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Neuroscience Letters 416 (2007) 17–21
Neuroscience Letters
www.elsevier.com/locate/neulet

Absence seizures are reduced by the enhancement of GABA-ergic inhibition in the hippocampus in WAG/Rij rats

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Received 25 September 2006; received in revised form 14 January 2007; accepted 19 January 2007

Abstract

Classical theories on absence epilepsy suggest that spike-wave discharge (SWDs) represent thalamo-cortical oscillations, where an abnormally excitable cortex interacts with thalamus and brain stem reticular formation. The limbic system is generally not included in any theory about the pathogenesis of absence seizures. However, some data demonstrated that the alterations in the limbic system attribute to the expression of absence epileptic phenotype in genetic models of absence epilepsy. The present study investigated whether local intrahippocampal administration of progesterone (a $GABA_A$-mimetic) and tiagabine (an inhibitor of GABA (re)uptake) might affect the occurrence of SWDs. Male WAG/Rij rats were implanted with permanent electroencephalograph (EEG) electrodes and bilateral cannulas in the CA1-CA3 region of the dorsal hippocampus. Control rats had bilateral cannulas in the cortical area above the hippocampus. Rats received intracerebral injections of progesterone (5 mg/ml), 45% β-cyclodextrin (CD), saline, or tiagabine (2 mg/ml). EEG recordings were made before and after injection. Progesterone, CD, and tiagabine administration to the hippocampus reduced SWDs for 60 min following administration without behavioral or electroencephalographic side-effects. Both progesterone administration into the cortex and saline injection into the hippocampus yielded no changes in the occurrence of SWDs. These data suggest that activation of GABA-ergic transmission in the hippocampus has an inhibitory effect on cortico-thalamo-cortical circuits underlying the generation of SWDs and might be critically involved in the regulation of absence seizures.

Keywords: Absence seizures; Hippocampus; Progesterone; Tiagabine; GABA; WAG/Rij rats

Absence epilepsy is a generalized, non-convulsive form of epilepsy, which is characterized by spontaneously occurring bursts of bilateral synchronous spike-wave activity accompanied by a decrease of consciousness. Episodes of this electroencephalographic activity, so-called spike-wave discharges (SWDs), can be recorded by EEG and may appear up to a few hundred times per day. Mechanisms underlying the generation of SWDs have being explored since the middle of the last century [16]. The most dominant theory, Gloor's classical concept of cortico-reticular epilepsy, presumes that SWDs represent a thalamo-cortical type of oscillation, where an abnormal excitable cortex interacts with the thalamus and brain stem reticular formation.

Whether a hyperexcitable cortex is indeed a sufficient condition for the occurrence of SWDs was recently tested in WAG/Rij rats [26], which are commonly considered to be a well validated genetic model of absence epilepsy [6]. We found that, in accordance with Gloor's theory, inbred WAG/Rij rats demonstrate higher cortical excitability in comparison with outbred Wistar rats, but not in comparison with non-epileptic inbred control rats of the ACI strain. These data suggest that in addition to a hyperexcitable cortex, the pathogenesis of absence epilepsy may involve other factors. Interestingly, in the same study we found that WAG/Rij rats exhibited a low threshold for the spread of epileptic activity into limbic structures in comparison with Wistar and ACI control rats. This limbic threshold decreased with age and showed an inverse correlation with the number of SWDs, that are also characterized by an age dependent increase [26]. Interestingly, the limbic system is generally not included in any theory about the pathogenesis of absence epilepsy. Indeed, neither recordings of field potentials, nor single unit activity in the

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0304-3940/$ – see front matter © 2007 Published by Elsevier Ireland Ltd.
doi:10.1016/j.neulet.2007.01.038

hippocampus in WAG/Rij rats, have indicated involvement of the hippocampus [9,10].

The limbic system and, especially, the hippocampus, is a particular steroid-sensitive area. Steroids hormones are well known to exert powerful effects on the nervous system development and functioning and modulate seizure susceptibility [17,21]. A series of recent studies in WAG/Rij rats also suggest an important role of steroids hormones, such as progesterone and corticosterone, in the regulation of absence seizures [22,25–27]. In an acute experiment it was shown that the effect of progesterone on absence seizures is mediated by its neuroactive metabolite, allopregnanolone, known to facilitate $GABA_A$ receptor inhibitory function [27]. However, neuroactive metabolites of steroid hormones, also known as neurosteroids, are able to modulate not only the GABA-ergic system but also the glutamatergic (NMDA), cholinergic and opioid system [17]—all involved in the regulation of absence seizures [6,7].

The present study investigated whether progesterone, $GABA_A$-mimetic, and/or tiagabine, a specific GABA (re)uptake inhibitor [13], injected into the hippocampus, an area rich in inhibitory GABA interneurons, would alter the occurrence of absence seizures. We hypothesized that if increased excitability of hippocampal neurons underlies the appearance of absence seizures, then facilitation of GABA-ergic inhibition (by local administration of progesterone and/or tiagabine) should result in a decrease of SWDs.

The experiments were performed in male WAG/Rij rats, 5–6 months of age, obtained from the breeding colony at the Department of Biological Psychology, Radboud University Nijmegen. All rats were group-housed prior to surgery and individually following surgery in a temperature-controlled room ($21 \pm 1\,^{\circ}C$), on a 12/12-h reversed light cycle (lights off at 8:00 a.m.). Food and water were available ad libitum. All manipulations with animals were approved by the Institutional Animal Care and Use Committee of Radboud University Nijmegen.

Surgery to implant a standard tripolar EEG-electrode set (MS333/1-A, Plastic One, Roanoke, VI, USA) was performed under isoflurane inhalation anesthesia. Electrodes were placed using the following coordinates: AP = +2.0, L = +3.0 and AP = −6.0, L = +4.0 as active electrodes (the ground electrode placed in the cortex of the cerebellum) and two cannulas (C311G, Plastic One). One group of rats (n = 16) had cannulas implanted into the CA3 region of the dorsal hippocampus AP = −3.8; L = ± 2.2; DV = 3.5. In order to ensure that effects of hippocampal manipulation were not due to non-specific effects of microinjection, a second group of rats (n = 8) had cannulas aimed to the cortex above the hippocampus (AP = −3.8; L = ±2.2; DV = 1.5). All stereotaxic coordinates were according to Paxinos and Watson [19]. The assembly of the three electrodes and two cannulas was attached to the skull surface using dental cement and jewelers screws. Following surgery, rats were allowed to recover for at least 2 weeks.

Progesterone (Sigma) (5 mg/ml) was dissolved in 45% 2-hydroxypropyl-γ-cyclodextrin (CD). Tiagabine [(*R*-)-*N*-(4,4-di(3-methylthien-2-yl)but-3-enyl) nipecotic acid hydrochloride] (Sigma) (2 mg/ml) was dissolved in saline. The solutions were prepared immediately prior to administration.

Hippocampal and cortical microinjections were performed through bilateral guide cannulas (C311I, 28-gauge, Plastic One) using injection needles (31-gauge) connected by a polyethylene tube to a 5-μl Hamilton micro syringe. The injection needles were inserted 0.5 mm beyond the tip of the cannulas. Then 1 μl of progesterone, tiagabine, vehicle (45% CD), or saline were injected bilaterally into CA3 of the hippocampus or the cortex above the hippocampus at a rate of 1 μl/45 s and needles were left in place for an additional 1 min. Rats were handled daily prior to experimental manipulations and subjected to two mock injections in order to habituate the animals to the procedure. Each rat was injected twice, with the order of drug or control injection counterbalanced, group size n = 8. Groups were: progesterone in cortex and hippocampus, CD, saline and tiagabine in the hippocampus. The behavior of the animals was monitored regularly, but not quantified.

Rats were familiarized with the recording leads for at least 3 days prior to the first day of experimental recording. EEG recordings were registered for 30 min before, and 2 h after, injections, between 10:30 h and 13:00 h. The EEG were amplified and filtered between 1 and 100 Hz, digitized at 200 Hz and stored for off-line analyses. SWDs were quantified in the EEG: the EEG data were pre-processed by a program, which searched in the EEG for the presence of steep and high-voltage activity with a minimal duration of 1 s. The selected periods of aberrant EEG activity were visually inspected to ensure that these periods contained SWDs on the basis of published criteria, and then quantified [5].

Upon completion of experiments, rats were anesthetized and given a microinjection of 2% cresyl violet to determine the site of drug administration. Rats were then exsanguinated with 0.9% saline solution and then perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) (pH 7.3). Following perfusion, brains were removed and post-fixed in 4% PFA in 0.1 M PB. Brains were later sectioned coronally at the level of cannulas to verify their placement by visual inspection. Only animals with a proper localization of cannulas in the (CA1-CA3) area of hippocampus and in the cortical area above the corpus callosum were included in statistical analyses.

Initial analyses revealed there was neither significant order effect, nor an interaction between order and condition. Hence, in all subsequent analyses, order was not included as a factor. The number of SWDs in 30-min periods was statistically analyzed by two-way ANOVAs, using time and condition as within and between factors, respectively. Orthogonal trends were used in order to show the changes over time. If the interaction between time and group was significant, separate ANOVAs were done to test the difference between groups for each 30 min period before and after an injection, and if appropriate, followed by post hoc t-tests. A p-level of <0.05 was considered to represent a significant effect.

There was a significant effect of time (F_{quad} = 16.6, d.f. 1.13, $p < 0.001$) with decreased SWDs at the first and second 30 min time periods. There was also a significant interaction between condition and time (F_{quad} = 10.03, d.f. 1.13, $p < 0.01$), which was due to a greater decrease in SWDs among rats that received progesterone to the hippocampus compared to rats that received

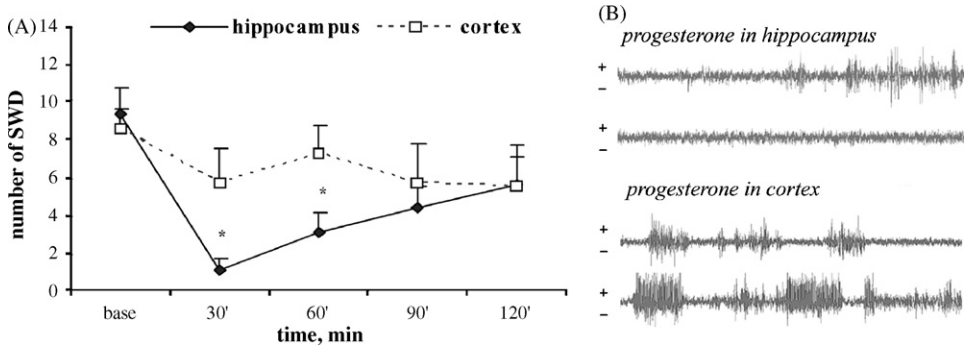

Fig. 1. (A) Number (mean ± S.E.M.) of cortical SWDs for five 30 min blocks (one before (=baseline) and four after injection) of rats that received intrahippocampal ($n=8$) or intracortical ($n=7$) progesterone injections. The stars indicate a significant difference between the groups ($^{*}p<0.05$ according to post hoc t-test). (B) Representative examples of EEG recordings at around 30 min after administration of progesterone in cortex and hippocampus in different animals. +/− indicate the polarity of the EEG recordings. Time mark 3 s, amplitude calibration 300 μV.

progesterone to the cortex. There was a significant difference between groups at the first 30 min ($t=2.45$, d.f. 13, $p<0.05$) and second 30–60 min ($t=2.30$, d.f. 13, $p<0.05$) periods after injection. Progesterone administered to the hippocampus, but not the cortex, decreased SWDs (Fig. 1A).

There was a significant effect of time ($F=12.33$, d.f. 5.70, $p<0.001$) with a significant quadratic ($F_{quad}=34.34$, d.f. 1.14, $p<0.001$) trend (a decrease followed by an increase), but no significant effect of condition. A subsequent paired sampled t-test for each data series showed a significant decrease in the number of SWDs in the first 30 min and between 30 and 60 min after both progesterone ($t=6.68$ and 5.18, d.f. 7, $p<0.001$) and CD injections ($t=3.5$ and 3.4, d.f. 7, $p<0.01$, Fig. 2A). The same was found if the data set were normalized for the base-line score (there was a small non-significant difference between the progesterone and CD group in the base-line).

This examination revealed a significant main effect of time ($F=6.34$, d.f. 4.48, $p<0.001$) and condition ($F=5.08$, d.f. 1.12, $p<0.05$). An orthogonal trend analysis showed a significant quadratic trend in the effect of time ($F_{quad}=26.5$, d.f. 1.12, $p<0.000$) as well as a significant quadratic trend in the interaction between time and condition ($F_{quad}=7.78$, d.f. 1.12, $p<0.016$). Tiagabine injected rats tended to have less SWDs in the first 30 min ($t=2.76$, d.f. 12, $0.05<p<0.06$), and had significantly less SWDs between 30 and 60 min after administration ($t=3.55$, d.f. 12, $p<0.01$) compared to saline injected animals (Fig. 3A).

No behavioral or electroencephalographic side-effects were observed after injections. Representative examples for EEG recordings of different groups at 30 min after administration are presented in Figs. 1–3B.

The present data suggest that activation of GABA-ergic neurotransmission in the hippocampus might be involved in the modulation of spontaneous absence seizures in genetically epileptic WAG/Rij rats. First, progesterone administration to the hippocampus, but not the cortex, significantly decreased SWDs for 60 min. Second, although both progesterone and its vehicle, CD, decreased SWDs when administered to the hippocampus, progesterone produced more robust decreases than did CD. Third, tiagabine, but not saline, administration to the hippocampus significantly decreased the occurrence of SWDs. The decrease in SWDs following CD administration was unexpected. However, evidence from both in vitro [23] and in vivo experiments [28] suggest that cyclodextrins, which are commonly used as solvents for many experimental drugs, may exert their own neuroactive effects. Cyclodextrins have direct effects on $GABA_A$ receptors [23]. Moreover, cyclic sugar molecules with a hydrophobic core region can simply sponge neuroactive steroids from endogenous recourses [23]. Both the sponging and the direct effect of cyclodextrins may alter

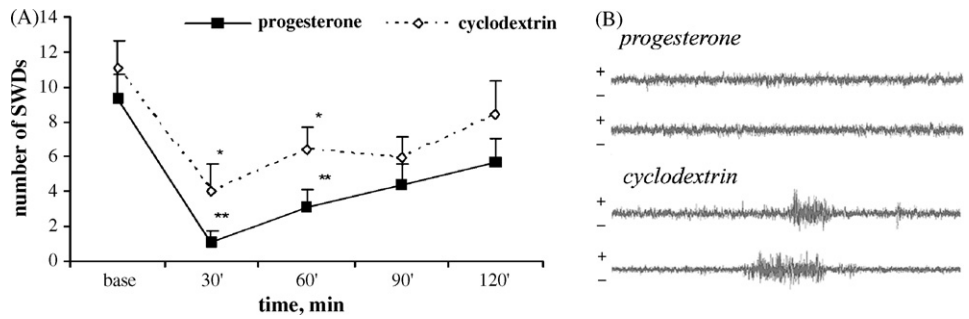

Fig. 2. (A) Number (mean ± S.E.M.) of cortical SWDs for five 30 min blocks (one before (=baseline) and four after injection) of rats that received intrahippocampal injections of progesterone ($n=8$) or cyclodextrin ($n=8$). The stars indicate a significant decrease compared to the basal level ($^{*}p<0.01$, $^{**}p<0.001$ according to t-test for paired samples). (B) Representative examples of EEG recordings at around 30 min after administration of progesterone and cyclodextrin in different animals. +/− indicate the polarity of the EEG recordings. Time mark 3 s, amplitude calibration 300 μV.

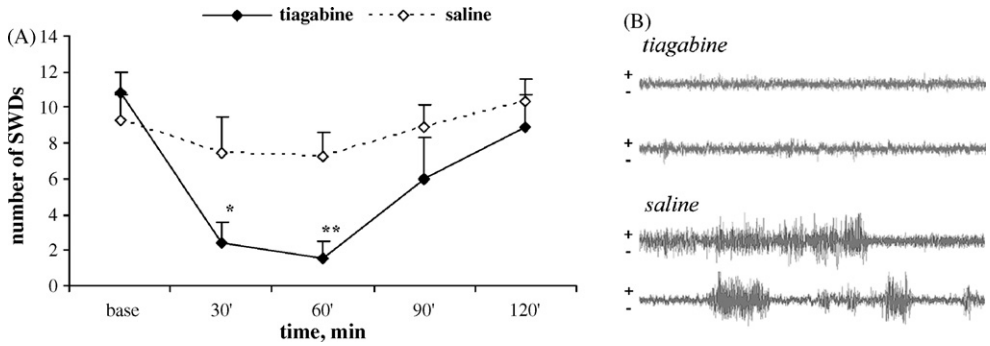

Fig. 3. (A) Number (mean ± S.E.M.) of cortical SWDs for five 30 min blocks (one before (=baseline) and four after injection) of rats that received hippocampal tiagabine ($n = 7$) and saline ($n = 7$) injections. The stars indicate a significant difference between the groups ($^{*}0.05 < p < 0.06$, $^{**}p < 0.01$ according to post hoc t-test). (B) Representative examples of EEG recordings at around 30 min after administration of tiagabine and saline in different animals. +/− indicate the polarity of the EEG recordings. Time mark 3 s, amplitude calibration 300 μV.

neuronal excitability and account for the reduction of SWDs found after injection of CD into the hippocampus. Hence, although these data support the hypothesis that actions of GABA-ergic compounds in the hippocampus might be involved in the regulation of absence seizures, the lack of difference between progesterone and CD does not allow us to parse out the effects of progesterone and its solvent. However, the effects of progesterone are also not specific for GABA and may influence a variety of different neurotransmitter systems [17]. Data from our third experiment suggest that drugs specific for activation GABA-ergic transmission can reduce the occurrence of SWDs when administered to the hippocampus. Indeed, the effect of tiagabine, a very specific drug known to inhibit the GABA (re)uptake process, was very prominent and also more similar to that of progesterone than CD. Taken together, these data suggest that compounds with more specific GABA-ergic activity may have more salient effects on the occurrence of SWDs and that these effects may be due to their actions in the hippocampus.

Interestingly, the anti-seizure (suppression of SWDs) effect of tiagabine (and most likely of progesterone) found in the present experiment is opposite to what has been found in previous studies with systemic injections, in which tiagabine (and progesterone) induced an increase in the number of absence seizures in the same model [5,27]. However, similarly to our findings, focal bilateral injections of pregnenolone sulphate and allopregnanolone into the peri-oral region of the primary somatosensory cortex also reduced the number and duration of SWDs in WAG/Rij rats [4]. General activation of the GABA-ergic system aggravates absence seizures in both humans and rats [7,20] and gave a rise to a general postulate regarding absence epilepsy as a condition associated with hyper-function of the GABA-ergic inhibitory system [20]. However, the local enhancement of GABA-ergic inhibition in the reticular thalamic nucleus (RTN) [1,7] or in the peri-oral region of the somatosensory cortex [4] results in a decrease in SWDs. Hence, the present findings suggest that the hippocampus is another structure, where hypo-, rather than hyper-function of GABA-ergic neurotransmission corresponds to an increased number of SWDs. How, and in which way, this affects the pathogenesis of absence epilepsy needs to be further established.

Cortical SWDs are accompanied by synchronized unit firing in cortex and thalamus and this was never found in the hippocampus in WAG/Rij rats [9,10] or in any other limbic structure (septum, amygdala, cingular and piriform cortex) in genetic absence epileptic rats from Strasburg (GAERS) [15]. Therefore, at first glance, the present effects are somewhat surprising. Nevertheless, the contribution of the limbic system in the regulation of SWDs generation might be mediated by the rostral pole of the RTN, which is regarded as part of the limbic system [14]. The RTN is a key structure in the generation of sleep spindles and SWDs, it controls switching from tonic to burst firing mode of thalamo-cortical neurons [3]. The afferents of the middle and caudal parts of the RTN are primarily sensory, while the rostral pole of the RTN is connected with various motor and limbic centres including the hippocampal formation [1]. We suggest that the hippocampus may provide a tonic excitatory input to the rostral part of RTN. However, there is currently no theory to evaluate the extent to which the limbic structures can engage the inhibitory network of the RTN and whether this would have any impact on the occurrence of SWDs.

Consistent with our hypothesis, Deransart et al. showed modulatory effects on absence seizures of dopaminergic neurotransmission in the nucleus accumbens, which is also part of the limbic system. The authors reported that both dopaminergic agonist and antagonist injections in the core of nucleus accumbens resulted in respectively a decrease and an increase in absence seizures without behavioral or electroencephalographic side-effects [8]. The nucleus accumbens receives direct projections from limbic structures, including the hippocampus, and might also play a role in the decrease in the occurrence of SWDs found in the present study. Further studies are required to determine whether activation of GABA-ergic neurotransmission in the hippocampus may indeed enhance dopaminergic activity in the core of the nucleus accumbens, which corresponds to the decreased incidence of SWDs.

In addition, there is also a number of functional alterations in limbic structures found in association with an absence epileptic phenotype in rats. Besides our own data on lower thresholds for limbic type of afterdischarges in WAG/Rij rats [26], Lason et al. found elevated levels of α-neoendorphin and up-regulation of the mRNA-encoding prodynorphin in the hippocampus of 6 month

old WAG/Rij rats in comparison with younger rats of the same strain and age matched ACI rats [12]. Aker et al. [2] found that WAG/Rij rats but also GAERS are more resistant to amygdala kindling. There are also data found in GAERS indicating that at postnatal day 21 (before the occurrence of SWDs), GAERS have higher brain metabolic activation in limbic regions, but not in the thalamo-cortical loop in comparison to non-epileptic control rats [18]. A decreased expression of one of the subunits of the $GABA_A$ receptor [24] as well as an up-regulation of the H-ferritin mRNA was found in the hippocampus of GAERS [11]. All these data show that the pathogenesis of absence epilepsy in WAG/Rij and GAERS involves a variety of alterations in the limbic part of the brain which might be, however, also a consequence of persistent absence seizures.

The present data show that activation of GABA-ergic neurotransmission by tiagabine and progesterone in the hippocampus has an inhibitory effect on cortico-thalamo-cortical circuits. We suggest that hormonal modulation of excitability of hippocampal neurons may play an important role in the pathogenesis of absence epilepsy and that it needs to be investigated whether this structure might serve as a new putative target for the treatment of absence epilepsy.

Acknowledgements

We would like to thank Drs. Madeline Rhodes and Boguslawa Budziszewska for sharing their expertise for performing this research, and biotechnicians Hans Krijnen, Saskia Hermeling and technical assistant Gerard van Oijen for their help in carrying out the experiments.

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