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Altered pharmacology and GABA-A receptor subunit expression in dorsal midline thalamic neurons in limbic epilepsy

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Abstract

The mediodorsal (MD) and paraventricular (PV) thalamic nuclei play a significant role in limbic epilepsy, and previous reports have shown changes in GABA-A receptor (GABAAR) mediated synaptic function. In this study, we examined changes in the pharmacology of GABAergic drugs and the expression of the GABAAR subunits in the MD and PV neurons in epilepsy. We observed nucleus specific changes in the sensitivity of sIPSCs to zolpidem and phenobarbital in MD and PV neurons from epileptic animals. In contrast, the magnitude of change in electrically evoked response (eIPSC) to zolpidem and phenobarbital were uniformly diminished in both MD and PV neurons in epilepsy.

Immunohistochemical studies revealed that in epilepsy, there was a reduction in GAD65 expression and NeuN positive neurons in the MD neurons. Also, there was a decrease in immunoreactivity of the $\alpha 1$ and $\beta 2/3$ subunit of GABAARs, but not the $\gamma 2$ of the GABAAR in both MD and PV in epilepsy. These findings demonstrate significant alterations in the pharmacology of GABA and GABAARs in a key region for seizure generation, which may have implications for the physiology and pharmacology of limbic epilepsy.

Keywords

Temporal lobe epilepsy; GABA-A receptor; mediodorsal thalamus; paraventricular thalamus; zolpidem; phenobarbital

Introduction

Temporal lobe epilepsy (limbic epilepsy) is a common form of human epilepsy that is frequently pharmacoresistant in that patients do not have their seizures completely controlled by current medications. The dorsal midline thalamus is an important component of the seizure circuit in limbic epilepsy (Bertram et al., 2001; Bertram et al., 2008). Previous experimental studies have found that focal inhibition of the mediodorsal (MD) thalamus by GABA agonists inhibited seizures and reduced seizure durations (Cassidy and Gale, 1998; Patel et al., 1988). Supporting clinical evidence suggests potential alterations in GABA binding, as revealed by [^{11}C] flumazenil positron emission tomography, in the MD nucleus

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of patients with limbic epilepsy (Juhasz et al., 1999). In a recent study, we provided evidence for contrasting alterations in synaptic GABA-A receptor (GABAAR) mediated sIPSCs in the two adjacent regions of the dorsal midline thalamus in limbic epilepsy (Rajasekaran et al., 2007). There was a significant decrease in the frequency of sIPSCs in the MD neurons of epileptic animals (referred hereafter as 'epileptic' MD neurons). In contrast, there was an increase in the number and magnitude of sIPSCs in the PV neurons of epileptic animals (referred hereafter as 'epileptic' PV neurons). The changes in IPSC kinetics obtained in the study suggested altered postsynaptic responses to synaptically released GABA.

Regional heterogeneity of GABAAR in the dorsal midline thalamus has been previously reported (Gao et al., 1995; Pirker et al., 2000b; Peng et al., 2002) suggesting the possibility of potential alterations in pharmacosensitivity to GABAergic agents in this region in epilepsy. The allosteric modulators of the GABAAR exert their actions through increasing the conductance of the receptor, which are in turn influenced by the subunit composition of the receptor. Because GABAA agonists are common antiepileptics, we wished to determine whether the pharmacology of the GABAAR was altered in epilepsy in this region by evaluating the modulation of the postsynaptic responses by the α -subunit preferential positive allosteric modulator, zolpidem (Pritchett et al., 1989), and the β -subunit specific (Smith and Olsen, 1995) anticonvulsant barbiturate, phenobarbital (PB). Using immunohistochemistry, we also investigated for evidence of changes in the expression of some GABAAR subunits in epilepsy. The report is the first examination of the potential alterations in the pharmacology of a key subcortical component of the limbic seizure circuit.

Methods

All experiments were performed on age-matched, adult (6–9 months old) male Sprague-Dawley rats housed on standard light / dark schedule with free access to food and water, and handled according to a protocol approved by the University of Virginia Animal Care and Use Committee.

Animal preparation

Adult male Sprague-Dawley rats were made epileptic using the continuous hippocampal stimulation method (Lothman et al., 1989). A bipolar electrode was implanted in the left mid-ventral hippocampus under ketamine-xylazine anesthesia using stereotaxic coordinates as described in Paxinos and Watson (Paxinos and Watson, 2005) (in mm): AP 5.3 posterior to bregma; L 4.9; DV 5.0 below dura; incisor bar at –3.3. One week after surgery, status epilepticus was induced by stimulating the hippocampus at 50Hz, 400 μ A using 1 ms biphasic square waves in 10 second trains applied every 11 seconds for 90 minutes. Self sustaining limbic status epilepticus developed during the stimulation and lasted 10–14 hours. Approximately 8–12 weeks later, animals developed spontaneous limbic seizures, which were documented by either continuous EEG monitoring or direct observation of behavioral seizures (Bertram and Cornett, 1994). Epileptic rats were used at least 4 months after stimulation to ensure that they had reached a comparable seizure maturity.

Electrophysiology

Thalamic slice preparation—Animals were anesthetized with halothane prior to decapitation, and brains were immersed into a low NaCl, high sucrose oxygenated dissection buffer (4°C) containing (in mM) 65.5 NaCl, 2 KCl, 5 MgSO₄, 1.1 KH₂PO₄, 1 CaCl₂, 10 Dextrose, 25 NaHCO₃ and 113 Sucrose (300 mOsm). The block containing the midline thalamus was mounted on a vibratome stage (Camden Instruments, UK) and cut at 300 μ m coronally. The slices were kept in oxygenated ACSF at 29°C for at least 1 hr before being

transferred to the recording chamber. The oxygenated ACSF contains (in mM) 127 NaCl, 2 KCl, 1.5 MgSO₄, 25.7 NaHCO₃, 10 Dextrose, 1.5 CaCl₂ (pH 7.4; 300 mOsm).

Recording and data acquisition—The slices were continuously perfused with oxygenated ACSF. The patch electrodes were prepared from thick walled borosilicate glass (World Precision Instruments, FL), pulled on a horizontal Flaming-Brown microelectrode puller (model P-97, Sutter Instruments, Novato, CA) using a two-stage pull protocol. Patch electrodes were filled with pipette internal solution containing (in mM) 153.3 CsCl, 1.0 MgCl₂, 10 HEPES, 5.0 EGTA, 2 ATP-Mg (buffered to pH 7.2 with CsOH, 285 mOsm) and had a resistance of 2–4 MΩ. Whole-cell voltage clamp recordings were made using an Axopatch 1D amplifier (Molecular Devices, CA). The temperature in the recording chamber was maintained at 24°C using an inline heating system coupled with an automatic temperature controller (Warner Instrument Corporation, USA). Attempts to record at physiological temperature resulted in reduced viability and recording quality.

The recordings were performed under visual control through a video monitor to identify neurons by position. Cells were voltage clamped to −60 mV and for the combination of the internal and external solutions, the calculated E_{Cl^-} was 0 mV and the experimental E_{Cl^-} was +5 mV. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of d-6-cyano-7-nitroquinoxaline-2, 3-dione (DNQX, 20 μM, AMPA/kainate antagonist) and DL −2-amino-5-phosphonopivalic acid (DL-AP5, 50 μM, NMDA antagonist) (both from Tocris, MO) to inhibit glutamate receptors. After access was obtained, the series resistance was compensated between 70–80% following capacitance compensation. The mean series resistance before compensation was 4–6 MΩ. Junction potential was not compensated for any experiment. Currents were low pass filtered at 5 kHz and digitized at a frequency of 10 kHz using 1322A Digidata (Molecular Devices, CA) A/D converter. The currents were recorded using pClamp 8.2 software (Molecular Devices, CA) 10 min after access was obtained to allow the pipette solution to equilibrate with the cell contents. Data for drug analysis were obtained from the recordings 10 min after the beginning of drug perfusion.

To obtain evoked IPSCs (eIPSCs), a bipolar concentric platinum electrode was placed adjacent to and within 500 μm of the recorded neuron. When recordings were performed from MD neurons, the stimulation electrode was positioned in the centrolateral nucleus, whereas the MD nucleus was stimulated when recording from PV neurons. The electrical stimuli consisted of single pulses (10–35 V, 100 μs duration) delivered at 0.1 Hz. In order to standardize the degree of receptor activation by the primary agonist (GABA) and facilitate comparison of drug effects, we adjusted the stimulus intensity for each cell such that it yielded a 475–500 pA postsynaptic response, which was at least 5 times greater than the average sIPSC amplitude of the MD and PV neurons. The responses were recorded using Clampex 8.02 (Molecular Devices, CA) such that the output current trace was a cumulative average of 10 consequent stimuli. Recordings in which access resistance changed > 20% before drug application were rejected.

Data Analysis—Tonic currents were determined using Clampfit 8.2 (Molecular Devices, CA) to measure the mean holding current sampled every 50 ms at 500 ms intervals. Fifty data points were collected from immediately before drug application and 5 min after drug application. The epochs containing synaptic events or unstable baselines were eliminated from the analysis. The drug effects on individual neurons were assessed by comparing the distribution of holding current before and after drug application by means of a Kolmogorov-Smirnov (KS) test (available online at http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html). The sIPSCs were analyzed using the MiniAnalysis (Synaptosoft, Decatur, GA). All events were identified visually to avoid errors in detection by automation. The threshold for detection of currents was set at 3

times the root mean square baseline noise, which was measured for each epoch of recording. An event was detected when there was at least a 100-ms interval between complete decay of the previous event and the rise of the next phasic current. All events were identified for determination of frequency, amplitude, half width, and 10–90% rise times; but decay constants were determined from events that did not have another overlapping event on the decay phase of the former. The 10–90% rise time was detected by the program by determining the time interval between the last data point with a value of 10% and the first data point with a value of 90% of the peak amplitude. Decay of sIPSCs was determined by fitting a 0.36 fraction of decay of current from the peak amplitude of the event. The decay constants were derived by the software by evaluating 0.36 fraction of peak current. Charge transfer for each event was calculated by the software as the integral under the current–time–trace (pAms) during an event. eIPSCs were analyzed using Clampfit 8.2 (Molecular Devices, CA). The charge transfer by the eIPSC was calculated as the area under the curve from the peak current to its return within 10% of the initial baseline holding current.

Immunohistochemistry

Tissue preparation—Four animals with limbic epilepsy and 4 age-matched controls were used. The procedures of tissue preparation were performed as described in detail previously (Sun et al., 2004). Briefly, animals were anesthetized with a lethal dose of pentobarbitone sodium (220 mg/kg i.p.) and perfused through the ascending aorta followed by 350–450 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and post-fixed in the same fixative for 2 hours at 4°C. After overnight incubation in 25% sucrose in 0.1 M phosphate buffer for cryoprotection, brains were frozen by immersion in –70°C isopentane. Coronal sections containing PV and MD nucleus were collected at 40 µm thickness. The sections were put into 5 vials sequentially, and 4–5 sections from each animal were used for double labeling of a subunit of GABAR and GAD65 or NeuN.

Silver staining—Silver staining experiments were performed on brain slices of rats 7 days after *status epilepticus* induced by continuous hippocampal stimulation using the manufacturer's protocol outlined in FD NeuroSilver™ Kit I (FD NeuroTechnologies, MD, USA). The sections were kept from light during and after the process. Stained slides were dehydrated and coverslipped for examination. Slides were examined qualitatively with a standard light microscope. Sections were examined at magnifications from 4X. The regions of positive-staining neurons were identified as grayish-black areas against a golden-brown background.

Immunohistochemistry—Following 3 washes in 0.1 M phosphate buffer and preincubation with blocking solution containing 5% normal goat serum (Jackson Immunoresearch Laboratories, INC. West Grove, PA) and 0.1% Triton X-100 in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 1 hour, tissue sections were incubated with the primary antibodies at 4°C for 72 hours on a shaker. The primary and secondary antibodies were diluted in PBS containing 2% normal goat serum, 0.2% bovine serum albumin (Jackson Immunoresearch Laboratories, INC. West Grove, PA). Subsequently, the sections were incubated in secondary antibodies of goat anti-rabbit conjugated with Alexa fluor 594 and goat anti-mouse conjugated with Alexa fluor 488 (5 µg/ml, Molecular Probes, Eugene, OR) for 1 hour on a shaker at room temperature in darkness. For each subunit and GAD65 (or NeuN) double-labeling experiment, sections from epileptic animals and age-matched controls were processed identically in the same time. Such experiments were repeated 3 times to ensure the reliability of the results. Sections were then mounted on slides with Gel/Mount* (Foster City, CA), the edge of each coverslip was sealed with clear nail polish, and slides were stored at –20°C. Controls in which the primary antibody was omitted provided only very weak nonspecific staining.

Antibodies and image acquisition—The antibodies against the GABAAR subunit $\alpha 1$ (1–16) and $\gamma 2$ (1–33) were obtained from Alomone labs (Israel). Mouse anti- $\beta 2/3$, mouse anti-GAD65, rabbit anti-GAD65, and mouse anti-NeuN antibodies were purchased from Millipore Corporation (Billerica, MA). Different concentrations of primary antibodies were titrated to arrive at the final concentrations (1.5 $\mu\text{g/ml}$ for $\alpha 1$; 2.0 $\mu\text{g/ml}$ for $\gamma 2$, $\beta 2/3$, and GAD65; 1:200 for NeuN) used in this study.

Fluorescent images of tissue sections were captured on a CoolSnap cf CCD camera (Roper Scientific Photometrics) mounted on an Eclipse TE200 fluorescent microscope (Nikon) driven by Metamorph imaging software (Universal Imaging Corp., Downingtown, PA). High-resolution digital images of each fluorochrome were acquired with a $\times 40/0.13$ NA lens (or a $\times 40/1.30$ NA lens for NeuN staining). All settings including light intensity and exposure time were kept constant for each pair of control and epileptic sections to yield data that are obtained in a standardized manner. High magnification laser scanning confocal microscopy (Sun et al., 2004) was performed in the MD and PV thalamic regions in some control slices to confirm that the antibody signals obtained were indeed specific and not due to autofluorescence. The sections were studied on a Nikon PCM2000 confocal microscope system equipped with a Nikon TE-200 inverted epifluorescence microscope (Nikon, Melville, NY) with an Argon/HeNe laser at the W.M. Keck Center for Cellular Imaging at the University of Virginia. Images were acquired through a 63×1.4 NA objective using an Orca-1 CCD Camera (Hamamatsu Photonics System, Middlesex, NY). Confocal and camera-based image acquisition and processing were driven by SimplePCI software (version 4.0.6, Compix, Cranberry Township, PA). Argon-ion and He/Ne lasers were used to visualize the fluorochrome.

Densitometric analyses—Expression levels of $\alpha 1$, $\beta 2/3$, $\gamma 2$ subunits, and GAD65 in control and epileptic animals were evaluated for staining intensity to determine the extent and patterns of change over time. For determining the levels of diffuse immunolabeling for each GABAAR subunits in the thalamus, the gray level values (or luminosity) were used for quantification. The mean luminosity of the immunoreactivity (IR) of GABAAR subunits and synaptic marker GAD65 in thalamic sections captured with 4x object were analyzed with Adobe Photoshop 6.0 (Adobe, San Jose, CA). Coronal sections from Bregma -2.2mm to Bregma -3.3mm were used for quantification in our study (Paxinos and Watson, 2005). A standardized $0.2\text{mm} \times 0.2\text{mm}$ area as shown in schematic image (Fig 7A, B) was selected to obtain the mean luminosity value. The area between the medial inferior corners of habenulae (from immediately below the third ventricle to 0.3mm below the third ventricle) was defined as the PV nucleus, and a 0.04mm^2 area was chosen for luminosity measurements. Two such regions were used for quantifying the (mean of region 1 and 2) luminosity in the MD, which was defined as lying between the lateral and medial edge of the habenula starting 0.2mm below the habenula. The chosen area was adjusted slightly to make sure that larger tissue-void areas (blood vessels) were excluded. To minimize the effect of staining variability on IR values between experimental groups, we normalized staining in MD and PV to the staining in the corpus callosum (CC) and derived intensity ratios for each. The CC lacks GABAR, and the staining intensity did not differ between experimental groups, staining in the region therefore represented non-specific background staining (Peng et al., 2004; Epsztein et al., 2006). The luminosity values for the MD and the PV were normalized against the values from the corpus callosum (CC) from the same section.

Digital images captured with a 40x object were used for neuronal counts for NeuN stained tissues. All neuronal NeuN-IR neurons visible on the $40\text{ }\mu\text{m}$ thick micrographs were counted for profile counting (Guillery, 2002). According to Abercrombie corrections, although an over count of 7% would be generated in our study; the degree of accuracy was enough to

discriminate the relative differences between control and epileptic animals. Two researchers blinded to the experimental grouping performed the profile count. Data were statistically analyzed by the unpaired t test using Prism 4.0 software (GraphPad Software, Inc. San Diego, CA), and $p < 0.05$ was considered significant. Unless specified otherwise, all values are reported as mean $\pm$ SEM.

Results

Electrophysiology

The IPSCs recorded from the MD and PV neurons in the presence of glutamatergic antagonists were completely blocked by 100 μ M bicuculline (data not shown) confirming that they were GABAAR mediated currents.

Baseline IPSC characteristics—Comparison of the baseline sIPSC data obtained from MD (control = 51 cells; epilepsy = 28 cells) and PV (control = 32 cells; epilepsy = 26 cells) neurons in the present study (Table 1,2) to that of our previous report (Rajasekaran et al., 2007) suggested that the baseline changes with respect to changes in frequency, amplitude and decay of the sIPSCs in the MD and PV neurons were similar; though the rise times of sIPSCs in the epileptic MD were similar to controls (2.48 ± 0.10 vs. 2.38 ± 0.05 ms; $p = 0.39$, unpaired t test), and that of epileptic PV were faster compared to controls (2.15 ± 0.11 vs. 2.56 ± 0.09 ms; $p = 0.006$, unpaired t test). However, the range of rise time values observed in the present study and our previous report were largely similar, and when rise time data of neurons from this and the previous study were pooled together, there was no significant difference between control and epilepsy groups suggesting that these differences may be principally related to sampling size.

We next examined changes in the decay kinetics of eIPSCs between control and epileptic MD and PV neurons. In contrast to sIPSCs which principally reflect activation of synaptically located GABAAR, eIPSCs, in view of increased multiquantal release of GABA, are likely generated by activation of both synaptic and extrasynaptic GABAAR (Overstreet and Westbrook, 2003; Scimemi et al., 2005); and they may represent activity modulated by the coordinated firing of multiple afferent synapses and represent a different point in the GABA dose response curve. While the baseline eIPSC characteristics were similar between control and epileptic MD neurons, the half width of the eIPSCs in epileptic PV neurons were shorter than that in control PV neurons (Table 3).

Effect of Zolpidem on GABAAR mediated currents—Application of zolpidem shifted the baseline holding current in both control and epileptic MD (Table 1) and PV (Table 2) neurons. This shift in baseline holding current was reversed by the co-application of 100 μ M bicuculline. Compared to controls, there was a significant increase in baseline holding current in epileptic MD neurons following application of 1 μ M zolpidem. The effect of zolpidem on sIPSCs was similar in both control and epileptic MD neurons at both concentrations (Table 1, Fig 1). In contrast, in the epileptic PV neurons, while shifts in baseline holding currents were similar to control neurons, there was a loss of zolpidem induced enhancement of sIPSC amplitude at both concentrations, and the net charge transfer following application of 1 μ M zolpidem was significantly attenuated compared to control PV neurons (Table 2, Fig 2). The sensitivity of eIPSCs to zolpidem was attenuated in both epileptic MD and PV neurons (Table 3, Fig 3).

Effect of Phenobarbital on GABAAR mediated currents—Application of PB (250 and 500 μ M) resulted in a consistent inward shift in baseline holding current of MD (Table 1) and PV (Table 2) neurons, which was reversed by stopping its application or by co-

application of 100 μ M bicuculline. In both control and epileptic MD neurons, the shift in baseline holding current was similar. However, there was diminished augmentation of sIPSC decay time constant by 250 μ M PB in epileptic MD neurons compared to controls (Table 1, Fig 4). In contrast, in epileptic PV neurons, there was a significant ($p < 0.002$) augmentation of baseline holding current by the higher concentration of PB, while effects on other sIPSC parameters remained unaltered (Table 2, Fig 5). PB-induced prolongation of eIPSC half width in control MD and PV neurons was significantly diminished in the epileptic MD and PV (Table 3, Fig 6) neurons.

Immunohistochemistry

Figure 8A shows the differential pathological changes in the MD and PV nucleus in epilepsy. Seven days after *status epilepticus*, significant neuronal degeneration, as evidenced by silver staining occurred in the MD, but not in the PV. We then examined the MD and PV nuclei in chronic limbic epilepsy for cell loss by counting the number of NeuN positive neurons. There was neuronal loss in the MD and a relative sparing of neurons in the PV in limbic epilepsy (Fig 8B–E). Quantitative analysis confirmed a significant reduction ($p < 0.05$, unpaired t test) in NeuN-IR neurons in the epileptic MD (12 ± 0.62 , $n = 25$) compared to controls (15 ± 0.66 , $n = 35$). In contrast, there was no difference in the number of the NeuN-IR neurons between control (28.12 ± 1.59 , $n = 17$) and epileptic PV (25.25 ± 1.68 , $n = 12$). Also, it is likely that we may have underestimated neuronal loss in the MD from a re-concentration of the neurons from shrinkage. However, a measure of the area of each nucleus was not feasible as the borders are not well defined.

We then examined for changes in the IR of GAD-65 (the presynaptic marker of GABAergic synapses), and the $\alpha 1$, $\beta 2/3$, and $\gamma 2$ subunits of the GABAAR in epilepsy. Fig 9A–D show low resolution images of GAD-65 and the GABAAR subunits IR demonstrating differential intensities for these markers in the thalamus and hippocampus. Fig 10A–D show high resolution confocal images confirm that there was diffuse staining in clusters and no well stained cell bodies. The intensity of CC staining was used in all subsequent examinations as a reference area for normalization of the IR staining in the MD and the PV because of the low and similar background staining together with the absence of the GABAAR in the CC. Diffuse GAD65-IR was found in both MD and PV nucleus of control animals (Fig 11A). The GAD65-IR was diminished in the MD nucleus in epilepsy (Fig 11B). Quantitative analysis demonstrated that the mean luminosity in epileptic MD, but not the PV was significantly reduced ($p < 0.05$, unpaired t test) compared to controls (Fig 11E).

Changes in the pharmacology of GABAAR modulators may also involve changes in the relative expression of the constituent receptor subunit. We therefore studied the expression of GABA-A receptor subunits – $\gamma 2$ (Fig 11C, D), $\alpha 1$ (Fig 12A, B), and $\beta 2/3$ (Fig 12C, D) – in the MD and the PV nuclei in limbic epilepsy. In epileptic animals, the IR of the $\gamma 2$ subunit (Fig 11D, F) was unaltered, whereas those of $\alpha 1$ (Fig 12B, E) and $\beta 2/3$ (Fig 12D, F) subunits of the GABAAR significantly diminished ($p < 0.05$, unpaired t test) in both the MD and the PV nucleus. Since the IR of GAD65 and number of NeuN positive neurons were significantly decreased in the MD in epilepsy, cell loss in epilepsy may be a confounding factor for diminished $\alpha 1$ IR in the MD. To address whether cell loss may have influenced $\alpha 1$ -IR in the MD, we therefore performed a blinded analysis to quantify the amount of tissue void area (predominantly capillaries) in a 4X image of the brain slice. Quantification was performed by aligning the image onto a grid of 99 equally spaced squares in Adobe Photoshop software and counting the number of intersections where there was a confluent area with no staining. The number of tissue void intersections in images was similar in both control and epileptic animals suggesting while cell loss may contribute to changes in epilepsy, it may not adequately explain the loss of IR in the MD in epilepsy. Further, while cell loss was found only in the MD, reduction in $\alpha 1$ -IR occurred in both MD and PV. Taken

together with changes in the IR of GAD65 and NeuN, these data suggest that the reductions in GABAR subunit IR in the epileptic MD and PV nucleus may not solely be a consequence of regional pathology, but likely due to altered receptor subunit expression.

Discussion

The primary finding of this study is that in limbic epilepsy there may be nucleus specific changes in the physiology and pharmacology of GABAAR mediated inhibitory transmission in the dorsal midline thalamus. In addition to the previously described nucleus specific changes in the frequency and kinetics of sIPSCs (Rajasekaran et al., 2007), we found reduction in the eIPSC half width in epileptic PV but not epileptic MD neurons, and a decrease in baseline holding current in epileptic MD but not epileptic PV neurons. There was a very broad reduction in the sensitivity to zolpidem in the PV, but a far more limited change in the MD. Phenobarbital also had a significant effect on tonic inhibition, with an enhanced sensitivity in epileptic PV, and a trend to reduction in sensitivity in the epileptic MD neurons. In addition, sensitivity to PB was broadly reduced in the epileptic MD neurons, whereas the changes were minimal in the epileptic PV neurons. These changes were associated with greater neuronal loss and reduced GAD65-IR in the epileptic MD; and reduced immunoreactivity to $\alpha 1$, $\beta 2/3$, but not $\gamma 2$ in both epileptic MD and epileptic PV neurons. These findings confirm and significantly extend the results of our previous study in the dorsal midline thalamic neurons in limbic epilepsy. In addition, these observations may have potential implications for the treatment of epilepsy.

Our observations from the electrophysiological studies of altered GABAergic currents and drug sensitivity of evoked responses in both the epileptic MD and PV suggest a less effective inhibitory transmission at the central level of the regional network. Unlike PV neurons, the epileptic MD neurons had a more positive baseline holding currents compared to control neurons, which may suggest reduced afferent GABAergic input, increased input resistance, or potential changes in the expression of potassium channels sensitive to cesium. At the same time, there were also clear nucleus specific differences between epileptic MD and PV neurons on the action of zolpidem and PB on sIPSCs and baseline holding current. In the present study, the alterations in drug sensitivity of sIPSCs and eIPSCs do not always parallel suggesting that changes in presynaptic properties may also potentially influence drug response in epilepsy independent of postsynaptic alterations. Alternatively, these observation may suggest that the differences between the sIPSC and eIPSC responses may be associated to differences in the postsynaptic responses at different points on the GABA concentration response curve because, the concentration of GABA in the synapse is higher during stimulation, yielding a greater postsynaptic response (500 pA) that was at least 5 times greater than that of the mean sIPSCs amplitude of control and epileptic MD and PV neurons. The differences in the degree of postsynaptic receptor occupancy or the potency and efficacy of GABA and its modulators in epilepsy may also mask potential changes in the drug sensitivity of the sIPSCs. We also found evidence for a shift in the input-output relationship in response to increasing stimulus in epileptic neurons; however, this was not investigated further because increasing stimulus intensity affected the integrity of the patch clamp recordings.

Previous studies have suggested that inhibitory afferents to the MD arise from the ventral pallidum, the reticular nucleus of the thalamus (Kuroda and Price, 1991; Kuroda et al., 1992), the reticular nucleus of the substantia nigra (Kuroda et al., 1998), and the anterior pretectal nucleus (Bokor et al., 2005). The reduction in GAD65 IR in the epileptic MD region suggests loss of inhibitory synapses, which may account for diminished sIPSC frequency. A shortened mIPSC decay observed in epileptic PV neurons (Rajasekaran et al., 2007) suggested an increase in $\alpha 1$ subunit expression and zolpidem sensitivity (Vicini et al.,

2001). However, similar to our previous report, we did not observe a change in the sIPSC decay kinetics in epileptic PV neurons, and there was a loss of zolpidem sensitivity of sIPSCs which was associated with a reduction in $\alpha 1$ subunit IR. Our study also provides evidence that the presynaptic effects of PB are distinct from that of the anesthetic barbiturates. The decrease in sIPSC frequency by PB may be related to increase in firing thresholds of presynaptic fibers (Schulz and Macdonald, 1981; French-Mullen et al., 1993), or due to a decrease in the release probability as a consequence of a direct action by reducing calcium entry to the synaptic terminal (Ondrusek et al., 1979).

The data from this study together with results of other studies suggest clear alterations in GABA physiology and pharmacology in limbic epilepsy. Also, the data indicates that two regions involved in potential circuit may have very different changes in their response to GABA as well as their pharmacosensitivity to the same drug. For instance, in this study, we observed attenuation of zolpidem sensitivity in the dorsal midline thalamic nuclei, whereas, there was a markedly enhanced sensitivity to zolpidem in the CA1 region of epileptic animals (Mangan and Bertram, III, 1997).

Drug sensitivity of the modulators is in part dependent on the subunit composition of the postsynaptic GABAAR, and there are significant differences in GABAAR subunit distribution between the MD and PV nucleus (Pirker et al., 2000b). While zolpidem sensitivity is principally influenced by the $\alpha 1$ – 3 subunits (Pritchett and Seeburg, 1990; Mohler et al., 2002; Rudolph and Mohler, 2004), it also depends on the $\gamma 2$ subunit of the GABAAR (Cope et al., 2005). Likewise, the $\beta 2/3$ subunit of the GABAAR is known to mediate the sensitivity of PB (Smith and Olsen, 1995). Zolpidem modulation of baseline holding current can be attributed to extrasynaptically present $\alpha 1$ subunit containing GABAARs (Liang et al., 2004; Shen et al., 2005), and an increase in zolpidem induced shift in tonic current in epileptic MD neurons maybe due a greater relative proportion of $\alpha 1$ subunit at the extrasynaptic domain. The modulation of baseline holding current by PB may be due to its action as a direct agonist (Schulz and Macdonald, 1981; French-Mullen et al., 1993) or an allosteric modulator, as observed with other anesthetic and anticonvulsant barbiturates (Pittson et al., 2004; Mathers et al., 2007). While it is known that anesthetic barbiturates activate GABAAR containing the δ (Adkins et al., 2001) or $\alpha 6$ subunit (Drahts and Fisher, 2006), similar reports do not exist for the anticonvulsant barbiturates. Immunochemical studies have previously reported the presence of the δ subunit of the GABAAR in both the MD and PV thalamus (Pirker et al., 2000b), and the modulation of tonic currents by PB in this study may be mediated by the δ subunit containing GABAARs; although it is also known that extrasynaptically located $\alpha \beta$ subunit containing GABAAR could also contribute to tonic inhibition (Mortensen and Smart, 2006). Further studies are necessary to confirm whether these data indicate a differential alteration in the expression of the δ subunit containing GABAAR in epileptic neurons. It is also necessary to note two limitations in our interpretation of changes in tonic currents in epileptic neurons: (1) that these recordings were performed at room temperature, where tonic currents can be enhanced by the summation of sIPSCs; and (2) that baseline shifts, and their alterations in epileptic neurons may be associated with the hyperpolarization of the recorded neuron due to drug effect on other cells that are synaptically connected to the recorded neurons.

Our observation of reduced $\alpha 1$ and $\beta 2/3$ subunit of the GABAAR in epileptic MD and PV neurons may partly explain the reduced sensitivity of zolpidem and PB, respectively in the epileptic neurons. A potential confound in our observation of decreased immunoreactivity of the GABAARs in epileptic MD and PV thalamus is cell loss in epilepsy. While the possibility of cell loss accounting for decreased GABAAR-IR cannot be excluded in the epileptic MD thalamus; however, in the epileptic PV thalamus, the unaltered number of NeuN positive neurons suggests that the reduced immunoreactivity to the $\alpha 1$ and $\beta 2/3$

subunit of the GABAAR may indeed be due to reduced expression of these subunits. Interestingly, despite cell loss in the epileptic MD, the $\gamma 2$ -IR was similar to that of control MD thalamus. One possibility for this observation could be an increased expression of the $\gamma 2$ subunit in the remaining MD neurons. However, previous studies (Fritschy and Mohler, 1995b; Fritschy and Mohler, 1995a; Pirker et al., 2000a) and our data demonstrate that the GABAAR-IR is diffuse and individual neurons could not be distinguished readily. Therefore, cell loss in epilepsy alone may not contribute to significant alterations in the global IR levels. While the present report provides clear evidence for alteration in the IR of the GABAAR subunits in the epileptic MD and PV thalamus, at the moment, we cannot distinguish the cellular localization of the subunits relative to the synaptic terminal to confirm whether changes in subunit expression occurred at the synapse or extrasynaptic regions. A detailed and focused anatomical study would be required to answer these questions.

Although, little is known about the intricacies of the primary seizure circuits of the dorsal midline thalamus, previous studies have shown that decreasing excitability of the MD thalamus, by either augmenting GABAergic or decreasing glutamatergic transmission, inhibits seizure activity (Cassidy and Gale, 1998; Patel et al., 1988). There is also evidence for pathology to the MD thalamus in chronic epilepsy in animal models (Bertram and Scott, 2000) and clinical studies (Dreifuss et al., 2001). A role for alterations in the pharmacology of GABAAR mediated inhibitory transmission in the MD thalamus is suggested by the report of Juhasz et al. (Juhasz et al., 1999) who found decreased benzodiazepine receptor binding in patients with chronic limbic epilepsy. Such changes may contribute to enhanced seizure spread and / or increased pharmacoresistance to antiepileptic drugs. The reduced efficacy of modulation by clinically important GABAAR modulators in the epileptic MD neurons in the present study may contribute to such changes. The results of the present study may also have implications on changes in circadian rhythms in epilepsy since the PV thalamus is an important component of the circadian timing system (Novak and Nunez, 1998; Salazar-Juarez et al., 2002) that has reciprocal connectivity with the suprachiasmatic nucleus of the hypothalamus (Berk and Finkelstein, 1981; Novak et al., 2000; Moga et al., 1995; Peng and Bentivoglio, 2004), and chronic seizures have clear diurnal distribution (Quigg et al., 1998; Bertram and Cornett, 1994) and also alter circadian rhythms in animals (Quigg et al., 1999; Bastlund et al., 2005) and humans (Quigg et al., 2006).

In summary, the results of the electrophysiological and anatomical studies revealed likely alterations in the GABAAR mediated neuronal transmission in chronic epilepsy and highlights complex changes in the pharmacology of GABAAR mediated transmission in the dorsal midline thalamic nuclei in limbic epilepsy. Together with previous studies, the present study demonstrates the significant variations in the GABA pharmacology in a seizure circuit in limbic epilepsy.

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Fig 1.

Effect of 100nM zolpidem on sIPSCs of control (A) and epileptic (B) MD neurons. The representative current traces are shown in A1 and B1. Cumulative probability plot of amplitude shows that drug application (black trace) did not change ($p > 0.05$, KS test) the amplitude of sIPSCs in control (A2) and epileptic (B2) neurons. Normalized average sIPSC revealed similar drug sensitivity in control (A3) and epileptic (B3) neurons. Figures C and D demonstrate the effect of 1μM zolpidem on control and epileptic MD neurons, respectively. Their representative current traces are shown in C1 and D1. Zolpidem application enhanced ($p < 0.05$, KS test) sIPSC amplitude and decay in both control (C2, C3) and epileptic (D2, D3) neurons.

Fig 2.

Effect of zolpidem on sIPSCs of control and epileptic PV neurons. Cumulative probability plots of amplitude and normalized average sIPSC from control neurons demonstrate that zolpidem application (black trace) at both 100nM and 1 μ M concentration significantly enhanced ($p < 0.05$, KS test) the amplitude (A1, C1) and decay (A2, C2) of sIPSCs. The drug sensitivity at both concentrations was lost in epileptic PV neurons (B1, B2; D1, D2).

Fig 3.

Effect of zolpidem on eIPSCs. Representative traces of electrically evoked IPSCs (eIPSCs) recorded from control and epileptic MD (A, B), and control and epileptic PV (C, D) neurons before (grey trace) and after (black trace) application of 1 μ M zolpidem. Note the absence of amplitude enhancement and attenuation of time course of eIPSCs following drug application in both epileptic MD and PV neurons. Area under the curve was measured from the peak current to its return to within 10% of the initial baseline holding current. Dotted lines represent the baseline holding current before and after drug application.

Fig 4.

Effect of PB on MD neurons. Representative averaged sIPSC traces show that PB at 250 μ M caused an increase in baseline holding current in control, the magnitude of which appeared to be reduced in epileptic neurons (A). The difference in the mean shifts in baseline holding currents in control (grey bar) and epileptic (black bar) MD neurons were however not statistically significant ($p > 0.05$, unpaired t test) (B). Representative normalized and averaged sIPSC reveals reduced drug sensitivity in epileptic MD neuron to 250 μ M PB (C). Representative averaged sIPSC show that PB at 500 μ M also caused similar shifts in baseline holding currents in both control and epileptic MD neurons (D). Statistical analysis revealed that the mean shifts in baseline holding current in control (grey bar) and epileptic (black bar) MD neurons following application of 500 μ M were similar ($p > 0.05$, unpaired t test) (E). Representative sIPSC after application of 500 μ M PB also show similar sensitivity of the drug in control and epileptic neurons (F).

Fig 5.

Effect of PB on PV neurons. Representative averaged sIPSC traces show that PB at 250 μ M caused similar shifts in tonic currents in control and epileptic PV neurons (A). Statistical analysis revealed that the mean shift of baseline holding current in control (grey bar) and epileptic (black bar) MD neurons following application of 250 μ M PB were similar ($p > 0.05$, unpaired t test) (B). Representative normalized and averaged sIPSC reveals unaltered drug sensitivity to 250 μ M PB in epileptic PV neuron compared to controls (C). Representative averaged sIPSC traces show that PB at 500 μ M enhanced tonic currents more in epileptic PV neurons compared to controls (D). Statistical analysis confirmed that there was a significant ($p < 0.0002$, unpaired t test) increase in the mean shift of baseline holding current in epileptic (black bar) neurons compared to control (grey bar) MD neurons following application of 500 μ M PB (E). Representative sIPSC after application of 500 μ M PB however showed similar sensitivity of the drug in control and epileptic neurons (F).

Fig 6.

Effect of PB on eIPSCs. Representative traces of electrically evoked IPSCs (eIPSCs) recorded from control and epileptic MD (A,B) and PV (C, D) neurons before (grey line) and after (black line) application of 250 μ M phenobarbital (PB). Note the attenuation of PB induced prolongation of time course in epileptic MD and PV neurons. Area under the curve was measured from the peak current to its return to within 10% of the initial baseline current. Dotted lines represent the baseline holding current before and after drug application.

Fig 7.

Area used for immunoreactivity quantification. The standardized boxed area ($0.2\text{ mm} \times 0.2\text{ mm}$) in the representative region for CC (A). PV was chosen 0.1 mm below the third ventricle, and MD was chosen from 2 adjacent boxes approximately 1 box below the third ventricle with the medial line of the boxes aligned with the medial edge of the medial habenular nucleus (B). Scale bar in B is $90\text{ }\mu\text{m}$.

Fig 8.

Upper panel. Representative silver stained section of dorsal thalamus from a 7 days post *status epilepticus* animal showing significant neuronal degeneration in the MD and a sparing of the PV (A). Scale bar in A is 100 μ m. *Lower panel.* IR of neuronal NeuN in the control and epileptic MD and PV. In the MD, compared to controls (B), the number of NeuN-IR neurons was decreased in epilepsy (C). In contrast, number of NeuN-IR neurons was similar in both control (D) and epileptic (E) PV. The scale bar in B is 15 μ m, and also applies to C, D and E.

Fig 9.

Distribution of $\alpha 1$, $\beta 2/3$, $\gamma 2$, of the GABAAR, and GAD-IR in thalamus captured at low magnification (2x, NA = 0.1) to demonstrate different intensity of staining at different areas. The white arrows point to PV and the white arrowheads point to MD. The $\alpha 1$, $\beta 2/3$, $\gamma 2$, and GAD-IR were expressed in most thalamic nuclei, and the entire thalamus appeared uniformly stained for these subunits, except for the reticular nucleus and the habenula or hippocampus (A–D). The staining was diffuse, and individual neurons could not be readily distinguished, except that of $\alpha 1$ -IR in hippocampal dentate gyrus (A). The scale bar in figure A is 200 μm , and also applies to B, C and D.

Fig 10.

High magnification (63X) images obtained with laser scanning confocal microscopy reveal the diffuse distribution of GABAAR $\alpha 1$, $\beta 2/3$, and $\gamma 2$ subunits and GAD65 immunoreactivity in control MD. The distribution of IR of $\alpha 1$ (A), $\beta 2/3$ (B), and $\gamma 2$ (C) subunits was similar and found to be present as many small diffuse clusters and some large clusters. The immunoreactivity of GAD65 (D) was mainly found as large intense clusters. The scale bar in figure A is 7 μm .

Fig 11.

GAD65-IR and $\gamma 2$ IR in MD and PV nucleus of control and epileptic animals. Compared to controls (A), the GAD65-IR was reduced in intensity in MD (MD is circled) of epileptic animals (B). In contrast, in both MD and PV, $\gamma 2$ IR was similar in control (C) and epileptic (D) animals. Quantification of GAD65-IR showed a significant ($p < 0.05$, unpaired t test) reduction in MD nucleus of epileptic animals (E). Scale bar in A is 80 μm , and also applies to B, C and D.

Fig 12.

Upper panel IR of $\alpha 1$ and $\beta 2/3$ in MD and PV nucleus of control and epileptic animals. Compared to their respective controls (A, C), the intensity of $\alpha 1$ - (B) and $\beta 2/3$ -IR (D) was reduced in PV and MD of epileptic animals. The location of PV and MD are delineated for comparison. *Lower panel* Quantification of $\alpha 1$ - and $\beta 2/3$ -IR showing a significant reduction in the IR ratio of the subunits in both MD and PV nucleus of epileptic animals (E and F). Scale bar in A is 80 μ m, and also applies to B, C and D.

Table 1
Effect of zolpidem and phenobarbital (PB) on sIPSCs in MD neurons in chronic limbic epilepsy

		Baseline (n=51, 28)	Relative difference to pre drug values			
			Zolpidem 100mM (n=6,5)	Zolpidem 1μM (n=5,6)	PB 250μM (n=8,7)	PB 500μM (n=13,8)
Difference in baseline holding current (pA)	Control	-50.71 ± 4.15	5.26 ± 3.13	3.95 ± 3.29	27.41 ± 5.8	43.23 ± 4.5
	Epilepsy	-33.88 ± 2.38 **	0.64 ± 3.70	15.22 ± 2.74 *	12.74 ± 3.3	45.55 ± 10.0
Frequency (Hz)	Control	0.47 ± 0.05	0.03 ± 0.04	0.03 ± 0.06	0.21 ± 0.08	0.20 ± 0.09
	Epilepsy	0.31 ± 0.03 *	0.01 ± 0.03	0.03 ± 0.07	0.12 ± 0.07	0.12 ± 0.05
Amplitude (pA)	Control	73.69 ± 2.83	6.09 ± 5.85	13.01 ± 3.54	3.05 ± 4.04	0.47 ± 4.30
	Epilepsy	76.38 ± 2.60	9.78 ± 9.35	18.84 ± 4.47	2.70 ± 3.90	0.70 ± 5.32
Decay (ms)	Control	14.77 ± 0.30	1.32 ± 0.34	3.82 ± 0.69	4.93 ± 0.69	5.26 ± 0.40
	Epilepsy	16.22 ± 0.50 **	1.24 ± 0.24	3.95 ± 0.61	1.79 ± 1.22 *	3.81 ± 0.58
Area (pAms)	Control	894.7 ± 40.67	223.5 ± 89.00	473.4 ± 70.11	496.1 ± 79.59	475.7 ± 83.47
	Epilepsy	1014 ± 50.33	342.6 ± 199.80	533.3 ± 117.30	296.3 ± 74.08	516.5 ± 129.20

* p<0.05,

** p<0.01 (unpaired t test) compared to control.

Table 2

Effect of zolpidem and phenobarbital (PB) on sIPSCs in PV neurons in chronic limbic epilepsy.

		Baseline (n=32, 26)	Relative difference to pre drug values			
			Zolpidem 100nM (n=6,5)	Zolpidem 1μM (n=5,6)	PB 250μM (n=8,7)	PB 500μM (n=13,8)
Difference in baseline holding current (pA)	Control	-45.17 ± 5.51	7.74 ± 3.23	8.94 ± 1.77	21.75 ± 5.59	33.25 ± 4.9
	Epilepsy	-44.93 ± 4.32	11.29 ± 8.50	2.58 ± 4.76	17.32 ± 3.84	71.80 ± 6.8 ***
Frequency (Hz)	Control	0.40 ± 0.04	0.03 ± 0.03	0.03 ± 0.06	0.12 ± 0.04	0.16 ± 0.05
	Epilepsy	0.91 ± 0.12***	0.05 ± 0.05	0.03 ± 0.07	0.31 ± 0.12	0.58 ± 0.13 **
Amplitude (pA)	Control	70.44 ± 2.82	18.22 ± 3.27	25.29 ± 6.70	4.68 ± 3.33	5.81 ± 2.95
	Epilepsy	96.03 ± 4.46***	0.13 ± 2.70 **	5.19 ± 10.70 *	0.97 ± 8.65	10.92 ± 5.32
Decay (ms)	Control	15.22 ± 0.43	1.95 ± 0.64	3.98 ± 0.78	3.99 ± 0.93	4.43 ± 0.64
	Epilepsy	14.61 ± 0.61	1.40 ± 0.61	2.33 ± 1.04	4.48 ± 0.68	3.55 ± 0.63
Area (pAms)	Control	891.30 ± 51.85	353.3 ± 106.40	667.6 ± 123.80	439.8 ± 102.00	605.5 ± 72.53
	Epilepsy	1110 ± 64.97 **	152.7 ± 68.11	178.6 ± 147.40 *	546.9 ± 164.70	786.3 ± 108.70

* p<0.05,

** p<0.01 (unpaired t test) compared to control.

Table 3

Effect of zolpidem and phenobarbital (PB) on eIPSCs in MD and PV neurons in chronic limbic epilepsy.

MD thalamus				
Parameter	Group	Baseline (n=19,13)	Relative difference to pre drug values	
			Zolpidem 1 μ M (n=9,7)	Phenobarbital 250 μ M (n=10,6)
Amplitude (pA)	Control	486.3 $\pm$ 39.60	205.4 $\pm$ 58.47	-14.09 $\pm$ 32.07
	Epilepsy	470.6 $\pm$ 22.62	42.63 $\pm$ 40.30 *	2.08 $\pm$ 45.03
Half width (ms)	Control	26.90 $\pm$ 1.874	16.67 $\pm$ 3.35	52.07 $\pm$ 5.69
	Epilepsy	30.27 $\pm$ 2.548	11.43 $\pm$ 2.46	34.25 $\pm$ 2.96 *
Area (pAms)	Control	19590 $\pm$ 2666	23600 $\pm$ 3163	30540 $\pm$ 4946
	Epilepsy	18640 $\pm$ 1919	10680 $\pm$ 2489 **	24730 $\pm$ 5694

PV thalamus				
Parameter	Group	Baseline (n=11,12)	Relative difference to pre drug values	
			Zolpidem 1 μ M (n=5,6)	Phenobarbital 250 μ M (n=6,5)
Amplitude (pA)	Control	497.9 $\pm$ 24.17	127.7 $\pm$ 35.35	-82.80 $\pm$ 63.52
	Epilepsy	512.7 $\pm$ 34.01	10.02 $\pm$ 33.52 *	29.85 $\pm$ 33.89
Half width (ms)	Control	32.73 $\pm$ 3.123	17.53 $\pm$ 1.90	54.97 $\pm$ 10.47
	Epilepsy	23.33 $\pm$ 2.336 *	6.17 $\pm$ 1.93 **	24.16 $\pm$ 4.41 *
Area (pAms)	Control	22570 $\pm$ 2298	23290 $\pm$ 3411	31930 $\pm$ 10940
	Epilepsy	17460 $\pm$ 3457	6291 $\pm$ 2094 **	15240 $\pm$ 12350

* p<0.05,

** p<0.01 (unpaired t test) compared to control.