Epilepsia, 50(1):33–43, 2009
doi: 10.1111/j.1528-1167.2008.01712.x

FULL-LENGTH ORIGINAL RESEARCH

Effect of stage 2 kindling on local cerebral blood flow rates in rats with genetic absence epilepsy

*Nihan Çarçak, †Arielle Ferrandon, †Estelle Koning, ‡§Rezzan Gülhan Aker, *Osman Özdemir, ‡§Filiz Yilmaz Onat, and †Astrid Nehlig

*Istanbul University, Faculty of Pharmacy, Department of Pharmacology, Istanbul, Turkey; †INSERM U 666, Strasbourg, France; ‡Marmara University, School of Medicine, Department of Pharmacology and Clinical Pharmacology, Istanbul, Turkey; and §Marmara University, Epilepsy Research Centre (EPAM), Istanbul, Turkey

Summary

Purpose: Genetic absence epilepsy rats from Strasbourg (GAERS) are resistant to the progression of kindling seizures. We studied local cerebral blood flow (LCBF) changes in brain regions involved in seizures in both GAERS and nonepileptic rats (NEC) to map the differences that may be related to the resistance to kindling.

Methods: Electrodes were implanted in the amygdala of adult NEC and GAERS male rats, which were stimulated to reach stage 2. Quantitative autoradiographic measurements of LCBF were performed by the [^{14}C]-iodoantipyrine ([^{14}C]IAP) autoradiographic technique allowing the precise mapping of regional perfusion changes. LCBF rates were measured bilaterally in 43 brain regions. The tracer infusion lasted for 60 s and started at 15 s before seizure induction.

Results: Rates of LCBF increased in stimulated GAERS and NEC groups compared to nonstimulated controls. The LCBF increase in stimulated GAERS was larger and more widespread than that observed in stimulated NEC. The LCBF increase in the somatosensory cortex, ventrobasal and anterior thalamic nuclei, hypothalamus, subthalamic nucleus, piriform, entorhinal and perirhinal cortex, amygdala, CA2 region of hippocampus, and substantia nigra was statistically significantly larger in stimulated GAERS compared to stimulated NEC rats.

Conclusion: The results show that more brain regions are activated by kindling stimulation in GAERS. This widespread activation in GAERS involves the somatosensory cortex and thalamus, which are both known to be involved in the expression of absence seizures as well as numerous limbic regions thought not to play a role in the expression of absence seizures, suggesting an interaction between corticothalamocortical and limbic circuitries.

KEY WORDS: Limbic seizures, Epileptogenesis, Kindling-resistance, Spike-and-wave discharges, [^{14}C]-iodoantipyrine.

The genetic absence epilepsy rats from Strasbourg (GAERS) are a well-validated model of human generalized absence epilepsy (Marescaux et al., 1992; Danober et al., 1998). GAERS are a fully inbred strain of rats, derived from an outbred Wistar colony, with 100% of animals displaying the electroencephalography (EEG) and behavioral characteristic of absence seizures. Increased synchronization and GABAergic inhibition in thalamocorticothalamic circuits, including the ventrobasal thalamus, reticular thalamic nucleus, and, as shown recently, the perioral region of primary somatosensory cortex are involved in the generation of synchronous, bilateral spike-and-wave discharges (SWDs) on EEG (Liu et al., 1991; Avanzini et al., 1992; Blumenfeld & McCormick, 2000; Meeren et al., 2002; Manning et al., 2003; Polack et al., 2007).

The kindling model of temporal lobe epilepsy is based on the daily repetitive electrical stimulation of a limbic structure such as the amygdala or hippocampus. The current, that is sufficient to produce an afterdischarge on the EEG, progressively induces the occurrence of motor seizures that are not initially triggered by the stimulation (Goddard, 1967). These seizures in turn recruit a progressively increasing number of structures and secondarily generalize over time (Racine, 1989). The kindling model

Accepted May 5, 2008; Early View publication July 24, 2008.
Address correspondence to Filiz Yilmaz Onat, Marmara University School of Medicine, Department of Pharmacology and Clinical Pharmacology, Haydarpasa, Istanbul, Turkey. E-mail: fonat@marmara.edu.tr

is a well-known animal model for investigating mechanisms of convulsive seizures and epileptogenesis (McIntyre, 2006). One of the great advantages of this model is that the process of epileptogenesis is well known, easily controlled, and can be reliably measured (Sutula & Ockuly, 2006).

In earlier studies of kindling in GAERS, we have shown that GAERS fail to reach generalized convulsive seizures stages 3, 4, and 5 and stay at stage 2 even after the maximum number of stimulations when they were stimulated at the afterdischarge threshold (Eskazan et al., 2002; Onat et al., 2005). Further, a study of amygdaloid kindling in WAG/Rij rats, another genetic model of absence epilepsy, demonstrated kindling-prone and kindling-resistant subgroups (Aker et al., 2006). The intensity of SWDs in the GAERS and WAG/Rij animals correlated negatively with the susceptibility to amygdala kindling. These findings raise the possibility that the corticothalamocortical activity in absence epilepsy contributes to the resistance to the secondary generalization of limbic seizures during amygdala kindling. Additionally, an increase in SWDs immediately after the kindling stimulation was observed in adult GAERS and WAG/Rij rats (Onat et al., 2007). In 20-day-old GAERS before the development of SWDs, kindling is almost as effective as in the nonepileptic Wistar age-matched animals (Onat et al., 2006), indicating that the kindling resistance in GAERS is related to the mechanism underlying the occurrence of SWDs.

These observations are part of increasing evidence for a mutual inhibitory interaction between the corticohalamocortical mechanisms that produce absence seizures and the limbic mechanisms that produce temporal lobe epilepsy. These observations relate to reports (Koutroumanidis et al., 1999; Nicholson et al., 2004) that the coexistence of idiopathic generalized absence and partial temporal lobe epilepsy in the same patient is extremely rare, again suggesting some mutual interactions between the two mechanisms. In order to investigate the structures involved in these mutual inhibitory interactions, which appear to characterize corticothalamocortical circuits acting in the production of absence seizures on the one hand and limbic circuits acting in the production of partial temporal lobe seizures on the other, we have used measures of local blood flow to reveal structures involved in the temporal lobe seizures that may not be revealed by the EEG records. We have studied the blood flow changes at the early stages of kindling because the GAERS rats do not progress beyond stage 2 during the kindling.

The measurement of local cerebral blood flow (LCBF) is frequently used to locate the areas involved in brain activation induced by epileptic seizures (Nehlig et al., 1995; Pereira de Vasconcelos et al., 1995, 2002; André et al., 2002; Chassagnon et al., 2005, 2006). The quantitative autoradiographic method using the freely diffusible tracer, [^{14}C]-iodoantipyrine ([^{14}C]IAP), allows the measurement of rates of LCBF over very short periods ranging from 30–60 s and is specifically adapted to the measurement of functional changes occurring during short events such as seizures. The rates of LCBF provide a precise follow-up of the structures recruited at different levels of severity of the kindling process (Chassagnon et al., 2005, 2006). The method is relevant, because in human patients with temporal lobe epilepsy, it provides critical information about the localization of the seizure focus and its degree of spatial extension and is a basic requirement before neurosurgery (Ho et al., 1997; Mariottini et al., 2001).

In the present study, we used the [^{14}C]IAP autoradiographic technique to map LCBF changes in GAERS and NEC rats subjected to kindling and undergoing a stage 2 seizure to clarify why the spike-and-wave discharge activity taking place in the corticothalamocortical circuitry would prevent the occurrence of fully generalized limbic seizures and better understand the relationships between the corticothalamocortical and limbic circuitries.

Materials and Methods

Adult Wistar nonepileptic (NEC; n = 14), and GAERS (n = 14) male rats, 4–6 months old weighing, at least 350 g, were used for the LCBF study. The strains were selected from Wistar rats that displayed spontaneous SWDs on routine cortical EEG for GAERS or no SWDs for the control NEC strain. Both phenotypes were obtained by cross-breeding after five or six generations. The animals were maintained under standard laboratory conditions on a 12/12-h light/dark cycle (lights on at 7:00 a.m.) and allowed free access to food and water. All experiments were conducted in conformity with the rules of the European Committee Council Direction of November 24, 1986 (86/69/EEC) and the French Department of Agriculture (License No. 97-67). All efforts were made to minimize the suffering and the number of animals used.

Stereotaxic surgery

Rats were anesthetized with ketamine [100 mg/kg intraperitoneally (i.p.)] and xylazine (10 mg/kg, i.p.) and implanted with a deep bipolar stimulating electrode (MS303/1 twisted; Plastic's One, Roanoke, VA, U.S.A.) in the right basolateral amygdala (2.6 mm posterior, 4.8 mm lateral, and 8.5 mm ventral from the bregma) (Paxinos & Watson, 1998) and three single-contact electrodes over the right and left frontal cortex and the left parietal cortex. The electrodes were fixed to the skull with dental acrylic cement. The rats were allowed a 1-week recovery period before the start of the kindling procedure.

Kindling

At least 1 week after stereotaxic surgery, the first stimulation delivered from an isolated constant current stimulator (Ari Bakim Ltd Şti., Kocaeli, Turkey) was applied

through the bipolar electrode (monophasic square-wave current; frequency, 80 Hz; duration, 2 s; pulse width, 1 ms). The afterdischarge threshold is defined as the minimal intensity able to trigger an autonomous spike discharge in the EEG lasting ≥2 s immediately after the stimulation (McIntyre & Racine, 1986). The afterdischarge threshold (100–400 μA) was determined by increasing the current intensity by 25 μA steps until an afterdischarge was recorded on the EEG. The right basolateral amygdala was stimulated twice daily, in the morning and late afternoon, at the current afterdischarge threshold until stage 2 was reached in both GAERS and NEC animals. After reaching their first stage 2 seizure, the animals received further stimulation if their afterdischarge duration was shorter than 20 s. The animals with afterdischarge durations longer than 20 s at the time of the first stage 2 seizure did not receive further stimulation until the time of LCBF measurement. Therefore, all rats underwent at least two stage 2 seizures. The maximum number of stimulations was six for all animals.

The progression of kindling was evaluated using the Racine scale (1972) as follows: stage 1, behavioral arrest, chewing, and eye blinking; stage 2, stage 1 plus rhythmic head movements and head nodding.

All rats were stimulated until they showed stage 2 seizure, with the afterdischarge duration reaching at least 20 s. Then, they were randomly assigned to the stimulated and nonstimulated group, depending on whether or not they received further stimulation at the time of LCBF measurement. Thus, there were four experimental groups comprising seven rats each: nonstimulated NECs, nonstimulated GAERS, stimulated NECs, and stimulated GAERS.

Measurement of LCBF

For the measurement of LCBF, polyethylene catheters were inserted into a femoral artery and a femoral vein under light sevoflurane anesthesia. Both catheters were threaded through a small opening of the skin at the upper part of the hindlimb. A loop was made with the ends of the catheters, which were put back under the skin, and the hole was closed with a suture. The animals recovered for a minimum of 3 h after surgery to allow for the elimination of the effects of anesthesia. LCBF rates were measured by means of the [^{14}C]iodoantipyrine method ([^{14}C]IAP) (Sakurada et al., 1978). The tracer, 4-iodo-*N*-methyl-[^{14}C]-iodoantipyrine (specific activity, 1.85–2.2 GBq/ mmol; New England Nuclear, Paris, France) was injected into the animals through the femoral vein at a concentration of 925 Bq/ml. The period of measurement of LCBF was 60 s, during which a total volume of 1.2 ml [^{14}C]IAP solution was administered at a progressively increasing rate in order to prevent back flux of the tracer from heavily labeled brain regions. The [^{14}C]IAP infusion was started at 15 s before the kindling stimulation. This time was chosen based on previous data showing that the best information on LCBF rates could be obtained when the infusion of the tracer was started before the onset of the seizure. Indeed, a stage 2 seizure is brief, and immediately after the seizure, rates of LCBF decrease below baseline. Starting the infusion before the onset of the seizure was designed to avoid the negative impact of the postictal depression on the measurement of LCBF (Chassagnon et al., 2005, 2006).

The EEG was recorded during the whole procedure. Throughout the period of [^{14}C]IAP administration, 20–22 timed arterial blood samples, freely flowing from the arterial catheter, were collected in glass capillary tubes. The last sample was taken at the time of killing and as long as blood could be withdrawn from the arterial catheter. The rats were killed by decapitation at 60 s after the onset of [^{14}C]IAP infusion, and brains were removed within 1 min, frozen in methylbutane chilled to −30°C, and stored at −80°C until sectioning. The rapid dissection and freezing of the brains is necessary to avoid a potential redistribution of [^{14}C]IAP from high to low perfused regions. This redistribution may significantly influence the data if the freezing of the brain exceeds 2 min. The content of each capillary tube was transferred to a preweighed scintillation vial that was immediately covered and reweighed after blood collection. The blood samples were then treated with 0.5 ml tissue solubilizer (Optisolv; Wallac, Loughborough, Leics, U.K.) and isopropanol (1:2, vol/vol) and 0.5 ml hydrogen peroxide (30%). Blood concentration of [^{14}C]IAP was then determined by liquid scintillation counting in 10 ml Optiphase Hisafe scintillation cocktail (Wallac, Loughborough, Leics, U.K.). The concentration of tracer per unit volume of blood in each sample was calculated from the measured amount of ^{14}C, the weight of the blood sample, and an assumed specific gravity of 1.06 g/ml for blood.

The frozen brains were cut into 20 μm coronal sections at −22°C in a cryostat. Sections were picked up on glass coverslips, dried on a hot plate (60°C), and autoradiographed on Kodak Biomax MR film (Eastman Kodak, Rochester, NY, U.S.A.) along with calibrated [^{14}C]methylmethacrylate standards (Amersham Biosciences, Piscataway, NJ, U.S.A.) calibrated for their ^{14}C concentration in brain sections. Adjacent sections were stained with thionine for histological identification of specific nuclei.

The autoradiographs were analyzed by means of quantitative densitometry with a computerized image-processing system (MCID; InterFocus Imaging Ltd, Linton, Cambridge, U.K.). Optical density measurements in 43 regions of interest were made bilaterally in four to six brain sections for each structure from every animal. A mean value was then calculated for each structure of interest in a given animal, and the final LCBF data represent the mean of seven animals in each group.

For quantification of rates of LCBF in the areas of interest, a calibration curve was first obtained from the autoradiographs of the standards containing a known ^{14}C amount. Tissue ^{14}C concentrations were determined from

the optical densities of the autoradiographic representations of the brain sections and the calibration curve obtained from the standards.

Calculation of LCBF

Throughout the period of measurement of LCBF, blood samples were collected at the distal end of the arterial catheter to determine the continuously changing [^{14}C]IAP concentration in the arterial blood at the proximal end of the catheter. The short duration of the experiment requires extremely precise timing of the samples. However, the relative length of the arterial catheter induces two types of arterial sampling distortion. The corrections for the time lag and the washout effect were taken into account for the calculation of LCBF (Nehlig et al., 1989), which was calculated according to the Fick equation by using a brain-blood partition coefficient of 0.8 (Sakurada et al., 1978).

Placement of the stimulating electrode

Electrode localization was verified in sections stained with thionine. Electrode placements were located in the basolateral amygdala in 26 out of 28 animals and in the medial and central amygdala in 2 out of 28 animals. All rats were included the study, since a previous study reported that the distribution of rates of glucose utilization were not affected by the location of the electrode within the amygdala complex (Ackermann et al., 1986).

Statistical analysis

Data were expressed as mean ± SEM for seizure stage and afterdischarge parameters and as mean ± SD for LCBF values in tables. LCBF values were determined in 43 brain regions bilaterally in four groups of animals. Rates of LCBF and percent of change in LCBF rates induced by kindled seizures in both GAERS and NECs were compared to their respective nonstimulated controls. Data were statistically evaluated by using a two-way analysis of variance (ANOVA) for repeated measures and a post hoc Bonferroni test. A two-way ANOVA followed by the post hoc Bonferroni test was also used to compare time-response curves for kindling rate and afterdischarge durations in NEC and GAERS groups. The level of statistical difference was considered significant at $p < 0.05$. The LCBF increases caused by stimulation were statistically evaluated with two factor analysis of variance and subsequent post hoc test according to Bonferroni ($p < 0.05$) to evaluate differences between groups.

Results

The kindling process and afterdischarge durations

All rats exhibited typical stage 1 and 2 behavioral manifestations after electrical stimulations. The mean afterdischarge threshold was 97 and 93 μA for NECs and GAERS, respectively. The mean number of stimulations to reach the first stage 2 seizure was 3.9 ± 0.7 in NECs and 3.8 ± 0.7 in GAERS (Fig. 1A). The differences in the threshold and mean number of stimulations between the NEC and GAERS groups were not statistically significant. The afterdischarge durations were longer in GAERS than in NEC (Fig. 1B). At the end of the stimulation protocol, the mean afterdischarge duration was 26.9 ± 1.6 s in NECs and 38.4 ± 3.5 s in GAERS. The difference in afterdischarge duration between the two strains was statistically significant for the fifth and sixth stimulations ($p < 0.01$).

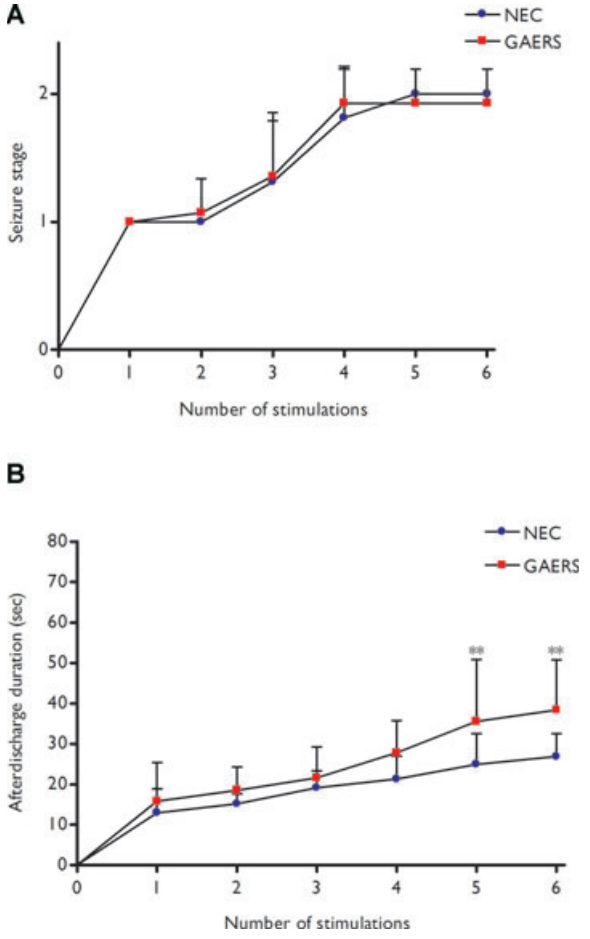

Figure 1.
Seizure stage (**A**) and duration of afterdischarge in the ipsilateral amygdala (**B**) of NEC (n = 14) and GAERS (n = 14) rats. Data are expressed as mean ± SEM. **, $p < 0.01$, significant differences between nonepileptic control rats (NEC) and GAERS.

Local cerebral blood flow rates

Comparison between nonstimulated (control) GAERS and NEC groups

As can be seen in Tables 1–3, LCBF rates tended to be slightly lower in most brain areas of nonstimulated

Table 1. Effects of kindling stage 2 seizures on LCBF rates in cerebral cortex, forebrain, and white matter areas of adult NEC and GAERS rats

		NEC			GAERS			
		Control (n = 7)	Stimulated (n = 7)	Difference (%)	Control (n = 7)	Stimulated (n = 7)	Difference (%)	F value
Cerebral cortex								
Frontal—motor M1	Right	109 ± 11	127 ± 23	16.5	94 ± 16	165 ± 37$^{\#\#\#}$	75.5	17 0.53$^{\bullet\bullet\bullet}$
	Left	112 ± 10	125 ± 28	11.6	93 ± 16	164 ± 34$^{\#\#\#}$	76.3	10.26$^{\bullet\bullet}$
Cingulate	Right	128 ± 20	131 ± 20	2.3	104 ± 18	175 ± 26$^{\#\#\#}$	68.3	17.98$^{\bullet\bullet\bullet}$
	Left	129 ± 21	142 ± 22	10.1	107 ± 21	176 ± 30$^{\#\#\#}$	64.5	9.68$^{\bullet\bullet}$
S1FL—somatosensory forelimb region	Right	129 ± 20	122 ± 20	−5.4	97 ± 21	161 ± 29$^{\#\#\#}$	66.0	16.95$^{\bullet\bullet\bullet}$
	Left	135 ± 13	126 ± 18	−6.6	95 ± 20	161 ± 35$^{\#\#\#}$	69.5	18.59$^{\bullet\bullet\bullet}$
S1BF—somatosensory barrel field-anterior	Right	144 ± 27	131 ± 21	−9.03	100 ± 17^{+}	167 ± 22$^{\#\#\#}$	67.0	23.06$^{\bullet\bullet\bullet}$
	Left	144 ± 20	132 ± 22	−8.3	97 ± 18	167 ± 27$^{\#\#\#}$	72.2	24.30$^{\bullet\bullet\bullet}$
S1BF—somatosensory barrel field-posterior	Right	152 ± 33	139 ± 13	−8.6	106 ± 16^{+}	200 ± 62$^{***,\#\#\#}$	88.7	14.96$^{\bullet\bullet\bullet}$
	Left	147 ± 22	141 ± 18	−4.1	105 ± 16	195 ± 65$^{**,\#\#\#}$	85.7	12.20$^{\bullet\bullet}$
S1HL—somatosensory hindlimb region	Right	119 ± 22	121 ± 15	1.7	104 ± 17	177 ± 50$^{**,\#\#\#}$	70.2	10.09$^{\bullet\bullet}$
	Left	127 ± 15	126 ± 17	−0.8	102 ± 16	181 ± 57$^{**,\#\#\#}$	77.5	11.15$^{\bullet\bullet}$
Piriform	Right	113 ± 27	146 ± 14	29.2	106 ± 15	191 ± 45$^{*,\#\#\#}$	80.2	5.96$^{\bullet}$
	Left	111 ± 19	128 ± 32	15.3	103 ± 19	157 ± 33$^{\#\#}$	52.4	3.38
Entorhinal	Right	98 ± 27	130 ± 15	32.7	110 ± 11	178 ± 36$^{*,\#\#\#}$	61.8	3.82
	Left	109 ± 24	134 ± 21	22.9	115 ± 25	163 ± 31$^{\#}$	41.7	1.42
Perirhinal	Right	102 ± 32	125 ± 17	22.6	110 ± 14	179 ± 33$^{**,\#\#\#}$	62.7	5.7$^{\bullet}$
	Left	112 ± 24	145 ± 21	29.5	118 ± 28	175 ± 33$^{\#\#}$	48.3	1.39
Forebrain								
Nucleus accumbens	Right	122 ± 23	134 ± 32	9.8	101 ± 17	171 ± 35$^{\#\#\#}$	69.3	7.67$^{\bullet}$
	Left	123 ± 32	136 ± 27	10.6	98 ± 15	161 ± 27$^{\#\#\#}$	64.3	6.46$^{\bullet}$
Lateral septum	Right	99 ± 20	126 ± 18	27.3	98 ± 17	155 ± 23$^{\#\#\#}$	58.2	4.08
	Left	95 ± 23	127 ± 22	33.7	96 ± 18	148 ± 18$^{\#\#}$	54.2	1.68
Medial septum		116 ± 15	135 ± 20	16.4	98 ± 15	161 ± 28$^{\#\#\#}$	64.3	8.29$^{\bullet\bullet}$
Diagonal band		108 ± 12	132 ± 18	22.2	98 ± 13	153 ± 26$^{\#\#\#}$	56.1	5.12$^{\bullet}$
Medial amygdala	Right	107 ± 23	132 ± 15	23.4	105 ± 29	188 ± 52$^{**,\#\#\#}$	79.1	5.47$^{\bullet}$
	Left	108 ± 16	126 ± 29	16.7	99 ± 13	165 ± 35$^{\#\#\#}$	66.7	6.47$^{\bullet}$
Basolateral amygdala	Right	106 ± 9	146 ± 11	37.7	108 ± 14	193 ± 57$^{*,\#\#\#}$	78.7	3.88
	Left	115 ± 13	132 ± 29	14.8	108 ± 15	175 ± 38$^{*,\#\#\#}$	62.0	6.53$^{\bullet}$
Central amygdala	Right	93 ± 11	128 ± 11	37.6	98 ± 16	190 ± 48$^{***,\#\#\#}$	93.9	8.11$^{\bullet\bullet}$
	Left	99 ± 13	138 ± 64$^{\blacklozenge}$	40.8	104 ± 21	160 ± 31$^{\#\#\#}$	53.9	0.35
Area CA1 of hippocampus	Right	69 ± 12	110 ± 15$^{\blacklozenge}$	59.4	96 ± 16	125 ± 27	30.2	0.74
	Left	78 ± 10	112 ± 11	43.6	98 ± 16	144 ± 22$^{\#}$	46.9	1.04
Area CA2 of hippocampus	Right	80 ± 15	112 ± 22	40.0	101 ± 19	128 ± 23	26.7	0.1
	Left	84 ± 13	122 ± 20	45.2	101 ± 14	165 ± 35$^{*,\#\#\#}$	63.4	2.37
Area CA3 of hippocampus	Right	97 ± 25	118 ± 23	21.7	105 ± 13	136 ± 32	29.5	0.29
	Left	109 ± 17	134 ± 24	22.9	109 ± 15	154 ± 30$^{\#}$	41.3	1.40
Dentate gyrus	Right	98 ± 20	125 ± 23	27.6	106 ± 16	150 ± 44$^{\#}$	41.5	0.64
	Left	106 ± 14	131 ± 16	23.6	104 ± 13	163 ± 35$^{\#\#\#}$	56.7	4.38$^{\bullet}$
White matter								
Genu of corpus callosum		49 ± 11	64 ± 9	30.6	58 ± 7	74 ± 14	27.6	0.01

Values, expressed as ml/100 g/min, represent means ± SD; the number of animals in parentheses.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, statistically significant differences between stimulated GAERS and stimulated NEC.
$^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, statistically significant differences between (nonstimulated) control GAERS and control NEC.
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, statistically significant differences between stimulated GAERS and control GAERS.
$^{\blacklozenge}p < 0.05$, $^{\blacklozenge\blacklozenge}p < 0.01$, $^{\blacklozenge\blacklozenge\blacklozenge}p < 0.001$, statistically significant differences between stimulated NEC and control NEC.
$^{\bullet}p < 0.05$, $^{\bullet\bullet}p < 0.01$, $^{\bullet\bullet\bullet}p < 0.001$, statistically significant interaction between strain and stimulation.

GAERS compared to nonstimulated NECs. Significant hypoperfusion in nonstimulated GAERS compared to nonstimulated NECs ($p < 0.05$) was observed in the barrel field of the somatosensory cortex (anterior and posterior) and superior colliculus.

Comparison between nonstimulated and stimulated NEC groups

In the NEC group, rates of LCBF tended to increase during the seizure, but this increase was not statistically significant in most brain regions. Significant

Table 2. Effects of kindling stage 2 seizures on LCBFrates in the thalamus and hypothalamus of adult NEC and GAERS rats

		NEC			GAERS			
		Control (n = 7)	Stimulated (n = 7)	Difference (%)	Control (n = 7)	Stimulated (n = 7)	Difference (%)	F value
Thalamus								
Reticular thalamic nucleus	Right	117 ± 21	146 ± 19	24.8	118 ± 29	208 ± 69$^{\#\#}$	76.3	4.06
	Left	117 ± 18	156 ± 34	33.3	113 ± 23	190 ± 63$^{\#}$	68.1	1.69
Anterodorsal	Right	122 ± 25	148 ± 22	21.3	122 ± 32	223 ± 64$^{*,\#\#\#}$	82.8	6.32$^{\bullet}$
	Left	131 ± 21	143 ± 29	9.2	121 ± 29	201 ± 58$^{\#\#}$	66.1	5.89$^{\bullet}$
Anteroventral	Right	169 ± 38	174 ± 33	3.0	123 ± 28	198 ± 41$^{\#}$	61.0	6.86$^{\bullet}$
	Left	160 ± 31	179 ± 40	11.9	124 ± 28	190 ± 23	53.2	3.99
Ventralanterior	Right	135 ± 26	157 ± 23	16.3	122 ± 25	194 ± 36$^{\#}$	59.0	5.59$^{\bullet}$
	Left	144 ± 20	179 ± 46	24.3	123 ± 24	182 ± 27	48.0	1.05
Anteromedial	Right	144 ± 27	167 ± 30	16.0	128 ± 17	211 ± 85$^{\#\#}$	64.8	2.75
	Left	144 ± 24	181 ± 53	25.7	125 ± 20	206 ± 81$^{\#\#}$	64.8	1.30
LD ventrolateral	Right	133 ± 25	159 ± 34	19.6	114 ± 18	191 ± 46$^{\#}$	67.5	4.31$^{\bullet}$
	Left	138 ± 22	153 ± 33	10.9	111 ± 12	201 ± 50$^{\#\#}$	81.1	9.33$^{\bullet\bullet}$
Mediodorsal	Right	145 ± 23	166 ± 33	14.5	122 ± 15	232 ± 80$^{\#\#\#}$	90.2	6.72$^{\bullet}$
	Left	155 ± 21	168 ± 39	8.4	121 ± 13	234 ± 78$^{\#\#\#}$	93.4	8.52$^{\bullet\bullet}$
Ventromedial	Right	151 ± 32	169 ± 30	11.9	124 ± 15	234 ± 69$^{\#\#\#}$	88.7	8.57$^{\bullet\bullet}$
	Left	144 ± 19	176 ± 42	22.2	125 ± 19	220 ± 60$^{\#\#\#}$	76.0	4.56$^{\bullet}$
Ventrolateral	Right	146 ± 25	167 ± 22	14.4	135 ± 30	251 ± 96$^{**,\#\#\#}$	85.9	5.62$^{\bullet}$
	Left	145 ± 18	181 ± 56	24.8	134 ± 37	235 ± 86$^{\#\#\#}$	75.4	2.41
Ventroposteromedial	Right	145 ± 24	160 ± 20	10.3	126 ± 23	219 ± 67$^{\#\#\#}$	73.8	7.1$^{\bullet}$
	Left	157 ± 22	175 ± 48	11.5	125 ± 24	235 ± 89$^{\#\#\#}$	88.0	5.25$^{\bullet}$
Hypothalamus								
Par anterior parvicellular	Right	138 ± 30	145 ± 22	5.1	116 ± 12	179 ± 25$^{\#\#}$	54.3	10.20$^{\bullet\bullet}$
	Left	136 ± 29	141 ± 21	3.7	116 ± 15	173 ± 28$^{\#\#}$	49.1	8.26$^{\bullet\bullet}$
Anterior	Right	122 ± 27	146 ± 18	19.7	107 ± 11	194 ± 63$^{*,\#\#\#}$	81.3	5.40$^{\bullet}$
	Left	123 ± 25	136 ± 27	10.6	108 ± 12	176 ± 41$^{\#\#\#}$	63.0	6.66$^{\bullet}$
Supraoptic nucleus	Right	105 ± 19	124 ± 10	18.1	105 ± 21	170 ± 36$^{*,\#\#\#}$	62.0	6.73$^{\bullet}$
	Left	109 ± 19	119 ± 23	9.2	104 ± 22	155 ± 38$^{\#}$	49.0	4.17
Dorsomedial	Right	127 ± 17	153 ± 24	20.5	118 ± 21	211 ± 69$^{**,\#\#\#}$	78.9	5.17$^{\bullet}$
	Left	130 ± 16	146 ± 21	12.3	114 ± 14	188 ± 37$^{\#\#\#}$	64.9	10.41$^{\bullet\bullet}$
Ventromedial	Right	117 ± 15	145 ± 15	23.9	106 ± 14	201 ± 65$^{**,\#\#\#}$	89.6	6.45$^{\bullet}$
	Left	121 ± 18	133 ± 21	9.9	107 ± 16	180 ± 47$^{*,\#\#\#}$	68.2	8.06$^{\bullet\bullet}$

Values, expressed as ml/100 g/min, represent means ± SD; the number of animals in parentheses.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, statistically significant differences between stimulated GAERS and stimulated NEC.
$^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, statistically significant differences between (non-stimulated) control GAERS and control NEC.
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, statistically significant differences between stimulated GAERS and control GAERS.
$^{\bullet}p < 0.05$, $^{\bullet\bullet}p < 0.01$, $^{\bullet\bullet\bullet}p < 0.001$, statistically significant interaction between strain and stimulation.

hyperperfusion in the stimulated over the nonstimulated NEC group was observed ipsilaterally in hippocampal CA1 area (59%), substantia nigra pars reticulata (37%), contralaterally in the central amygdala (41%), caudate putamen (32%), and bilaterally in the ventral tegmental area (32%) (Tables 1–3 and Fig. 2).

Comparison between stimulated and nonstimulated GAERS groups

In GAERS, widespread, large, and significant hyperperfusion was observed in most brain regions during the stage 2 seizure. The rates of LCBF were significantly increased over nonstimulated control levels bilaterally by a mean value of 60%–94% in most brain structures. The increase in LCBF rates was limited to about 30% over control levels only in hippocampal CA1, CA2, and CA3 pyramidal cell layers ipsilateral to the stimulation (Tables 1–3 and Fig. 2).

Comparison between stimulated GAERS and NEC groups

The seizure-induced increase in LCBF rates was larger and more widespread in GAERS than in NECs. We have found significant interactions between strain and stimulation. The areas in which the LCBF increase is different in the two strains are listed in Tables 1–3, which also shows the relevant F values (Tables 1–3 and Fig. 2). The values for LCBF were significantly different for stimulated NEC and GAERS in the following regions: bilaterally in the

Table 3. Effects of incomplete amygdala kindling on LCBFrates in central gray nuclei and midbrain-brainstem areas of adult NEC and GAERS rats

		NEC			GAERS			
		Control (n = 7)	Stimulated (n = 7)	Difference (%)	Control (n = 7)	Stimulated (n = 7)	Difference (%)	F value
Central gray nuclei								
Globus pallidus	Right	77 ± 11	100 ± 10	29.9	77 ± 14	124 ± 19$^{\#\#}$	61.0	5.18$^{\bullet}$
	Left	74 ± 9	98 ± 16	32.4	76 ± 13	113 ± 11$^{\#}$	48.7	1.88
Caudate putamen	Right	114 ± 23	133 ± 20	16.7	105 ± 25	163 ± 21$^{\#\#\#}$	55.2	5.33$^{\bullet}$
	Left	108 ± 17	143 ± 27$^{\blacklozenge}$	32.4	103 ± 25	164 ± 19$^{\#\#\#}$	58.2	2.36
Subthalamic nucleus	Right	127 ± 37	146 ± 18	15.0	120 ± 16	197 ± 47$^{**,\#\#\#}$	64.2	5.66$^{\bullet}$
	Left	130 ± 39	158 ± 35	21.5	122 ± 21	204 ± 59$^{**,\#\#\#}$	67.2	3.06
Midbrain-brainstem								
Mammillary nucleus	Right	136 ± 35	142 ± 18	4.4	113 ± 21	172 ± 32$^{\#\#}$	52.2	6.52$^{\bullet}$
	Left	141 ± 35	136 ± 25	−3.6	111 ± 24	166 ± 22$^{\#\#}$	49.6	8.66$^{\bullet\bullet}$
Substantia nigra reticulata	Right	97 ± 19	133 ± 10$^{\blacklozenge}$	37.1	104 ± 14	172 ± 31$^{*,\#\#\#}$	65.4	4.43$^{\bullet}$
	Left	101 ± 19	133 ± 21	31.7	104 ± 16	165 ± 33$^{\#\#\#}$	58.7	2.74
Ventral tegmental area	Right	112 ± 16	148 ± 16$^{\blacklozenge}$	32.1	114 ± 11	170 ± 14$^{\#\#\#}$	49.1	3.37
	Left	114 ± 17	149 ± 19$^{\blacklozenge}$	30.7	120 ± 20	165 ± 16$^{\#\#}$	37.5	0.53
Superior colliculus	Right	142 ± 25	147 ± 23	3.5	113 ± 18$^{\bullet}$	175 ± 45$^{\#\#\#}$	54.9	6.49$^{\bullet}$
	Left	147 ± 13	152 ± 24	3.4	113 ± 20	177 ± 41$^{\#\#\#}$	56.6	8.62$^{\bullet\bullet}$

Values, expressed as ml/100 g/min, represent means ± SD; the number of animals in parentheses.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, statistically significant differences between stimulated GAERS and stimulated NEC.
$^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, statistically significant differences between (non-stimulated) control GAERS and control NEC.
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, statistically significant differences between stimulated GAERS and control GAERS.
$^{\blacklozenge}p < 0.05$, $^{\blacklozenge\blacklozenge}p < 0.01$, $^{\blacklozenge\blacklozenge\blacklozenge}p < 0.001$, statistically significant differences between stimulated NEC and control NEC.
$^{\bullet}p < 0.05$, $^{\bullet\bullet}p < 0.01$, $^{\bullet\bullet\bullet}p < 0.001$, statistically significant interaction between strain and stimulation.

barrel field of somatosensory cortex (posterior part), hindlimb region of somatosensory cortex, basolateral amygdala, subthalamic and ventromedial hypothalamic nuclei, ipsilaterally in the piriform, entorhinal and perirhinal cortex, central and medial amygdala, anterior and dorsomedial hypothalamic nuclei, supraoptic nucleus, ventrolateral and anterodorsal thalamic nuclei, substantia nigra pars reticulata, and contralaterally in the CA2 region of hippocampus (Tables 1–3).

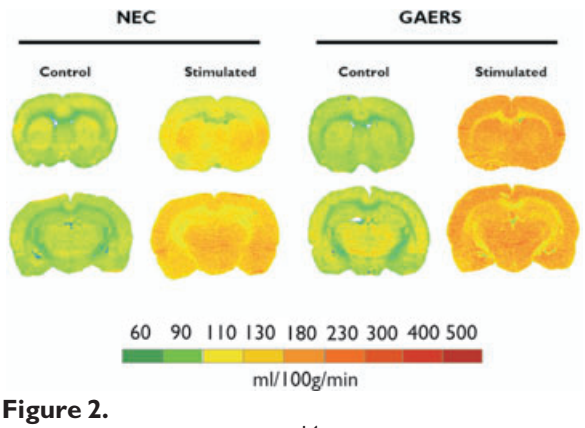

Figure 2.
Color-coded images from [^{14}C]iodoantipyrine-labeled brain sections showing the distribution of cerebral blood flow rates during a stage 2 kindled seizure in NECs and GAERS. The seizure-induced hyperperfusion is larger and more widespread in GAERS than in NECs.
Epilepsia © ILAE

Discussion

The present study shows that stage 2 kindling induced widespread increases in LCBF rates in both strains, but they were of higher amplitude in GAERS than in NECs. In GAERS, rates of LCBF were activated both in limbic regions that are stimulated by kindling and in the cortico-thalamocortical pathway involved in the generation of absence seizures. The same tendency towards an increase in blood flow rates was noticed in NECs.

In NECs, there was a widespread, though not statistically significant, increase in blood flow rates during a stage 2 seizure. This observation is in line with our data on kindled stage 0 and stage 1 seizures, which showed that LCBF increases were already largely distributed, despite the very discrete clinical expression of the seizures (Chassagnon et al., 2006). In the present study, although blood flow changes are of lower amplitude than in our previous study, they tended to be at least as widespread as during a stage 1 seizure. Conversely, in stimulated GAERS, the stage 2 seizure strikingly increased blood

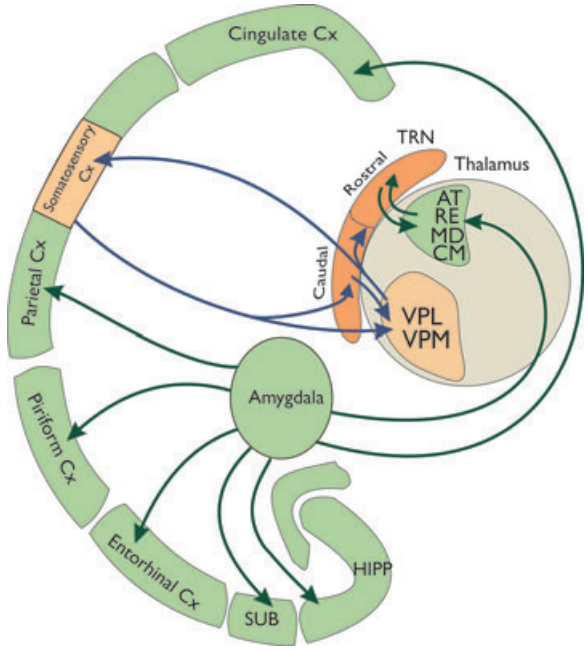

Figure 3.
Schematic view of the interactions between cortico-thalamocortical and limbic circuitries. The thalamocortical network can be activated through the propagation of kindling seizures outside the amygdala to the limbic thalamic nuclei (AT, RE, MD, CM) and cortex. The amygdaloid nuclei are connected with the limbic thalamic nuclei, which have two-way links to the rostral part of the thalamic reticular nucleus, limbic reticular nucleus. Brain areas in green show limbic structures, and areas in light orange show structures of cortico-thalamocortical circuitry. The dark green lines show limbic circuitry, which is also linked to the rostral part of the reticular nucleus. The blue lines show cortico-thalamocortical network. Sub, subthalamic nucleus; Hipp, hippocampus; TRN, thalamic reticular nucleus; AT, anterior thalamic nucleus; RE, reuniens nucleus; MD, mediodorsal thalamic nucleus; CM, centromedial thalamic nucleus; VPL, ventroposterolateral thalamic nucleus; VPM, ventroposteromedial thalamic nucleus.

flow rates all over the brain in a way that is closer to a stage 5 kindled seizure in NECs (Chassagnon et al., 2005).

Comparison between stimulated GAERS and NECs

In both strains, LCBF rates increased in most limbic structures during stage 2 amygdala stimulation. These structures are considered to be critical for the production of the kindling effect (Löscher & Ebert, 1996; Chassagnon et al., 2005, 2006). The most striking result is the degree of activation induced by a stage 2 kindled seizure in GAERS compared to NECs and the nature of the structures activated. Statistically significant differences between GAERS and NECs were found in central amygdala, hippocampal CA1 and CA2 subfields, piriform, entorhinal, and perirhinal cortices.

The amygdala is the area of stimulation and of origin of the seizure. During the stage 2 seizure, hippocampal pyramidal cell layers were activated in NECs and even more in GAERS. In NECs, milder kindled seizures (stage 0 and 1) do not recruit hippocampal pyramidal cell layers (Chassagnon et al., 2006). A facilitatory role of hippocampus on amygdala kindling via the activation of the entorhinal cortex has been hypothesized (Savage et al., 1985). This facilitation occurs via the dentate gyrus, which is the first relay of the trisynaptic pathway connecting the entorhinal cortex (that is also more activated in GAERS than in NECs) and hippocampus.

The entorhinal, perirhinal, and pirifom cortices were also more activated in GAERS than in NECs. These three cortices were also activated by a stage 1 seizure (Chassagnon et al., 2006). The entorhinal and pirifom cortices play a role in the propagation of limbic seizures to subcortical and cortical areas (Löscher & Ebert, 1996). The perirhinal cortex participates in the secondary generalization of kindled seizures via the recruitment of the perirhinal/frontal cortices into the piriform/insular cortical kindling network at the early kindling stages (Ferland et al., 1998).

Thalamic nuclei and the somatosensory cortex that are responsible for the expression of absence seizures and SWDs were also significantly more activated in GAERS than in NECs by the stage 2 seizure (Avanzini et al., 1992; Marescaux et al., 1992; Danober et al., 1998; Blumenfeld, 2005). A cortical focus initiating SWDs was identified within the perioral region of the somatosensory cortex in WAG-Rij rats (Meeren et al., 2002), and a parallel increase of cerebral blood flow and neuronal activity was observed in the somatosensory cortex of WAG-Rij rats (Nersesyan et al., 2004). In addition, studies with ethosuximide, lidocaine, and phenytoin in WAG/Rij and GAERS support the view of the cortical origin of absence seizures (Manning et al., 2003; Sitnikova & van Luijtelaar, 2004; Gurbanova et al., 2006).

Finally, during stage 2 seizures, some hypothalamic nuclei were more strongly activated in stimulated GAERS than in NECs. These nuclei also underwent a strong activation during fully kindled seizures in NECs (Chassagnon et al., 2005). The hypothalamus plays a vital role in the regulation of homeostatic processes, blood pressure, and heart rate (Di Micco et al., 1996). The hypothalamus and the limbic system represent a whole complex of interconnected elements. The increase of LCBF rates in hypothalamic regions may reflect the autonomic responses during stage 2 seizures, since intrahypothalamic bicuculline-induced increases in blood pressure and heart rate are more prominent in GAERS than in NECs (Aker & Onat,

2002). These data suggest either (1) that the hypothalamic function is differently regulated in GAERS as a result of the ubiquitous genetic mutations underlying this epilepsy and/or (2) that the hypothalamus may play a role in the mechanisms underlying absence epilepsy.

LCBF rates were also more strongly activated in the substantia nigra pars reticulata and subthalamic nucleus of stimulated GAERS than NECs. Studies in GAERS suggest that basal ganglia provide a subcortical system controlling absence seizures, the so-called ''system of remote seizure control,'' via a modulation of activity of the substantia nigra pars reticulata, the main basal ganglia output structure (Danober et al., 1998; Deransart et al., 1998b). The substantia nigra acts also to reduce the propagation of limbic motor seizures (Gale, 1992; Deransart et al., 1998a). Likewise, bilateral excitotoxic lesions of the subthalamic nuclei supress absence seizures in GAERS (Vercueil et al., 1998). Thus, the activation of both structures in stimulated GAERS might reflect their role in both absence and limbic seizures.

Specificity of GAERS in the response to stage 2 seizures and resistance to kindling

Whereas a critical role has been assigned to the circuitry of the cortex and thalamus in the production and maintenance of SWDs, limbic regions have not previously been involved in absence seizures. However, although no SWDs were recorded in the hippocampus and other limbic structures, such as the amygdala and septum, some significant changes occur in limbic structures in absence epilepsy. Cerebral glucose utilization rates in adult GAERS, in which absence seizures are fully expressed, increased not only in the corticothalamocortical circuit but also in limbic and motor regions (Nehlig et al., 1991). Likewise, in humans with typical childhood absence epilepsy, increases in cerebral functional activity were recorded in all brain areas whether or not they express SWDs (Engel et al., 1985; Ochs et al., 1987). In addition, in 3-week-old GAERS, before the occurrence of absence seizures, glucose metabolism is increased only in limbic regions and in structures belonging to the remote system of seizure control, which is most likely indicative of preictal activity present in the brain before the occurrence of SWDs (Nehlig et al., 1998). In the present study, stage 2 limbic seizures led to the marked activation of LCBF rates in limbic regions of adult GAERS, which are resistant to the generalization of amygdala kindling. These data point to the critical role played by limbic structures, possibly in preventing the spread of hyperactivity and hypersynchronization.

In GAERS, hippocampal activity is disturbed, independently from or dependently of SWDs. Thus, basal hippocampal extracellular levels of glutamate are larger in GAERS than in NECs (Richards et al., 2000). Glutamate concentration is decreased in mossy fiber terminals (Sirvanci et al., 2005). Glutamate metabolism is increased in the hippocampus and cortex of adult GAERS. Changes are more pronounced in the cortex than in the hippocampus, while glutamate metabolism is identical in the thalamus of GAERS and NECs (Melo et al., 2006). Thus, genetically induced changes in metabolism of both cortex and hippocampus of GAERS may be critical in the control and genesis of absence seizures. Although the density of GABA in GABAergic nerve terminals is comparable in NECs and GAERS in hippocampal CA3 area (Sirvanci et al., 2003) or dentate gyrus (Sirvanci et al., 2005), the activation of GABAergic transmission in the hippocampus reduces the occurrence of SWDs in WAG-Rij rats (Tolmacheva & van Luijtelaar, 2007). In WAG/Rij rats, increased levels of prodynorphin and α-neoendorphin opioid peptides were found in hippocampus, and in hippocampus and striatum, respectively (Lasoń et al., 1992, 1994). In the same strain, the activation of the κ opioid receptor tonically inhibits absence seizure activity (Przewłocka et al., 1995). In addition, the intensity of SWDs in genetic absence epilepsy models correlates negatively with the kindling rate (Aker et al., 2006). A relationship between the daily occurrence of SWDs and the resistance to kindling points to an interaction between the limbic system and the corticothalamocortical circuitry (Onat et al., 2007). Electrical stimulations delivered simultaneously in the thalamic reticular nucleus and the hippocampus suppress seizure generalization (Nanobashvili et al., 2003).

These findings point to interactions between the corticothalamocortical and limbic circuitry, but do not show how they occur. Amygdala and hippocampus are both connected with thalamic nuclei close to the midline (Zhang & Bertram, 2002), such as the mediodorsal, centromedial, and anterior nuclei, which have two-way links to the rostral thalamic reticular nucleus (Cavdar et al., 2008). Thus, the thalamic reticular nucleus can receive excitatory input through its limbic connections from the amygdala, enthorhinal, and piriform cortices during kindling and, in turn, can send inhibitory inputs back to the thalamocorticothalamic network as seen in Fig. 3. Taken together, connections of the rostral thalamic reticular nucleus with limbic structures through mediodorsal, centromedial, and anterior thalamic nuclei may be particularly relevant for understanding the interplay within absence epilepsy and limbic circuitry.

In conclusion, functional activity was largely increased in the limbic system of GAERS compared to NECs during stage 2 kindling seizures. These results support the tight interconnection between the thalamocortical circuitry, in which SWDs originate and occur, and the limbic system. The reasons underlying the strong enhancement in functional activity to stage 2 kindled seizures in GAERS compared to NECs are not clear. It will be important to understand if the dysfunction of the corticothalamocortical

pathway in GAERS is responsible for the larger functional activation as a result of limbic stimulation. Our data also suggest that limbic structures relate to events underlying absence epilepsy even though the limbic circuitry is not directly involved in the expression of absence seizures. The clear understanding of the exact role of limbic structures in this idiopathic epilepsy will need further investigation.

Acknowledgments

This study was supported by the Turkish Association of Brain Research, INSERM U 666, and Janssen Cilag. The authors would like to thank Altug Bayraktar and Ari Bakim (Turkey) for their technical help.

Conflict of interest: We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. The authors have no conflicts of interest to report.

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