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Age-dependence of the anticonvulsant effects of the GABA uptake inhibitor tiagabine in vitro

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Abstract

Epileptic syndromes frequently start at childhood and therefore it is crucial to test new anticonvulsants at immature stages of the nervous system. We compared the effects of the γ-aminobutyric acid (GABA) uptake inhibitor tiagabine [(*R*)-*N*-(4,4-bis(3-methyl-2-thienyl)but)3-en-1-yl nipecotic acid] on low-Mg^{2+}-induced epileptic discharges in brain slices from rat pups (p 5–8) and juvenile animals (p 15–20). In tissue from rat pups, tiagabine slightly reduced epileptiform activity in hippocampal area CA1 but had no effect in the entorhinal cortex. In juvenile rats, epileptiform discharges were unaffected in CA1 but suppressed by 60% in the entorhinal cortex. While tiagabine increases its efficacy with age, in-situ hybridisation and PCR analysis show that mRNA coding for the neuronal GABA-transporter GAT-1 is already present at p 5. We therefore conclude that the increasing efficacy of tiagabine during ontogenesis is due to functional maturation of GABAergic synapses rather than to up-regulation of GAT-1 expression.

Keywords: Epilepsy; Brain slice; Tiagabine; GABA (γ-aminobutyric acid) uptake; Development; Hippocampus; Cortex, entorhinal

1. Introduction

Many epileptic syndromes in man start during childhood or adolescence and it is generally assumed that the immature brain is especially susceptible for seizure generation and for the chronification of epilepsy (Moshé et al., 1983; Lothman and Bertram, 1993). Various mechanisms have been suggested to contribute to this age-dependence, including depolarising $GABA_A$ receptor-mediated potentials (Kriegstein et al., 1987; Ben-Ari et al., 1988), immature regulation of K^+-homeostasis in the developing brain (Hablitz and Heinemann, 1987), changes in the function and expression of NMDA receptors (Brady et al., 1991) or enhanced recurrent excitatory connections (Swann et al., 1993). These developmental characteristics may be mirrored by differences in the potency or efficacy of anticonvulsant drugs. In juvenile rats, various γ-aminobutyric acid (GABA) mimetic substances show little efficacy against experimentally induced afterdischarges and tonic–clonic seizures (Velísek and Mares, 1995; Polásek et al., 1996). Microinfusion of $GABA_A$ receptor agonists into the substancia nigra of rat pups exerts proconvulsant effects on flurothyl-induced seizures whereas they are anticonvulsant at low doses in adults (Garant et al., 1995). Therefore, GABAergic agents should be thoroughly tested throughout development including very immature stages.

Tiagabine [(*R*)-*N*-(4,4-bis(3-methyl-2-thienyl)but)3-en-1-yl nipecotic acid] is a derivative of nipecotic acid with good anticonvulsant activity both in vitro and in vivo (Faingold et al., 1994; Suzdak, 1994; Walton et al., 1994; Smith et al., 1995; Pfeiffer et al., 1996). It is effective as add-on therapy in complex partial seizures in adults (Gram, 1994; Richens et al., 1995; Luer and Rhoney, 1998) while results from children with epilepsy are not yet sufficient to allow for a comparison with established anticonvulsants (Adkins and Noble, 1998). Tiagabine acts by blocking the uptake of synaptically released GABA into neurons and glia cells, thereby prolonging the postsynaptic inhibitory effect of this transmitter (Rekling et al., 1990; Roepstorff and Lambert, 1994). However, in a recent study on inhibitory postsynaptic currents (IPSCs) in dentate granule

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cells we found that tiagabine is ineffective during the first postnatal week of development, whereas in tissue from juvenile (third postnatal week) rats inhibitory transmission is potently prolonged (Draguhn and Heinemann, 1996). This ontogenetic change in the effects of tiagabine on the cellular level raises the question whether its anticonvulsant efficacy is also age-dependent.

We have previously shown that tiagabine is highly potent and effective against electrographic seizure-like events (SLEs) in the low-Mg^{2+} model of temporal lobe epilepsy in adult rat brain slices in vitro (Pfeiffer et al., 1996). Here we report the effects of tiagabine on the same form of epileptiform activity in brain slices from early postnatal (p 5–8) and later juvenile (p 15–20) rats.

2. Materials and methods

2.1. Slice preparation

Wistar rats of both sexes from our own breeding colony were kept under standard conditions (12/12-h light–dark cycle, free access to rodent food pellets and water). For experiments, animals aged 5–8 and 15–20 postnatal days (day of birth = p 0) were deeply anaesthetised with ether, decapitated and the brain removed (approved by the local government of Berlin). Horizontal slices containing the hippocampal formation, the perirhinal, entorhinal and part of the temporal cortex were cut on a vibratome at 400 μm (p 15–20) or at 400–500 μm (p 5–8) thickness. The slices were transferred to an interface recording chamber where they rested at least 1 h before onset of recordings and were continuously perfused with artificial cerebrospinal fluid (ACSF, 2.0–3.0 ml/min) at 35°C containing (in mM): NaCl 124, KCl 3, $MgSO_4$ 1.8, $CaCl_2$ 1.6, NaH_2PO_4 1.25, $NaHCO_3$ 26 and glucose 10 (pH 7.4, saturated with 95% O_2/5% CO_2). In order to elicit epileptiform activity, $MgSO_4$ was omitted (Zhang et al., 1995).

2.2. Recordings

Extracellular field potentials were recorded using glass electrodes of 2–8 MΩ resistance filled with 150 mM NaCl. One electrode was placed in stratum pyramidale of the hippocampal CA1 subfield and one electrode in the deep layers (5–6) of the medial entorhinal cortex (mEC). DC-coupled field potentials were measured with a high-impedance voltage amplifier and written on a chart recorder (Dash IV, Astromed, West Warwick, RI, USA). In most slices, population spikes in CA1 stratum pyramidale were recorded before onset of the experiment. Slices with population spike amplitudes below 4 mV were discarded. Then, slices were exposed to Mg^{2+}-free solution and the extracellular potential in CA1 and the mEC was continuously monitored and recorded. When stable epileptiform activity had established, tiagabine was added at 1 to 100 μM (diluted from a stock solution of 10 mM in distilled water). Tiagabine was a kind gift from Novo Nordisk, Maloev, Denmark.

2.3. Data analysis

SLEs were counted for equal periods (20 to 30 min) immediately before application of tiagabine and at the end of the application period. The more frequent late recurrent discharges, LRDs (see Section 3), were counted during 4-min periods before and at the end of application. Data were only accepted if changes during the application of tiagabine could at least be partially reversed upon washout of the substance. Data are given as mean ± S.E.M. (standard error of the mean). Significance of changes in epileptiform activity under tiagabine was determined by the Wilcoxon's matched-pairs signed rank test (SPSS program; $P < 0.05$ regarded as significant).

2.4. PCR-amplification of GAT-specific mRNA

Tissue samples containing CA1 or the mEC were prepared from freshly cut combined hippocampal–entorhinal cortex slices (400 μm, see above) under a binocular microscope and subsequently transferred into a reaction tube containing 100 μl TRI-Reagent™ (Molecular Research Center, Cincinnati, USA). After 5 min we added 40 μl chloroform, vortexed the samples for 15 s, waited 15 min at room temperature and centrifuged the samples at 12 000 × *g*. The supernatant containing the RNA was precipitated in 100 μl propanol at −20°C and stored at −80°C. Subsequently, the RNA was reversely transcribed as described by Lambolez et al. (1992). Briefly, the RNA was incubated at 42°C with random hexamers (Pharmacia Biotech, Uppsala, Sweden) and M-MLV Reverse Transcriptase (200 U, Gibco BRL, Paisley, Scotland). PCR was performed with GoldStar™ DNA Polymerase (Eurogentec, Seraing, Belgium) in 36 cycles at 92°C (45 s), 50°C (45 s) and 72°C (90 s). Specific oligonucleotide primers were constructed based on the sequence published by Guastella et al. (1990): 5′: GCC CCC TCA TCA CCC CTA CAC T and 3′: GCT TGT GGC TTT TCT TTT TCT C. The expected length of the amplification product was 625 bases. Subsequently, 12 μl of the PCR-product were analysed by agarose gel electrophoresis (1.5% agarose).

2.5. In-situ hybridisation

The rats were perfused with ice-cold DEPC-treated saline under deep anaesthesia (thiobutabarbital sodium, 100 mg/kg KG i.p.). After removal, the brains were grossly dissected and tissue blocks from both hemispheres were frozen in isopentane at −35°C and stored at −80°C. Horizontal slices were cut at 12 μm on a cryotome at −20°C, mounted on cover slips and fixed in 4% paraformaldehyde. For in-situ *hybridisation*, we inserted the

PCR-amplified GAT-1 specific sequence (see above) into a TA cloning vector (Invitrogen, Leek, Netherlands) in both orientations to produce sense and antisense probes. Transcripts were labelled with digoxigenin (Boehringer Mannheim, Germany). The slices were incubated for 16 h at 55°C with In-situ *hybridisation* buffer (Amersham Life Science, Buckinghamshire, England) containing 3 μg/ml of the labelled probe. Slides were then washed following the instructions included in the hybridisation buffer and processed for immunodetection using a Nucleic Acid Detection kit (Boehringer Mannheim). The incubation in BCIP/BT colour solution was performed in the dark overnight. Then the sections were rinsed in water, air dried and mounted with Entellen (Merck, Darmstadt, Germany).

3. Results

3.1. Epileptiform activity in early postnatal and juvenile tissue

Washout of the extracellular Mg^{2+}-ions resulted in the appearance of spontaneous epileptiform discharges in CA1 and the medial endorhinal cortex (mEC; Fig. 1) most frequently appearing as seizure-like events (SLEs). SLEs typically displayed an initial negative-going DC-shift superimposed by repetitive short negative potential deflections. These ''clonic'' events sometimes outlasted the initial ''tonic–clonic'' phase (Fig. 1). In some slices we also observed repetitive discharges lacking any DC-shift. Recordings with K^+-sensitive microelectrodes revealed that these events were accompanied by similar changes in the ionic environment like ''normal'' SLEs (not shown) and they were therefore counted as SLEs unless explicitly stated. Positive going short repetitive discharges in CA1, which are typical for adult tissue in this model (e.g., Pfeiffer et al., 1996) were not regularly observed. The epileptiform activity started between 6 and 40 min after omitting Mg^{2+}. In animals aged 5–8 days, the SLEs persisted over several hours at relatively constant frequency. In animals aged 15–20 postnatal days, SLEs in the entorhinal cortex transformed after 16 to 107 min into short, repetitive, negative-going field potentials (Fig. 1, bottom right). This pattern is known from adult tissue as late recurrent discharges, LRDs (Zhang et al., 1995; Pfeiffer et al., 1996). The mean frequency of the LRDs was $125 \pm 15/4$ min and they were never observed in the early postnatal group (p 5–8).

3.2. Effect of tiagabine in hippocampal CA1

Stable SLEs were recorded for 20–30 min prior to drug application in 20 slices from 17 animals aged 5–8 postnatal days and in nine slices from seven animals aged 15–20 days. Mean ''seizure'' frequency corresponded to $7.5 \pm 0.8/30$ min and $5.1 \pm 1.7/30$ min, respectively. Tiagabine (10 μM) slightly reduced the frequency of SLEs to $72 \pm 12\%$ of control in the younger group ($P = 0.047$) but was ineffective in the older group (p 15–20) where the frequency of SLEs remained at $107 \pm 24\%$ ($P = 0.92$). However, the appearance of the discharges changed under the drug: while SLEs without an initial negative DC-potential shift became more abundant, events with a clearly discernible DC-shift were reduced in both groups (to $46 \pm$

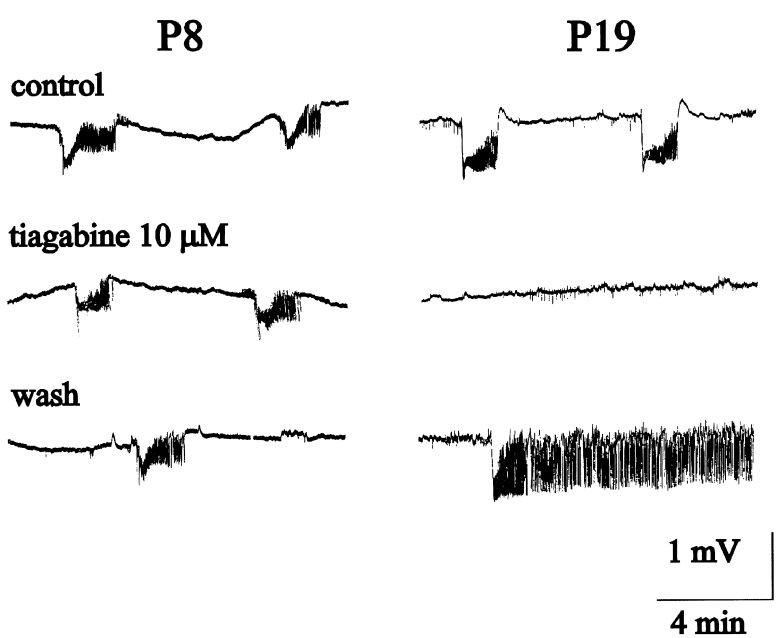

Fig. 1. Effect of 10 μM tiagabine on SLEs in the mEC in slices from an animal aged 8 days (left) or 19 days (right). The substance shows no marked alteration of the seizures in the younger tissue but suppresses SLEs in the slice from older animal. Bottom trace (right) shows the transition from SLEs to LRDs after prolonged exposure to Mg^{2+}-free solution and washout of tiagabine.

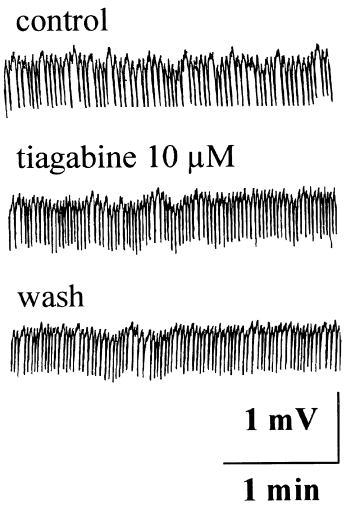

Fig. 2. Tiagabine does not suppress the LRDs in mEC of an animal aged p 18. High-frequency negative going field potentials develop upon prolonged washout of Mg^{2+}. This activity cannot be influenced by tiagabine.

13% in the younger and to 38 ± 14% in the older group; $P < 0.05$). At 1 μM, tiagabine did not change the frequency of SLEs in the early postnatal group ($n = 9$ slices from six rats; $P = 0.068$). Surprisingly, at a higher concentration (100 μM) tiagabine also lost its efficacy in the younger group ($n = 8$ slices from five animals) and likewise did not influence SLE-frequency in the late juvenile tissue ($n = 4$ slices from three rats; $P > 0.5$).

3.3. Effect of tiagabine in the mEC

In the immature mEC (p 5–8) the frequency of SLEs was 7.3 ± 1.0 SLEs/30 min and remained at 113 ± 17% of control under 10 μM tiagabine ($n = 12$ slices from 10 rats; $P > 0.8$; cf., Fig. 1, left). There was also no significant effect of 100 μM tiagabine ($n = 8$ slices from six animals; $P > 0.15$). In the group aged p 15–20, however, SLEs were effectively suppressed by 10 μM tiagabine to 43 ± 15% of control frequency (i.e., 5.5 ± 0.4 SLEs/30 min; $n = 14$ slices from nine rats; $P = 0.005$) and this suppression was partially reversible upon washout in eight experiments where SLEs could be restored before development of the late, high-frequent pattern of activity (LRDs, cf., Fig. 1, bottom right). Similar to CA1, 100 μM tiagabine failed to block the activity ($n = 4$ slices from three rats, $P > 0.5$). In 12 experiments, we exposed juvenile (p 15–20) slices to Mg^{2+}-free medium for a prolonged period of time before application of tiagabine. After an initial phase of repetitive SLEs, these slices generated LRDs. As shown in Fig. 2, LRDs could not be influenced by tiagabine (frequency 107 ± 7% of control; $n = 8$ slices from seven rats; $P > 0.1$ for 10 μM) which was also true at 100 μM (111 ± 8%; $n = 4$ slices from four rats, $P > 0.05$). The effects of 10 μM tiagabine on SLE frequency at both age groups and both locations are summarised in Fig. 3.

3.4. Age-dependence of the expression of GAT-1

The effect of tiagabine is based on a highly potent block of the GABA transporter GAT-1. We therefore examined whether the age-dependence of the effect of tiagabine is caused by a developmental up-regulation of the expression of GAT-1. Micropreparations of the respective areas (CA1 and mEC) from brain slices were used to amplify the specific sequence for GAT-1 and display the resulting cDNA on agarose gels (see Section 2). Fig. 4 shows that GAT-1 mRNA is present throughout all ages tested, including very young animals where tiagabine has no effect on epileptiform activity in the entorhinal cortex. In total, we analysed tissue from 5 to 6 animals in each age group (p 5–8, p 15–20, adult (> 6 weeks)). GAT-1 specific mRNA could be detected in two to five samples within each group and region without any developmental trend. In order to reveal more detailed data on the spatial distribution of GAT-1 we also performed in-situ hybridisations on brain sections from an immature (p 7) and an adult animal. Hybridisation with sense-probes did not yield detectable staining (not shown). Hybridisation with antisense probes showed GAT-1 mRNA positive cells in both the adult and young animal. In CA1 (Fig. 5a,b) we found clearly labelled, isolated cells in all layers with some bias for the border between stratum radiatum and stratum lacunosum–moleculare. This pattern is well compatible with the published distribution of interneurons in the hippocampus (Houser and Esclapez, 1994) In the entorhinal cortex the appearance of stained cell bodies was constricted to the grey matter with scattered distribution throughout all cell layers (Fig. 5c,d). Age-dependent differences in the expression or distribution of GAT-1 mRNA were not obvious. Thus, the tiagabine-sensitive GABA uptake molecule

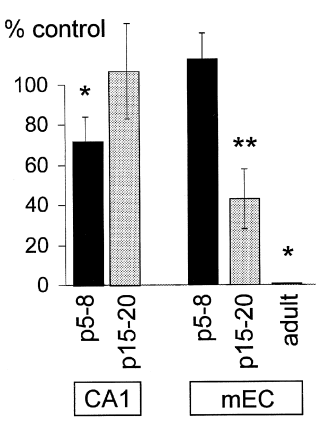

Fig. 3. Summary of changes in frequency under 10 mM tiagabine. At p 5–8, SLEs in CA1 are slightly reduced ($n = 20$), whereas tiagabine has no effect in the entorhinal cortex ($n = 12$). In older (juvenile, p 15–20) animals, tiagabine has no effect in CA1 but suppresses SLEs in the mEC ($n = 9$ and 13, respectively). In six slices from adult (> 6 weeks) rats, tiagabine completely and reversibly abolished epileptiform activity (data reported from Pfeiffer et al., 1996). $^{*}P < 0.05$; $^{**}P < 0.01$.

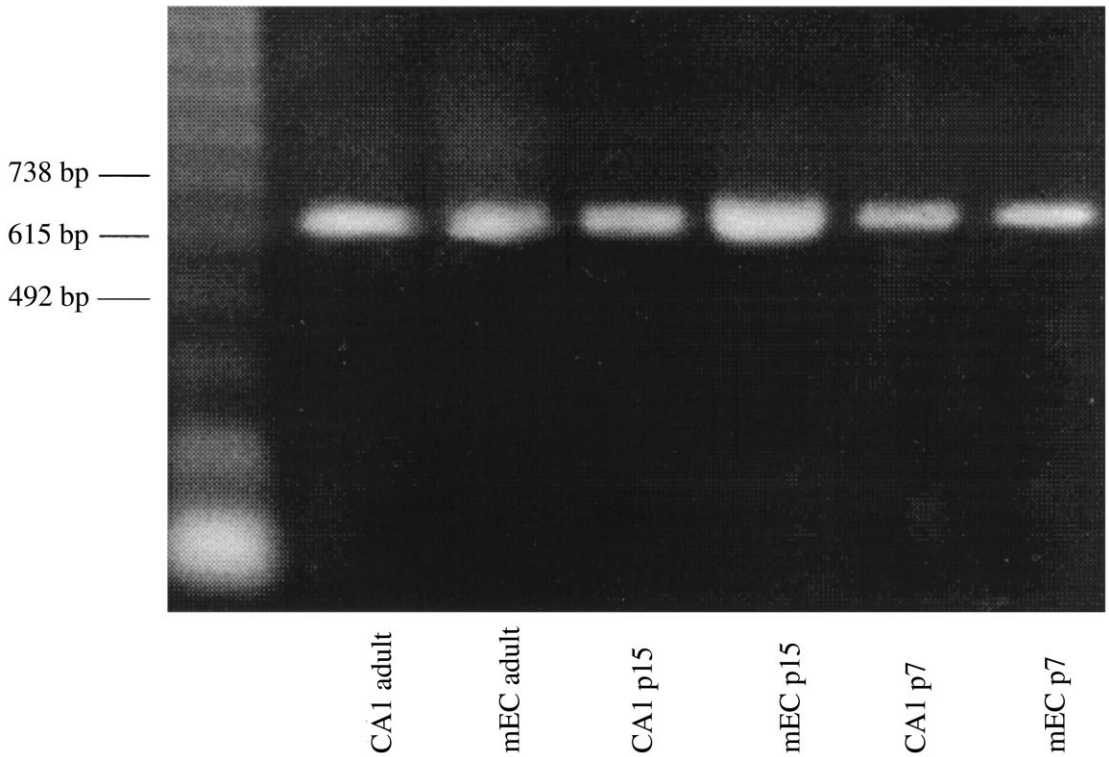

Fig. 4. Presence of GAT-1 mRNA in CA1 and mEC of rats at different age. Fluorescence-photograph from an ethidium bromide-stained agarose gel showing PCR-amplification products of cDNA specific for GAT-1. Lane 1: DNA-length standards. Lanes 2 and 3: adult animal, CA1 and mEC, respectively; lanes 4 and 5: animal at p 15, CA1 and mEC, respectively; lanes 6 and 7: animal at p 7, CA1 and mEC, respectively.

GAT-1 is expressed throughout development without clear changes in its tissue distribution.

4. Discussion

Our data show marked ontogenetic differences in the anticonvulsant effects of tiagabine in the low-Mg^{2+} model of acute temporal lobe epilepsy. In the entorhinal cortex, which is the key input and output structure of the hippocampus, the GAT-1-specific GABA uptake blocker was ineffective during the first postnatal week and showed more than 50% reduction of seizure-like activity in juvenile tissue. In adult animals, SLEs were completely and consistently suppressed (Pfeiffer et al., 1996). The anticonvulsant effect in mature brain slices is in line with findings from various animal models of epilepsy (Faingold et al., 1994; Suzdak, 1994; Walton et al., 1994; Smith et al., 1995). Meanwhile, the drug is successfully being used as add-on therapy in complex partial seizures (Gram, 1994; Richens et al., 1995; Suzdak and Jansen, 1995; Luer and Rhoney, 1998). However, data about tiagabine in childhood epilepsy are still scarce (Adkins and Noble, 1998).

In our study, the anticonvulsant efficacy of tiagabine clearly increased with age in the entorhinal cortex , but we found a more complex pattern in CA1. Here, tiagabine showed a moderate (ca. 30%) reduction of SLE frequency during the first week but had no effect in juvenile (p 15–20) tissue. When we counted only SLEs with a clear DC-shift (which are more reminiscent of the electroencephalographic equivalents of seizures) tiagabine reduced the activity equally in both groups. Our control measurements revealed, however, that also the ''flat'' events lacking a DC-shift go along with a pathologic rise in extracellular K^+ and thus cannot be ignored. It is difficult to compare the complex behaviour of tiagabine in immature CA1 with the effects in adult animals because hippocampal SLEs almost exclusively occur during infancy (Gloveli et al., 1995). In adult tissue, the predominant pattern of synchronised electrical activity in CA1 consists of short positive deflections (reminiscent of interictal spikes) which cannot be directly compared to the long-lasting SLEs (Pfeiffer et al., 1996). In this study, tiagabine could not suppress the short recurrent discharge pattern in adult CA1 (Pfeiffer et al., 1996) and showed a paradoxical (frequency-enhancing) effect at higher concentrations. A similar dose-dependency was found in the present experiments where the initial anticonvulsant effect of 10 μM tiagabine in CA1 of the younger animals was lost at 100 μM. The mechanism behind this behaviour remains unknown but it is feasible that the block of the GAT-1-mediated (neuronal) GABA uptake leads to a fading of synaptic inhibition due to depletion of interneurons of GABA (Schousbou et al., 1983; Gonsalves et al., 1989).

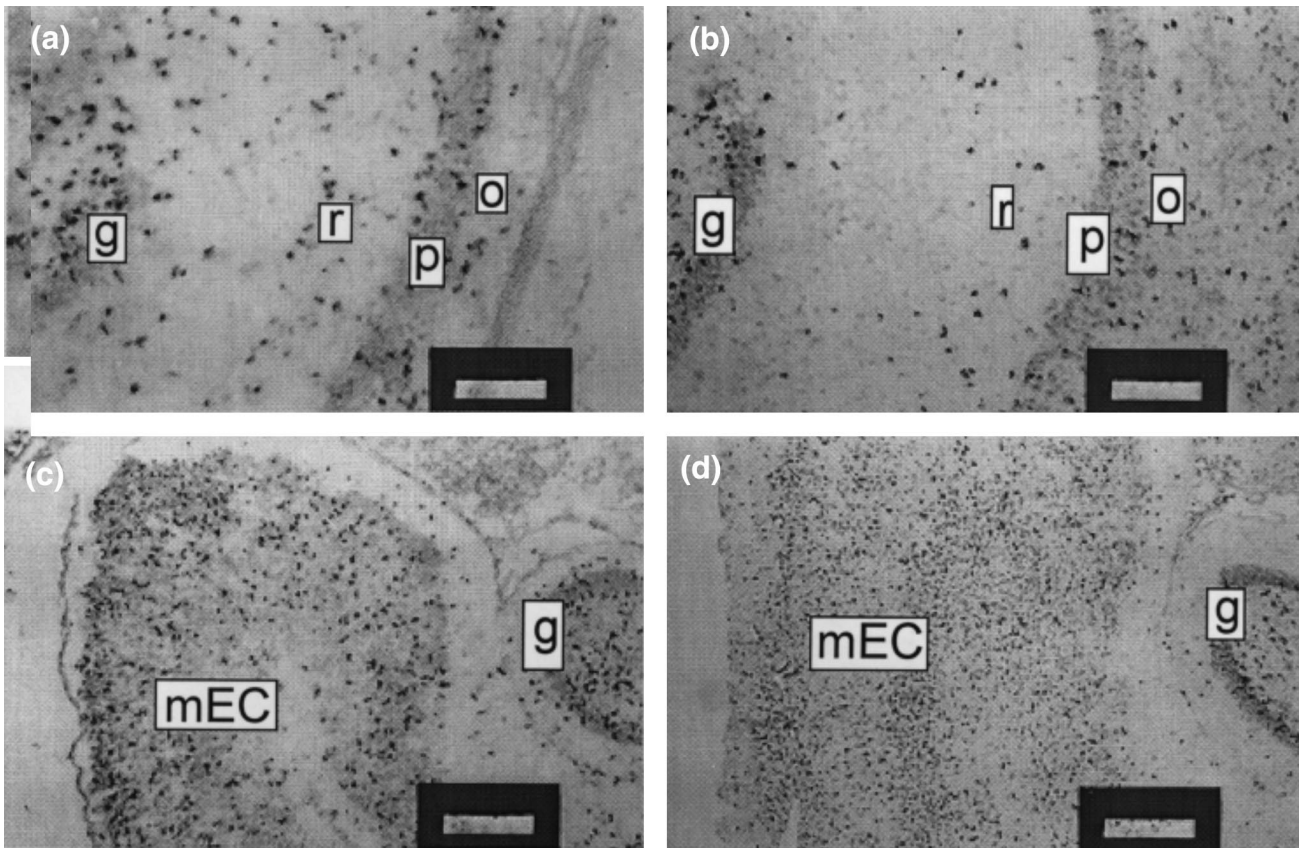

Fig. 5. Presence of GAT-1 specific mRNA in CA1 and mEC (in-situ hybridisation, antidigoxigenin immunostaining). (a) GAT-1 is clearly expressed in CA1 of an animal aged 7 days. Strong signals are visible in single cells throughout stratum radiatum, pyramidale and oriens. (b) GAT-1 mRNA in the hippocampus (CA1) of an adult animal showing qualitatively the same distribution which is typical for interneurons. (c) Expression of GAT-1 mRNA in the mEC of an animal at p 7. D: Expression of GAT-1 mRNA in the mEC of an adult animal. Calibration bar: 200 μm in (a) and (b), 400 μm in (c) and (d).

The same hypothesis has been put forward in our previous study (Pfeiffer et al., 1996) which showed that LRDs in the entorhinal cortex do not react to tiagabine. This electrographic pattern is generally resistant towards all clinically established antiepileptic drugs and therefore provides a model for drug-resistant forms of epilepsy (Zhang et al., 1995). GABA uptake blockers exert their anticonvulsant effects by prolongation of GABAergic inhibitory potentials and hence are comparable to the action of benzodiazepines and barbiturates. The failure of tiagabine to influence LRDs is therefore consistent with the general failure of GABAergic drugs in the mEC under these conditions, either due to a reduced availability of presynaptic GABA after prolonged seizure activity (Schousbou et al., 1983; Gonsalves et al., 1989) or due to a rundown of $GABA_A$ receptor function following impaired Mg^{2+}-dependent phosphorylation (Whittington et al., 1995).

Why does the efficacy of tiagabine depend on postnatal age? Tiagabine acts by blocking the GABA transporter subtype GAT-1 (Borden et al., 1994) which usually re-imports GABA into the axonal endings of inhibitory interneurons but is also found at low levels in hippocampal glia cells (Ribak et al., 1996). Block of GAT-1 results in prolongation of inhibitory postsynaptic potentials or currents which has been directly measured in brain slices (Rekling et al., 1990; Thompson and Gähwihler, 1992; Oh and Dichter, 1994; Roepstorff and Lambert, 1994; Draguhn and Heinemann, 1996). However, this mechanism can only be exploited by anticonvulsant drugs if GABA uptake by GAT-1 is indeed time-limiting for the decay of inhibitory postsynaptic potentials. We have previously shown that in dentate granule cells GABA uptake is not responsible for the decay of IPSCs during the first postnatal week but becomes time-limiting after the second postnatal week (Draguhn and Heinemann, 1996). This developmental change in the functional importance of GABA uptake is probably caused by the large extracellular volume fraction in very immature brain tissue which allows for the transmitter to diffuse very rapidly from the synaptic cleft. At later developmental stages, the extracellular space becomes progressively smaller (Lehmenkühler et al., 1993) and thereafter GABA uptake dominates the IPSC decay time course. It is well possible that this mechanism explains why in the mEC tiagabine gains more anticonvulsant efficacy from the first postnatal week to adulthood. Alter-

natively, the reduced efficacy of tiagabine in immature brain tissue may be caused by other developmentally regulated features of GABAergic inhibition. In the substancia nigra, age-dependence of the pharmacological control of flurothyl-induced seizures in vivo (Garant et al., 1995) has been attributed to developmental changes in the expression pattern of $GABA_A$-benzodiazapine and $GABA_B$ receptors (Velisek et al., 1995; Veliskova et al., 1998). Similar reasons might underlie the reduced efficacy of GABA mimetic drugs against electrically induced afterdischarges (Polásek et al., 1996) and NMDA-induced seizures (Velísek and Mares, 1995) in immature rats. It is also feasible that excitatory effects of GABA at early developmental stages (Kriegstein et al., 1987; Ben-Ari et al., 1988) contribute to the reduced efficacy of tiagabine. In this case a potentiation or prolongation of GABAergic potentials may be inefficient or even proconvulsant.

Our data show that the age-dependence of the effect of tiagabine is not likely to reflect an ontogenetic regulation of the expression of GAT-1. It has recently been shown that in mice GAT-1 is already present at a very early postnatal age (Evans et al., 1996). Our own results are in line with an early onset and continuous expression of GAT-1 in the rat hippocampus and entorhinal cortex. The expression pattern of GAT-1 mRNA in the immature animal did not show any obvious deviation from the distribution of GAT-1 positive cells in adult tissue which very much resembles the distribution of interneurons (Houser and Esclapez, 1994). Thus, we exclude that general changes in the GAT-1 expression pattern underlie the developmental differences in the efficacy of tiagabine. However, more subtle ontogenetic changes in the heterogeneous interneuron population are well feasible. We have recently shown a high degree of regional variance in the kinetic effects of tiagabine when different populations of interneurons are activated in adult rat CA1 (Engel et al., 1998). Similar data do not yet exist for immature tissue or for the mEC, but the variing effects of tiagabine may well be due to minor ontogenetic or regional differences in the amount of GAT-1 at certain interneuron terminals.

In summary, our data show that tiagabine does not suppress epileptiform activity in very immature mEC and shows low efficacy in CA1. The underlying developmental alterations in the maturing GABAergic system remain to be elucidated. Similar experiments should be carried out with animal models of chronic epilepsy in order to establish the effect of tiagabine in early postnatal and juvenile animals in vivo.

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