (columns B) and dopamine (columns C) during intrahippocampal administration of 600 $\mu$M lamotrigine via the microdialysis probe. Each column represents a 20 min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD *post hoc* test. Asterisks denote the values significantly different from corresponding baseline values; $^{*}P<0.05$, $^{**}P<0.01$. (b) Hippocampal (H) microdialysate concentrations (mean ± s.e.mean) ($n=6$) of glutamate, GABA and dopamine during (H16–H17) and after (H18–H23) simultaneous intrahippocampal administration of 600 $\mu$M lamotrigine and 10 mM pilocarpine via the microdialysis probe. The 100% baseline level (lamotrigine) was taken as the mean ± s.e.mean of the 8 preceding dialysate concentrations under lamotrigine perfusion presented in (a) (H8–H15). Each column represents a 20 min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD *post hoc* test. Asterisks denote the values significantly different from corresponding baseline values $^{*}P<0.05$; $^{**}P<0.01$.

increased to about 200% for 80 min ($P=0.0498$). This dopamine increase was significantly lower than the dopamine increase observed in the control animals ($P=0.028$). During collection H19 (i.e. 80 min after the start of perfusion with pilocarpine) we noticed a significant increase in extracellular glutamate overflow to 210% ($P=0.0007$). No further changes in extracellular hippocampal GABA concentrations were observed.

### *Group 5 (n=6): intrahippocampal perfusion with lamotrigine followed by co-administration of pilocarpine*

No specific behavioural and electrocorticographic changes were noticed during intrahippocampal lamotrigine (600 $\mu$M) and simultaneous lamotrigine/pilocarpine perfusion. The rats were protected from pilocarpine-induced seizures. The intrahippocampal lamotrigine administration (Figure 7a) increased hippocampal dopamine release to about 400% ($P=0.0001$) and exerted no changes on extracellular hippocampal glutamate or GABA overflow, i.e. a very similar course as that during the local perfusion with MK-801 (Figure 6a). During the simultaneous lamotrigine/pilocarpine perfusion (Figure 7b) the extracellular glutamate and GABA levels decreased to 17% ($P=0.0001$) and 52% ($P=0.0001$), respectively. The extracellular hippocampal lamotrigine-induced dopamine levels increased to about 200% for 60 min ($P=0.0063$). From 80 min to 120 min after the start of perfusion with pilocarpine, extracellular GABA levels increased to 135% ($P=0.0185$). No further changes in extracellular hippocampal glutamate concentrations were observed.

### *Group 6 (n=3): peroral pretreated animals with lamotrigine followed by intrahippocampal pilocarpine administration*

The rats which were pretreated per os with lamotrigine 10 mg $kg^{-1}$ $day^{-1}$, were not protected against the focally evoked pilocarpine-induced seizures. ECoG recordings clearly showed patterns of seizure activity (Figure 8). Moreover, the limbic convulsions were more severe than the seizures observed in the control group and resembled status epilepticus. The changes in

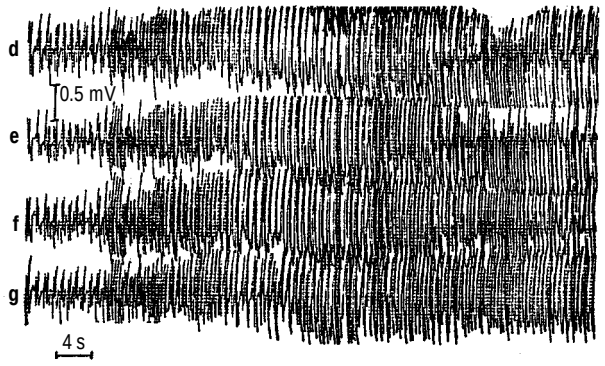


**Figure 8** ECoG recording obtained from a rat premedicated perorally with 10 mg $kg^{-1}$ $day^{-1}$ lamotrigine. This recording was registered after 10 mM pilocarpine administration and during the limbic convulsions. Monopolar ECoG recordings were determined between monitoring electrodes (d–g) and the prefrontal reference electrode (b).

extracellular neurotransmitter concentrations were similar to the extracellular transmitter changes obtained for the control group (data not shown).

## Discussion

The pilocarpine rat model for temporal lobe epilepsy produces morphological changes, altered membrane properties and altered synaptic responses of hippocampal neurones, similar to what has been observed in human hippocampal neurones surgically removed for the treatment of complex partial seizures (Isokawa & Mello, 1991). However, very few studies have monitored possible changes in extracellular neurotransmitter levels during pilocarpine-induced seizures (Al-Tajir & Starr, 1993; Millan *et al*., 1993). The presumed mechanism of action is muscarinic receptor stimulation being responsible for seizure initiation and for driving amino acids to sustain epileptic activity and to induce neuronal damage (Turski *et al*., 1989). This combined microdialysis-electrocorticography (ECoG) study provides *in vivo* data concerning changes in extracellular glutamate, GABA and dopamine levels in the hippocampus of freely moving rats during focally evoked pilocarpine-induced seizures. The effects of the non-selective muscarinic receptor antagonist atropine, the voltage-dependent sodium channel blocker tetrodotoxin, the NMDA receptor antagonist MK-801 and the effectiveness of lamotrigine, were tested in this rat model.

During the intrahippocampal administration of pilocarpine, we observed a decrease of the extracellular glutamate and GABA levels, and a simultaneous slowing of the rhythmic activity recorded on the ECoG, showing theta and delta waves. This effect was blocked by co-perfusion with the non-selective muscarinic receptor antagonist. The cholinergic nature and involvement of cholinoceptors in hippocampal theta rhythm has been previously described *in vitro* (Konopacki *et al*., 1988). A presynaptic muscarinic $M_2$ receptor on hippocampal glutamatergic nerve terminals that decreases the release of glutamate, has been described in *in vitro* studies (Marchi *et al*., 1989; Marchi & Raiteri, 1989). Intra- and extracellular single cell recordings demonstrated that acetylcholine exerted a rapid and powerful muscarinic inhibitory effect upon both excitatory and inhibitory afferents to hippocampal neurones and it was thought that this effect was mediated by a decrease in the amount of released neurotransmitter (Valentino & Dingledine, 1981). Segal (1989) demonstrated in hippocampal cultures under patch-clamp conditions that this presynaptic cholinergic inhibition is mediated by muscarinic $M_2$ receptors. Our microdialysis results provide *in vivo* evidence for these former *in vitro* observations. Moreover, muscarinic receptor stimulation significantly enhanced the spontaneous firing of the hippocampal GABA-ergic interneurones, resulting in an increased frequency of spontaneous-activity-dependent inhibitory postsynaptic potentials (Pitler & Alger, 1992). This might result in hyperpolarization of the postsynaptic glutamatergic pyramidal cells of the hippocampus and a subsequent diminished glutamate release.

In the control group, the decrease of the extracellular glutamate levels due to intrahippocampal pilocarpine perfusion, was followed by a significant and sustained enhancement of the extracellular glutamate concentrations. These elevations were associated with the onset of the limbic seizures, as evidenced from the patterns recorded on the ECoG. Seizure related elevations of the extracellular glutamate concentration have also been observed in patients with complex partial seizures subjected to epilepsy surgery (Carlson *et al*., 1992; During & Spencer, 1993). Our results can also be related to a current- and voltage-clamp study showing that muscarinic receptor stimulation produced an initial suppression of the excitatory postsynaptic potentials, followed by a long-lasting facilitation of NMDA receptor-mediated excitatory postsynaptic potentials (Markam & Segal, 1990). The enhanced extracellular glutamate concentrations are probably involved in seizure spread and maintenance, in morphological changes of the neurones, in the NMDA receptor-mediated processes (see below) and in selective loss of hippocampal neurones (de Lanerolle *et al*., 1994).

The initial GABA decrease was followed by a sustained increase of the extracellular GABA levels in the control rats and thus followed a similar course as extracellular glutamate. A rise in the extracellular hippocampal GABA concentration was also observed during spontaneous seizures in human brain (During & Spencer, 1993) and extracellular GABA enhancement lasted for several hours after amygdaloid kindling (Ueda & Tsuru, 1995). The enhanced GABA release may compensate for the excessive firing of glutamatergic neurones. Indeed, it has been shown that GABA exerts a tonic inhibition on glutamate release in the hippocampus (Rowley *et al*., 1995) and there exists a dynamic equilibrium between excitatory and inhibitory processes in which synaptic mechanisms play an essential role for stabilizing neuronal networks (Lopes da Silva *et al*., 1992). However, prolonged stimulation of GABA receptors is presumed to result in GABA desensitization and fading of the inhibitory effect (Krnjevic, 1990). The reduction in GABA inhibition is not primarily due to a reduction in GABAergic cell density (Lopes da Silva *et al*., 1992) nor to a reduction in GABA release (as shown in this study), so the progressive reduction of inhibition is possibly caused by impairment of the response and/or down regulation of GABA receptors (Ueda & Tsuru, 1995). Thus, in the end, excessive stimulation of GABA receptors and desensitization, will be insufficient to overcome excitatory NMDA receptor-mediated responses and may even aggravate the seizures. We observed seizures until the end of the experiments. Our results showing sustained elevations of both glutamate and GABA during the pilocarpine-induced limbic convulsions support the hypothesis of the cholinergic system being responsible for driving amino acids to support sustained seizure activity.

We observed an increase of the extracellular hippocampal dopamine levels during intrahippocampal pilocarpine perfusion and during the convulsions. We suggest that the increase in hippocampal dopamine release during pilocarpine administration may be the result of presynaptic muscarinic receptor stimulation, as was also shown in rat striatum (Raiteri *et al*., 1982). The long lasting dopamine increase during the convulsions can be interpreted as an anticonvulsant mechanism. Although the dopaminergic innervation of the hippocampus is sparse, the release of dopamine elicited in this region by some anticonvulsants can be large (see below). This release, coupled with poor dopamine reuptake facilities and the wide distribution of dopamine receptors in the hippocampus (Starr, 1996), suggests that dopamine is a functionally important transmitter

in the hippocampus. Indeed, behavioural and electrophysiological studies have indicated that the endogenous hippocampal dopamine system is actively engaged in curbing neuronal hyperexcitability and that hippocampal dopamine exerts a profound inhibitory effect on epileptogenesis and that this action is mediated via dopamine $D_2$ receptors (Suppes *et al*., 1985; Alam & Starr, 1993). Dopamine acts in the hippocampus preventing epileptic discharges and at the level of the limbico-striatal interface limiting propagation through the basal ganglia (Starr, 1996).

Since we presumed that all observed effects in this rat model for focal epilepsy are primarily mediated via muscarinic receptor activation, we must provide evidence that the effects can be blocked by muscarinic receptor antagonists. During the intrahippocampal application of the non-selective muscarinic receptor blocker atropine, extracellular hippocampal glutamate dialysate levels fell below the detection limit, hippocampal dopamine release increased and no significant alterations were observed for GABA. Administration of the non-selective muscarinic receptor agonist pilocarpine resulted in a decrease of extracellular hippocampal glutamate and GABA levels and also increased dopamine overflow. Whether these responses represent pre- or postsynaptic events, and direct or indirect mechanisms, remains to be elucidated, although we can suggest possible explanations. Intrahippocampal perfusion with atropine blocks the muscarinic $M_2$ receptors on the glutamatergic and GABA-ergic hippocampal nerve terminals, the $M_2$ autoreceptors on the septohippocampal cholinergic neurones, and the muscarinic receptors on dopamine nerve endings and on the GABA-ergic interneurones. The presynaptic inhibition of glutamate and GABA release by $M_2$ receptor stimulation is thus abolished, but the spontaneous firing of GABA-ergic interneurones keeps on exerting an inhibitory control over glutamatergic pyramidal nerve cell excitability. Indeed, Pitler & Alger (1992) demonstrated on GABA-ergic interneurones that addition of atropine after agonist-induced increases in inhibitory postsynaptic potentials rapidly reduced the frequency of the inhibitory postsynaptic potentials to previous control levels. Glutamate has been shown to depress excitatory synaptic transmission by activating presynaptic metabotropic glutamate receptors for which L-AP4 is a selective agonist (Forsythe & Clements, 1990). The net effect of these interactions during atropine perfusion might result in a decreased glutamate overflow, while simultaneously no changes were noticed in extracellular GABA concentrations. The observed neurotransmitter changes can also be due to more complex reciprocal transmitter interactions in the hippocampus. For instance, it has been demonstrated that atropine stimulates the hippocampal acetylcholine release by blocking the muscarinic autoreceptors (Chesselet, 1984). Dual modulatory effects of synaptic inhibition mediated by distinct metabotropic glutamate receptors on hippocampal inhibitory nerve cells have been described (Poncer *et al*., 1995). NMDA receptors and adrenoceptors participate in the regulation of GABA-ergic inhibition (Andreasen & Lambert, 1991; Benardo, 1995). 5-Hydroxytryptamine (5-HT) reduces the efficacy of glutamatergic hippocampal neurotransmission via $5\text{-HT}_3$ receptors (Zhang *et al*., 1994). Dopamine modulates the hippocampal acetylcholine release (Imperato *et al*., 1993; Day & Fibiger, 1994) and there is evidence for 5-hydroxytryptaminergic/cholinergic interactions (Cassel & Jeltsch, 1995).

After intrahippocampal perfusion with atropine for three hours, no further significant changes in extracellular hippocampal GABA and dopamine levels were observed during and after co-administration of pilocarpine, and no specific limbic convulsions were noticed, indicating muscarinic receptors as the primary site of action. This confirms the behavioural study by Malanski *et al*., (1994) who showed that atropine can block pilocarpine-induced seizures but is unable to interrupt already established convulsions, again suggesting a role for cholinergic receptors in the initiation but not the maintenance of limbic seizures.

Neurotransmitters can be released in the extracellular space via exocytosis. This vesicular release is tetrodotoxin-sensitive and calcium-dependent. In recent years, it has become clear that tetrodotoxin-insensitive and calcium-independent carrier-mediated release represents a functional, pharmacological and physiopathological important alternative mechanism to exocytosis for raising extracellular transmitter concentrations (Attwell *et al*., 1993). Furthermore, there is evidence for non-synaptic neurotransmission, i.e. information transmission by diffusion through the extracellular space and activation of extrasynaptic receptors (Bach-y-Rita, 1993). Intrahippocampal administration of tetrodotoxin decreased extracellular dopamine concentrations in the hippocampus, sustaining vesicular dopamine release from functionally active nerve terminals. Tetrodotoxin infusion in the hippocampus did not have any significant effect on basal glutamate or GABA release. The latter findings agree with those of Rowley *et al*. (1995), who observed no effects of perfusion with nickel, a T-type calcium channel blocker, on basal hippocampal glutamate and GABA levels. The basal amino acid overflow in hippocampus is calcium-independent and tetrodotoxin-insensitive and thus of non-vesicular origin. However, after co-administration of tetrodotoxin and pilocarpine, all increases in extracellular glutamate, GABA and dopamine levels observed in the control group, were completely prevented. This finding suggests that the stimulated hippocampal transmitter levels reflect neuronal vesicular release.

During perfusion of the hippocampus with MK-801, a selective non-competitive NMDA receptor antagonist, we observed a sustained approximately 3 fold increase in hippocampal dopamine release. Since the drug was administered locally, one could argue that the change in dopamine dialysate levels may not affect seizure processes as it occurs in the immediate vicinity of the dialysis membrane. However, systemic administration of MK-801 also clearly increased hippocampal dialysate levels of dopamine, but decreased the striatal dopamine overflow (Whitton *et al*., 1992). As described above, it is presumed that increased dopaminergic activity in hippocampus mediates an anticonvulsant effect. Therefore, it is possible that the anticonvulsant action of MK-801 is in part mediated via increased hippocampal dopamine release. Extracellular single unit recordings demonstrated that MK-801 produced dose-dependent increases in the firing rate of ventral tegmental A10 dopamine neurones (French *et al*., 1993). It is known that MK-801 possesses affinity for the dopamine transport sites (Clarke & Reuben, 1995), which can result in elevated dopamine levels due to direct inhibition of dopamine uptake. However, there is more evidence that the dopamine increase may be a consequence of NMDA receptor inhibition (Maurice *et al*., 1991). It has been suggested that MK-801 facilitates the activation of dopaminergic mechanisms via an indirect (perhaps by reduced glutamatergic activity) rather than a direct effect on dopamine nerve cells (Liljequist *et al*., 1991).

The intrahippocampal administration of MK-801 protected all animals completely from pilocarpine-induced seizures and no sustained elevations of the extracellular glutamate levels were observed. These findings provide evidence for the secondary involvement of excitatory amino acids and NMDA receptor activation in the generation of epilepsy by cholinomimetics. Our results confirm the study by Isokawa & Mello (1991) who showed NMDA receptor-mediated hyperexcitability in dendritically-deformed hippocampal neurones of pilocarpine-treated rats, indicating the participation of NMDA receptors in both structural and functional alterations. Moreover, the pilocarpine rat model has important features in common with human complex partial seizures, since NMDA receptor-mediated excitotoxicity has been proposed as being one of the underlying mechanisms of this disease. NMDA receptors do not participate in normal low frequency synaptic hippocampal transmission (Harris *et al*., 1984), but recruitment of normally dormant NMDA receptors may be a feature of the epileptic human brain

(Urban *et al*., 1990). The increase in hippocampal dopamine release after simultaneous MK-801/pilocarpine perfusion was attenuated compared to the dopamine increase in the control rats. This might be the result of a depletion of the dopamine pool or a blunted response due to the fact that baseline dopamine concentrations were elevated by MK-801 administration, or as a result of the lack of seizure activity in the presence of MK-801.

During intrahippocampal perfusion with lamotrigine, no alterations in extracellular glutamate or GABA concentrations compared to baseline levels were observed, but a significant increase of the extracellular dopamine level was noticed. These results were very similar to the effect obtained by intrahippocampal MK-801 administration. To our knowledge, elevations of hippocampal dopamine release during lamotrigine administration have never been obtained before. Therefore, the anticonvulsant effect of lamotrigine may – besides by stabilizing voltage-dependent sodium channels and neuronal membranes – also be mediated by increasing extracellular hippocampal dopamine levels.

Epileptic seizures were not observed on the ECoG recordings from rats which received lamotrigine via intrahippocampal perfusion. This result indicates protective effects of lamotrigine, when administered locally, against focally evoked pilocarpine-induced seizures. Lamotrigine was practically equipotent in inhibiting veratrine-induced [$^3$H]-GABA, [$^3$H]-dopamine and endogenous glutamate release in an *in vitro* slice preparation (Waldmeier *et al*., 1995). In our *in vivo* experiments, lamotrigine was able to suppress pilocarpine-induced glutamate release, but could not prevent increases in hippocampal GABA and dopamine release. These results confirm that lamotrigine is a less potent inhibitor of induced acetylcholine, noradrenaline, dopamine and GABA release, compared to inhibition of glutamate release (Leach *et al*., 1991).

Rats pretreated perorally with lamotrigine, were not protected against the focally evoked pilocarpine-induced seizures. A species variation in metabolism of lamotrigine has been described (Gram, 1989). However, it is unlikely that in rats a proconvulsant metabolite was formed, since the same dose of lamotrigine after oral administration was effective against the maximal electroshock test in rats (Miller *et al*., 1986). It is known that not all antiepileptic drugs can prevent seizures induced by pilocarpine (Turski *et al*., 1987; Smolders *et al*., 1996b) and these contradictory results obtained by using different routes of administration of lamotrigine, confirm that it is important to recognize the limits of the existing models of epilepsy.

In conclusion, pilocarpine-induced seizures are initiated via muscarinic receptors and further mediated via NMDA receptors. Sustained increases in extracellular glutamate overflow after pilocarpine perfusion are related to the limbic seizures and are probably involved in NMDA receptor-mediated excitotoxicity. Hippocampal dopamine release is functionally important in epileptogenesis and may participate in the anticonvulsant effects of MK-801 and lamotrigine. The pilocarpine-stimulated hippocampal neurotransmitter levels might reflect neuronal vesicular release.

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