Pharmacologically induced absence seizures versus kindling in Wistar rats

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ABSTRACT

OBJECTIVE: In this study, it was aimed to investigate the effect of gamma-butyrolactone (GBL), a prodrug of gamma-hydroxybutyric acid-induced absence seizures on the development of kindling in Wistar rats.

METHODS: Three groups of adult male Wistar rats under anesthesia were implanted with bilateral cortical recording electrodes for the GBL group (GBL) and/or bipolar stimulation electrodes into the right basolateral amygdala for the Kindling group (KI) alone and Kindling plus GBL group (GBL+KI). Rats in the KI and GBL+KI groups were stimulated twice daily at the afterdischarge threshold until they reached Racine’s stage 5 seizure state. The animals in the GBL + group had an i.p injection of GBL 20 minutes before each electrical stimulation, and the effect of GBL-induced seizures on the development of kindling was investigated. The animals in the GBL group were injected GBL twice daily i.p. for 15 days without receiving any electrical stimulation.

RESULTS: The KI animals reached stage 5 seizure stage at 12th stimulations, whereas the GBL+KI rats reached at 27th stimulations. The mean numbers of stimulations needed for the development of the first stage 3, 4 or 5 generalized seizures were significantly higher in the GBL+KI group than the KI group.

CONCLUSION: The resistance to amigdala kindling in the GBL model can be modulated by the absence seizure mechanism alone, without the intervention of an abnormal genetic background.

Keywords: Amygdala; experimental limbic epilepsy; genetic absence epilepsy; gamma-butyrolactone; kindling.

is related to the development and also to the total duration of the SWDs, which are the electroencephalogram (EEG) hallmark of the unique neural circuitry of typical absence epilepsy [7, 9-12]. To date, data concerning the resistance to temporal lobe epilepsy conveyed by SWDs have been generated only from genetic absence epilepsy rat models. This raises a question as to whether the resistance to kindling depends on the genetic background of these specific models or relate to the neural mechanisms of absence epilepsy itself.

Therefore, we used the genetically normal Wistar strain of rats and a pharmacological model that is reproducible and predictable for the EEG and behavioral characteristics of generalized typical absence seizures in humans, namely the gamma-hydroxybutyrate (GHB) model [13, 14]. GBL model is based on the systemic administration of gamma-butyrolactone (GBL), the prodrug of GHB, known to produce exactly the same electrographic and behavioral effects of experimental typical absence seizures comparable to human absence epilepsy [15-17]. Further, GBL is used in lieu of GHB to induce this model of absence seizures because of the consistency, predictable dose response and, rapidity of the onset of the action of GBL [17-19]. The systemic administration of GBL produces absence seizures through the potentiation of the GHB-related neuromodulation and/or interaction with GABAergic inhibitory and excitatory neurotransmission within cortico-thalamo-cortical circuitry [18].

The objective of our kindling study in genetically normal Wistar rats undergoing GBL-induced absence seizures was to determine whether the resistance to the development of kindling observed in experimental absence epilepsy, can be independent of the genetic background.

**MATERIALS AND METHODS**

**Animals**
Adult (6-8 months-old) Wistar male rats weighing 270-300 g were used for all experiments. Animals were housed in a temperature controlled room (20±3°C) with a 12-h light–dark cycle in groups of four per cage and separated individually after the surgery with free access to commercial rat pellets and tap water. The experimental protocol was approved by the local Ethical Committee of animal research.

The animals were randomly organized in three groups, Group 1 was treated with GBL only (GBL, n=6); Group 2 was treated with GBL and received kindling stimulation (GBL+KI, n=6) and finally, Group 3 received kindling stimulation only, without GBL treatment (KI, n=7).

**Surgery**
Animals were anaesthetized with ketamine (100 mg/kg, intraperitoneally (i.p.)) and xylazine (10 mg/kg, i.p.). Each animal of the GBL, GBL+KI and KI groups was placed in a stereotaxic instrument (Stoelting Model 51600, Stoelting Co., Illinois, USA) with the skull surface flat and bregma 0.0. The scalp was longitudinally incised for the implantation of stainless steel screws, and screw electrodes were placed bilaterally in the skull over frontal and parietal cortices for EEG recordings. Plastic's One bipolar stimulating and recording electrodes (Roanoke, VA, USA, MS303/1) were implanted into the right basolateral amygdala (BLA) of the GBL+KI and KI groups. The stereotaxic coordinates [20] for the localization of BLA (2.6 mm posterior, 4.8 mm lateral from bregma and 8.5 mm ventral from the skull). All of the electrodes were fixed to the skull with dental acrylic and linked by insulated wires to a connector for the EEG recordings. The animals were allowed to recover from surgery for one week before any experimental protocols were started.

**Experimental Protocol**
On the first day of the experiment, all animals were placed in Plexiglas recording cages. After an adaptation period, a baseline EEG was recorded for 20 min as indicated in the experimental design (Fig. 1). Electrical activities of the cortex and/or BLA were amplified (BioAmp ML 136) and recorded with a PowerLab 8S System running Chart v.5, (ADI Instruments, Oxfordshire, U.K.) before and after each GBL injection and/or each electrical kindling stimulation at their after-discharge threshold. GBL was purchased from Sigma Aldrich (St Louis, U.S.A) and prepared in saline for the i.p. injections. For the GBL and GBL+KI groups, a dose of 100 mg/kg of GBL was chosen based on the previous literature [18, 19]. GBL is readily hydrolyzed in vivo to its active congener, GHB [21].

**The GBL group**
The rats in the GBL group were administered 30 i.p. injections of GBL twice daily from day Monday to Friday.
at the same time as the GBL+KI and KI groups, but did not receive electrical stimulation over the 3 weeks period (Fig. 1). The EEG was recorded continuously 90 min after the first GBL injection to determine the duration of the acute GBL effect. Then for the rest of the week, EEG was recorded 20 min before and 40 min after GBL injections to evaluate GBL-induced discharges with chronic administration of GBL throughout the 3 weeks. In order to monitor spontaneous SWDs, the baseline EEG on the subsequent Monday mornings after the first, second and third weekends were recorded for 90 min.

The GBL+KI and KI groups
To determine the afterdischarge threshold of the GBL+KI or KI groups, the right BLA was stimulated with an initial stimulus of 50 μA (monophasic, square-wave pulses of 80 Hz, each 1 ms in duration, for a total duration of 2 s). This was continued with 50 μA increments until an initial afterdischarge in EEG was obtained for KI or GBL+KI groups, before the beginning of the experimental protocol. The minimal duration of the afterdischarge activity was accepted as a spike discharge lasting ≥2 s immediately after the stimulation [1]. The animals in the GBL+KI and KI groups were stimulated at the afterdischarge threshold current, twice daily from Monday to Friday (Fig. 1). The GBL+KI group received kindling stimulation 20 min after the i.p. GBL injection. Seizure stages observed after each stimulation were classified for both groups by using Racine’s standard five-stage scale: stage 1, facial movements; stage 2, rhythmic head movements, head nodding; stage 3, unilateral forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, falling and clonic convulsion [22]. As indicated, the animals of GBL+KI or KI groups were stimulated until they reached a stage 5 seizure state. If the animal had at least three consecutive stage 5 seizures it was accepted as the end point of the experiment for that rat. The EEG of the GBL+KI group was recorded continuously for 20 min before and after the GBL administration and subsequently for 40 min after the kindling stimulation to analyze afterdischarge and SWD durations. The EEG of the KI group was monitored continuously for 20 min before and 40 min after the kindling stimulation to analyze afterdischarge durations (Fig. 1).

Evaluation of GBL-induced discharges after acute and chronic administration
A GBL-induced discharge was identified visually as such if its duration was ≥1 s with a complex of spike-and-wave (5-6 Hz) and the amplitude at least three times the background amplitude of the EEG was the inclusion criteria (Fig. 2A). The GBL-induced discharges were referred to as SWDs captured on the EEG. The cumulative and mean duration of SWDs in the GBL group and only cumulative duration of SWDs in the GBL+KI group were evaluated.

The spectral characteristics of SWDs in the GBL group were analyzed by computing power spectra using the Fast Fourier Transform (FFT) (The MathWorks MATLAB 7.6, USA). The power spectra of the mid-

<table>
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<th>Time table</th>
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<th>Day 2nd</th>
<th>Monday (After the 1st Weekend)</th>
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<td>Day 2nd</td>
<td>9:00 am</td>
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<td>GBL+KI</td>
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<td>GBL inj.</td>
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**Figure 1.** Design of the Gamma-butyrolactone (GBL), Kindling (KI) and GBL+KI experiments. Experimental protocol of GBL injections and/or kindling stimulations along with the EEG recording periods are seen for the three experimental groups: GBL, GBL+KI and KI alone. EEG recording periods are presented by rectangular clear boxes either of 20 or 30 min duration. Solid black arrows indicate the time of GBL injections and the clear arrows show the kindling stimulation time points. A total of 30 GBL i.p injections were performed in all groups, over a period of three weeks.
sucrose mixture. The brains were cut as 40μm frozen sections in a cryostat and stained with thionine to determine the electrode placement. Only the animals with a correct electrode placement in the BLA were included in the study.

Statistical analysis
Data were expressed as mean±SEM and statistically evaluated by analysis of variance of repeated measures (ANOVA). A one-way ANOVA followed by the post hoc Bonferroni test was used to analyze the cumulative duration of SWDs after the first GBL injection as well as the cumulative and mean duration of spontaneous SWDs after the first, second and third weekends in the GBL group. Paired t-test was used to analyze the differences between frequencies and the power of the fundamental frequency of SWDs and the relative strength of the first harmonic of SWDs after 1st vs 20th injections of GBL in the GBL group.

A two-way ANOVA followed by the post-hoc Bonferroni test was used to analyze the kindling rate, afterdischarge duration and the mean number of stimulations needed to reach the first stage 2, 3, 4 or 5 seizures of the GBL+KI and KI groups. A one-way ANOVA followed by the post- hoc Bonferroni was used to compare the cumulative duration of SWDs in GBL and GBL+KI groups. The level of statistical significance was considered to be p<0.05.

RESULTS
The baseline EEG recordings of all Wistar rats showed no abnormal discharges.

The effect of acute and chronic GBL administration (GBL group)
The administration of GBL caused a rapid onset of bilaterally synchronous discharges in the cortical EEG accompanied by immobility, vacant-staring, and vib-rissal twitching. After the first injections, these discharges appeared as series of SWD complexes with a mean duration of 1.3±0.2 sec with sudden initiation and termination (Fig. 2A). The SWDs are appeared 4.8±0.7 min after the first injection of GBL, reached maximum numbers and durations within 20 min and disappeared by 90 min (Fig. 2B). These complexes became regular, waxing and waning rhythmic SWDs with the repeated chronic administration (Fig. 3A). The mean duration of

**FIGURE 2.** The representative electrographic pattern of SWDs from the left (L) and right (R) cortical (Cx) regions, induced by the 1st injection of GBL in Wistar rats. The magnification of the EEG signal from the gray rectangle shows a burst of SWD at the frequency of 5-6 Hz at the visual inspection (A) and the cumulative duration of SWDs after the first GBL injections over a 90 min period (B). **p<0.01, revealed by one-way ANOVA followed by the post hoc Bonferroni test. Voltage/time scale: 0.5 mV/1 sec.**

**Evaluation of the kindling-induced stages and afterdischarges in the EEG**
Seizure stages (kindling rate), and the total duration of the afterdischarges were evaluated immediately after the electrical stimulation in the GBL+KI and KI groups. The mean number of stimulations needed to reach the first stage 2, 3, 4 or 5 were also evaluated in the GBL+KI and KI groups.

**Histological verification**
After the KI and GBL+K experiments, the animals were decapitated and the brains were put into a formalin/
SWDs after the 20th injection was 3.0±0.8 sec that was significantly longer than those observed in the initial administrations (p<0.01). The latency for the onset of the first SWD complex was found to be delayed by repeated injections of GBL and reached to 10.5±2.7 min after the 30th injection.

The power spectra of the SWD complexes after the 1st injection (acute administration) consisted of a peak at the fundamental frequency of 5.2±0.2 Hz, and a secondary peak with a small power at the first harmonic frequency of 10.7±0.2 Hz (Fig. 3B). After the 20th injection (chronic administration), the fundamental frequency of the SWD complex increased significantly to 6.0±0.3 Hz (p<0.05) and in addition to a more pronounced peak at the first harmonic frequency of 11.9±0.5, a third clearly visible peak appeared at the second harmonic frequency of 18.0±0.6 Hz (Fig. 3B). Additionally, the power at the fundamental frequency increased significantly after the repeated injections of GBL (p<0.05) during the chronic administration. Because the peak at the first harmonic was observed in both conditions, the relative strength of the first harmonic was calculated by dividing the power at the first harmonic frequency by the power at the fundamental frequency, and this ratio was compared between both conditions. Statistical analysis revealed that this ratio was increased significantly after 20th injection (0.2±0.4 after the 1st injection vs 0.4±0.1 after the 20th injection; p<0.05).

As shown in Figure 4A, by repeated GBL injections, animals displayed spontaneous bilateral synchronous SWDs in the baseline EEG on the Monday morning session after the GBL-free weekend period (60 h after the friday afternoon injection). The cumulative and mean duration of spontaneous SWDs were increased throughout the 3-weeks experimental period in the chronic administration. The statistical analysis revealed a significant increase in the cumulative duration of spontaneous SWDs after the third weekend (Fig. 4B).

**Figure 3.** The representative electrographic pattern of SWDs induced after the 20th injection of GBL in Wistar rats. The magnification of the EEG signal from the gray rectangle shows a 5 sec burst of SWD at the frequency of 6 Hz at the visual inspection. Voltage/time scale: 0.5 mV/1 sec. Abbreviations: Left (L) and right (R) cortex (Cx) (A). The power spectra of the GBL-induced SWDs after 1st and 20th injections, with a dotted line for the first GBL injection compared to that after the 20th GBL injection represented with the solid line. There is a primary peak at the frequency of 5.16±0.16 Hz followed by a secondary peak with a small power at the first harmonic frequency of 10.67±0.21 Hz (B). The frequency and the power at the fundamental frequency of SWDs were increased significantly after the repeated injections of GBL (p<0.05).

**Figure 4.** The representative pattern of spontaneous bilateral synchronous SWDs in the baseline EEG on the Monday morning session after the GBL-free weekend period with a slow speed in order to demonstrate the subsequent SWD complexes together. The magnification of the 5 sec EEG signal from the gray rectangle shows a burst of SWD at the frequency of 6.8 Hz. Abbreviations: Left (L) and right (R) cortex (Cx) (A). The cumulative and mean duration of spontaneous SWDs throughout the 3-weeks experimental period, during the chronic GBL systemic administration (B). The statistical analysis revealed a significant increase in cumulative duration of spontaneous SWDs after the 3rd weekend (p<0.05), one-way ANOVA. Voltage/time scale: 0.5 mV/1 sec.
The development of kindling in the GBL+KI and KI groups

All rats in the KI and GBL+KI groups reached stage 5. There was a difference however in the number of stage 5 triggering stimulations: the KI group revealed stage 5 seizures by 12 stimulations, whereas the not all animals of GBL+KI showed stage 5 seizures until the 27th stimulation (Fig. 5A). This seems to indicate that kindling was less effective in the GBL+KI group compared to the KI group. Furthermore, there was a marked increase from stage 2 to stage 3-4 in the GBL+KI group after the 15th to 18th stimulation (Fig. 5A). The mean number of stimulations for the occurrence of the first seizures of stage 3, 4 or 5 were significantly higher in the GBL+KI animals than in the KI group (p<0.001), whereas the number of stimulations needed to produce the first stage 2 seizure was similar in both KI and GBL+KI groups (Fig. 5B). As indicated in figure 5C, the afterdischarge durations of the GBL+KI and KI groups showed no significant difference. The mean afterdischarge threshold was 185.7±28.3 μA in the KI group and 205.6±29.4 μA in the GBL+KI group, therefore, it was not statistically significant.

The effect of kindling on GBL-induced discharges in the GBL+KI group

The GBL-induced SWD complexes appeared 3.9±1.1 min after the first injection of GBL (acute administration) in the EEG of the GBL+KI group, which is similar to that observed in the GBL group after the first injection. The latency to onset of the first SWD complex was delayed by repeated injections of GBL and reached to 14.9±3.7 min after the 30th consecutive injection.

The GBL+KI group showed the longer cumulative duration of SWDs at the 10-20 min period of the 12th post-injection relative to the same time period of the GBL group, therefore, it was considered statistically significant (p<0.01; Fig. 6A and B). In the GBL+KI group, the GBL-induced discharges disappeared in both post injection and post stimulation periods when they became kindled at the 27th and/or 30th stimulations.

DISCUSSION

The present study demonstrates that 1) in non-epileptic animals which received chronic administration of GBL, the GBL-induced discharges consisting of spike/spike-and-wave complexes became more typical or ma-
In accordance with our findings, electrical co-stimulation of the thalamic reticular nucleus during the rapid hippocampal kindling procedure suppresses the development of generalized limbic seizures in the adult control Wistar rats [23]. The similar protective interaction was described also in genetic mouse model of absence epilepsy [24]. Furthermore, such seizure type interaction was observed with the development of focal limbic epilepsy in GAERS and largely impaired the expression of absence seizures. Following the application of lithium-pilocarpine, the spontaneous absence seizures were delayed in GAERS compared with Wistar rats [25].

A better understanding of the mutual cross interaction between these two types of epilepsy raises the question as whether the partial or full resistance to the electrical kindling, when animals are stimulated at their afterdischarge, is attributed to the genetic absence epilepsy rats or to the absence seizures in general. Our findings here demonstrate that the resistance to the kindling in the GHB model is influenced by the mechanism of absence epilepsy itself without the intervention of an abnormal genetic background and therefore, is circuitry-dependent. This is apparently the case, at least to some degree, in the development of kindling by electrical stimulation of limbic structures in rats with genetic absence epilepsy or chemically-induced absence seizures.

In a previous work, electrical stimulation of the neocortex of Spraque-Dawley rats at 400 μA resulted in generalized kindled cortical seizures [26]. When the effect of several cataleptic and depressant anesthetics were evaluated based on the behavioural and afterdischarge duration responses of kindled cortical seizures, a dose of 100 mg/kg of GBL affected neither the afterdischarge duration nor the behavioural seizure activity [26]. A further study of the same group demonstrated that the doses of 200 or 450 mg/kg of GBL produced a delay on the kindling process when the Spraque-Dawley rats were stimulated at 400 μA [27]. However, the findings in these studies do not necessarily interfere with our results due to the methodological differences, including the higher doses of GBL than the dose in the present work (100 mg/kg) and the stimulation current used (400 μA vs 205.6±29.4 μA in the present work).

It is also of interest to investigate the cellular and/or molecular mechanisms underlying the interaction of chemical absence epilepsy with kindling. GBL is converted rapidly and irreversibly to GHB after parenteral administration and is biologically inactive [18, 19]. GHB
has multiple mechanisms of action in the brain, including the activation of both the γ-aminobutyric acid type B (GABAB) receptor and a separate GHB-specific receptor as well as the suppression of the glutamate-specific subunit B (GluR2) [16, 28, 29]. The findings in GluR2 null mutant mice highlight the involvement of the GluR2 subunit in the initiation and maintenance of absence seizures induced by GBL [29]. Further, an analysis of the mouse thalamic proteome using fluorescence 2D difference gel electrophoresis combined with mass spectrometry showed reversible changes in the expression of particular proteins corresponding to the appearance of SWDs 10 min after the i.p. administration of 50 mg/kg GBL [30]. Indeed, a number of further studies addressing the neural correlates of this mutual cross interaction on the basis of GBL-related changes in the brain will provide evidence for the mechanisms underlying the issue. For instance, it is conceivable that GBL mediated mechanisms of absence seizures might have ameliorated the reported changes in neurogenesis in our GBL+KI group [31].

Additionally, the present study reproduced the acute and characterized the chronic effects of the systemic administration of GBL. The EEG and behavioral characteristics of GBL-induced discharges with the dose of 100 mg/kg are in line with the previous observations [18, 32, 33]. The initial electrographic change in the baseline EEG recorded from the right and left frontoparietal cortices was brief burst of spikes which thereafter quickly progressed to the typical SWDs of absence seizures as reported previously [18]. These GBL-induced bilateral synchronous discharges in the acute administration show irregularity in the rhythmicity, duration, and amplitude. These electrographic discharges after the first injections were similar to the immature SWD paroxysms observed in postnatal 30 days old GAERS [7]. With repeated chronic administration of GBL, the SWD complexes appeared to mature with an increase in the power at the fundamental frequency and similar to that in adult GAERS or WAG/Rij rats [34]. As shown in previous studies carried out on genetic animal models of absence epilepsy, the typical SWD pattern is characterized by the presence of strong peaks at the harmonics of the fundamental SWD frequency in the power spectrum [34, 35]. The ratio of the power of the first harmonic to the power of the fundamental frequency reflects higher weights of the faster components in a single SWD cycle and increases with the SWD maturation [7, 34]. Therefore, the significantly higher frequency of the SWDs and the significantly higher power ratio of the spectral peak at the first harmonic frequency show that the GBL-induced SWD pattern became more typical and mature after 20th injection of GBL.

It is accepted that the major disadvantage of the GHB and GBL models is that they represent an acute model of absence seizures rather than a chronic model of absence epilepsy such as the genetic models [14, 17]. However, the maturation of GBL-induced SWDs over the time and spontaneous SWDs after the week ends in the present study indicated a fully synchronized paroxysmal activity, suggesting an augmentation in the cortico-thalamic-cortical network with repeated GBL administrations. The brief spontaneous bursts of bilaterally synchronous SWDs have been previously observed [28] following the acute administration of GBL as late as 72 h. Indeed, the involvement of the cortico-thalamo-cortical circuitry has been well accepted in the pathophysiology of absence epilepsy based on the studies in the genetic and pharmacological absence epilepsy models [36-38]. This synchronized paroxysmal activity with the chronic administration of GBL suggests a change over time in the functional organization of absence seizure circuit that possibly occurs at the cellular and/or synaptic level in the thalamo-cortical circuitry. Taken together the chronic systemic application of GBL may represent an epileptogenesis model for absence epilepsy itself, deserving a particular attention in further studies.

Finally, we studied the interaction between kindling and a pharmacological model of absence in non-epileptic Wistar rats and encounter another clue for the mutual cross interaction between absence epilepsy and limbic epilepsy, without the existence of an abnormal genetic background.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received no financial support.

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