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Cellular neuropathology of absence epilepsy in the neocortex: a population of glial cells rather than neurons is impaired in genetic rat model

Evgenia Sitnikova*, Sofia Kulikova, Lidia Birioukova and Vladimir V. Raevsky

Institute of Higher Nervous Activity RAS, Moscow, Russia; *Email: jenia-s@mail.ru

It is well accepted that absence epilepsy is not accompanied by structural brain abnormalities. In the present report, we challenged this view based on microscopic analysis of neocortex in a genetic model of absence epilepsy, WAG/Rij rats. Density of neurons and glial cells was measured in the motor, somatosensory and cingular cortical areas in epileptic WAG/Rij rats and in non-epileptic control ACI rats. More extensive and significant differences between two strains were found in a population of glial cells and less significant - in neurons. In contract to ACI rats, WAG/Rij rats showed (1) a deficit of glial cells and a lower glia-neuron index in the somatosensory and cingulate areas (deep layers); (2) a reduced number of neurons locally in the motor cortex. The somatosensory cortex (deep layers) is known to play a key role in triggering of epileptic discharges, and an impairment of glia-neuron interactions in this area might underlie pathological processes in a primary epileptic focus. In the motor cortex, epileptiform activity is known to reach the highest amplitude, and this may cause or result from a deficit of neurons. Our data suggest the critical role of glial cells and glia-neuron interactions in pathogenesis of absence epilepsy.

Key words: absence epilepsy, glial cells; glia-neuron index, neocortex, somatosensory cortex, WAG/Rij rats

Absence epilepsy is a non-convulsive generalized form of epilepsy, which is characterized by a brief impairment of consciousness (so-called episodes of 'absences') with minimal myoclonic jerks of eyes and perioral automatisms. Absence seizures in human patients are accompanied by 3 Hz spike-and-wave discharges in the electroencephalogram (EEG; Panayiotopoulos 1999). Absence-like behavioral manifestations and characteristic spike-and-wave discharges have been reported in some inbred rodent strains, such as Genetic Absence Epilepsy Rats from Strasbourg, GAERS, and Wistar Albino Glaxo from Rijswijk, WAG/Rij rats (Coenen and van Luijtelaar 2003, Depaulis and van Luijtelaar 2005). Both strains have genetic predisposition to absence epilepsy and they are regarded as reliable animal models of this disease. One of the most remarkable findings in rat models of absence epilepsy is determining a focal epileptic zone in neocortex (Meeren et al. 2002, reviewed in van Luijtelaar and Sitnikova 2006). In particular, spike-and-wave discharges are known to be initiated by neurons located in deep layers of the somatosensory cortex (D'Antuono et al. 2006), and a single microinjection of penicillin in this region is capable of inducing epileptiform activity (Tutkun et al. 2010). Within a few ms epileptic discharges became generalized: they quickly spread over the cortex and invade the thalamus (Meeren et al. 2002).

No structural brain abnormalities have been reported in patients with absence epilepsy and, therefore, absence epilepsy is considered as a purely functional disorder. Histological examination of brain tissue in patients with typical absence epilepsy is almost impossible for ethical reasons as these patients are not amenable to surgical treatment or any invasive investigations. A solely report (Meencke 1989) in childhood absence epilepsy has demonstrated an increased number of dystrophic neurons in the frontal cortex and in subcortical white matter of the frontal lobe. These data suggest that cortical microdysgenesis could either

Correspondence should be addressed to E. Sitnikova
Email: jenia-s@mail.ru

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result in (or be caused by) absence epilepsy or be caused by medical treatment. Recently non-invasive neuroimaging methods (e.g., SPECT, PET and MRI) provided some evidences for the structural abnormalities in cortex and thalamus in patients with absence epilepsy (reviewed in Koepp and Duncan 2004, Richardson 2010). However, data obtained with advanced neuro-visualization techniques still need to be confirmed and clarified in experimental studies and this encouraged us to examine presumable changes in cortical tissue that might associate with absence seizures.

In a pioneering study of cortical cytoarchitecture, Karpova and coworkers (2005) described morphology of pyramidal cells in the sensori-motor region of neocortex in WAG/Rij rats versus non-epileptic control rats (ACI[1] strain). It was shown that pyramidal neurons in superficial layers (I–III) in WAG/Rij rats displayed severe abnormalities in respect to dendritic arborization, branching and orientation of dendrites. Recently we found some evidences that absence epilepsy in WAG/Rij rat model could associate with microstructural disorder in neocortex (Kulikova et al. 2009). In particular, a population of glial cells in epileptic rats appeared to be impaired.

Reciprocal interactions between neurons and glial cells are known to play a vital role in neuronal plasticity and metabolism. Unfortunately, basic theories of epilepsy mainly concentrate on neuron-specific processes, but the role of glial cells in epilepsy has scarcely been acknowledged. Although it is well known that the most abundant type of neuroglia, astrocytes, cooperate with neurons on several levels. These cells supply neurons with oxygen and nutrition, keep chemical homeostasis, e.g., regulate ion concentration (Herrup and Yang 2007), protect neurons from injury in case of stroke or head trauma, remove dead neurons (Roitbak 1999). In order to clarify the role of glial cells and glia-neuron interactions in pathophysiology of absence epilepsy in the current study we investigate local densities of glial and neuronal cells as well as glia-neuron ratio in epileptic WAG/Rij and in non-epileptic ACI rat strains.

Light microscopic analysis was performed in the somatosensory cortex (SmI, projection area of vibrissae, S1BF)[2], motor (M1) and cingulate (Cg1-2) areas. These areas were chosen based on their involvement in absence epilepsy. The SmI is known to be primarily involved in the initiation of absence seizures (Meeren et al. 2002). The motor cortex plays a role in amplification and spreading of the seizure activity (Sitnikova and van Luijtelaar 2006), also epileptic discharges appear in this area with the highest amplitude (Coenen and van Luijtelaar 2003). The cingulate cortex, in which spike and wave discharges are absent, does not seem to be effectively involved in epileptic processes.

Five WAG/Rij rats and five ACI rats (males, six months old) were used in this study. Rats were born and raised at the laboratory of Biological Psychology, Donders Institute for Brain, Cognition and Behavior of Radboud University Nijmegen (The Netherlands). The experiments were conducted in accordance with the European Communities Council Directive 86/606/EEC and were approved by the Ethical Committee on Animal Experimentation of the Radboud University Nijmegen. Rats were sacrificed with an overdose of sodium pentobarbital (200 mg/kg i.p.). The brains were removed and fixed in 4% formaldehyde. Serial coronal slices (20 μm thick) were made with a cryostat, mounted on slides (Superfrost®Plus Gold, Menzel GmbH, Germany) and stained with 0.1% cresyl violet.

Digital microphotographs were taken using Nikon Eclipse E200 microscope (40× magnification) and Canon F640 camera. In microphotographs, neurons were distinguished from glial cells by their size, morphology and staining pattern using the following criteria:

(1) Cytoplasmic staining intensity. In neurons, cytoplasm was stained in violet, and cytoplasmic staining pattern was heterogeneous because of the presence of Nissl substance. In glial cells, cytoplasm was not visible (it remained almost unstained).

(2) Staining pattern of nucleus and nucleolus. In neurons, nucleus was stained in light blue and nucleolus - in dark blue. In glial cells, nucleus was colored intensively in dark blue with almost black thick nuclear membrane; nucleolus was not visible.

Neurons with indistinct or obscure nucleoli were distinguished from glial cells by darkly stained cytoplasm. Eight cells with uncertain anatomical traits were excluded from analysis. In the population of glial cells, we did not differentiate between astrocytes and oligodendrocytes.

[1] Agouti Copenhagen Irish rats, which are commonly used as a control strain, since these rats showed no or at least very few spike-and-wave discharges (Inoue et al. 1990).

[2] This and following abbreviations are given in accordance to the atlas of rat brain (Paxinos and Watson 1986).

For the present study, we improved our previous experimental design (Kulikova et al. 2009) in respect to scoring procedure and statistical analysis. Quantitative microscopic analysis was performed in the vibrissae projection area in the SmI (S1BF), motor (M1) and cingulate (Cg1-2) cortices on two levels: AP 1.0 mm and -1.4 mm, according to Paxinos' rat brain atlas (Paxinos and Watson 1986). Neuronal and glial cells were scored in square fields of 0.01 mm^2 (100×100 μm) in layers II/III, V and VI (Fig. 1). Laminar boundaries were determined visually based on depth and cytoarchitectural features of each layer (details can be found in Paxinos 2004). Two microphotographs were taken in each area of interest. In each microphotograph, two or three 0.01 mm^2 fields were analyzed. In each rat, we analyzed 4-5 fields at the chosen layer of the area of interest. Group statistics (n=5 rats of each strain) is based on 23 measurements per layer and per area. The value of glia-neuron index was computed for each square field by dividing the number of glial cells by the number of neurons.

Statistical analysis was performed using two-way ANOVA for evaluation of between-subject factor 'strain' (two levels: WAG/Rij and ACI) and within-subject factor 'layer' (three levels: II/III, V and VI). Fisher LSD test was used for *post-hoc* analysis when appropriate.

Factor 'layer' showed a high level of significance in all measures (Table I, factor 'layer'), suggesting a non-homogeneous distribution of neurons and glial cells

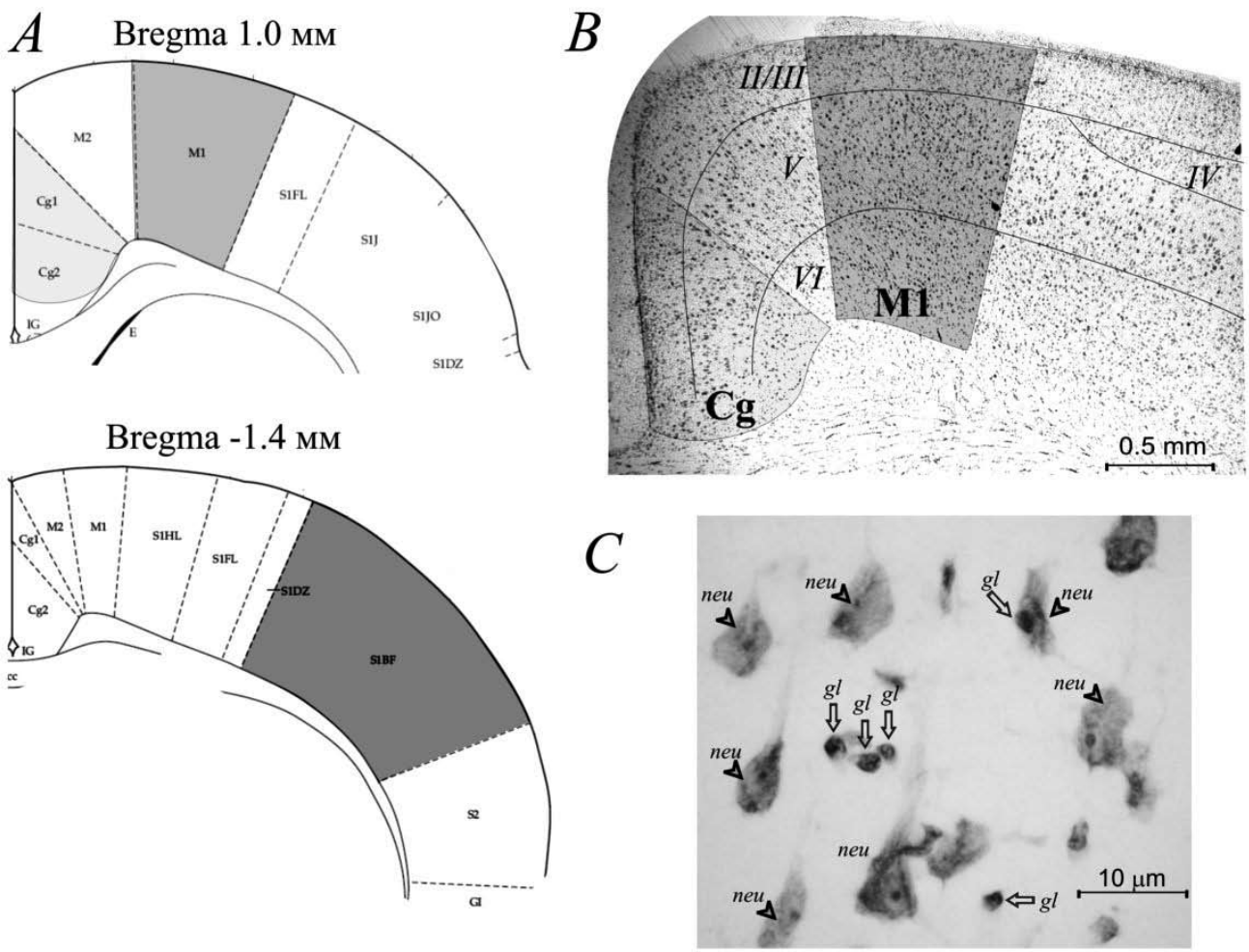

Fig. 1. Details of microstructural analysis of the selected areas in the neocortex. (A) Areas of interest (filled up in gray) as determined by rat brain atlas (Paxinos and Watson 1986): S1BF - somatosensory cortex, barrelfield area; M1 - motor cortex; Cg1-2 - cingular cortex. (B) Low magnification microphotograph (x40) of the area of interest (AP=1.0 mm). (C) High magnification microphotograph (x2000, layer V of S1BF in WAG/Rij rat) in which neurons (neu, arrowheads) and glia (gl, arrows) could be recognized.

Table 1

Density of neurons and glial cells (per 10^{-2} mm^2, mean±SD) and results of two-factors ANOVA for significant differences between two rat strains.

		Neurons		Glial cells		Index glia-neuron	
		ACI	WAG/Rij	ACI	WAG/Rij	ACI	WAG/Rij
Somatosensory cortex (SmI)							
II/III		27.5±3.0	28.4±5.2	7.6±1.7	6.5±2.6	0.28±0.07	0.23±0.10
V		15.2±3.2	13.7±1.5	**11.2±2.1**	**6.3±2.8***	**0.77±0.23**	**0.47±0.22***
VI		22.3±4.5	25.3±4.0	**8.1±2.2**	**5.5±2.8***	**0.38±0.14**	**0.24±0.10***
Mean		21.7± 6.2	22.5±7.4	9.0±2.6	6.1±2.6	0.48±0.26	0.31±0.19
ANOVA, factors	'strain'	Ns		$F_{1,132}$=52.1; p<0.0001		$F_{1,132}$=38.9; p<0.0001	
	'layer'	$F_{2,132}$=159.8; p<0.0001		$F_{2,132}$=9.7; p<0.0005		$F_{2,132}$=72.7; p<0.0001	
	interaction 'strain'×'layer'	ns		$F_{2,132}$=7.7; p<0.0005		$F_{2,132}$=7.8; p<0.001	
Motor cortex							
II/III		**35.0±4.5**	**21.9±2.2***	**8.3±2.0**	**11.6±2.8***	**0.24±0.07**	**0.53±0.14***
V		16.3±3.7	17.0±3.2	7.4±1.6	7.2±2.4	0.47±0.12	0.42±0.18
VI		23.6±3.7	22.2±4.1	7.4±1.7	7.0±2.2	0.32±0.10	0.33±0.13
Mean		25.0±8.7	20.6±3.7	7.7±1.8	8.6±3.3	0.35±0.14	0.43±0.17
ANOVA, factors	'strain'	$F_{1,132}$=49.5; p<0.0001		$F_{1,132}$=5.5; p<0.05		$F_{1,132}$=14.2; p<0.0005	
	'layer'	$F_{2,132}$=114.2; p<0.0001		$F_{2,132}$=23.8; p<0.0001		$F_{2,132}$=9.8; p<0.0005	
	interaction 'strain'×'layer'	$F_{2,132}$=52.0; p<0.0001		$F_{2,132}$=10.5; p<0.0001		$F_{2,132}$=23.4; p<0.0001	
Cingulate cortex							
II/III		32.5±5.1	34.7±6.5	9.03±1.6	8.7±2.0	0.28±0.07	0.26±0.08
V		16.9±3.2	17.6±2.7	**11.2±2.6**	**7.9±2.4***	**0.68±0.20**	**0.46±0.15***
VI		24.6±3.6	23.4±2.8	**9.6±2.1**	**6.9±1.7***	**0.40±0.10**	**0.29±0.07***
Mean		24.7±7.5	25.2±8.4	10.1±2.9	7.8±2.2	0.45±0.22	0.34±0.13
ANOVA, factors	'strain'	ns		$F_{1,132}$=34.4; p<0.0001		$F_{1,132}$=32.1; p<0.0001	
	'layer'	$F_{2,132}$=173.2; p<0.0001		$F_{2,132}$=4.4; p<0.05		$F_{2,132}$=76.0; p<0.0001	
	interaction 'strain'×'layer'	ns		$F_{2,132}$=6.4; p<0.001		$F_{2,132}$=7.7; p<0.0005	

Significant whitin-layer differences are asterisked (*; *post-hoc* LSD test, p<0.05), and, correspondingly, bold font indicates significantly different values.

across cortical layers. The most significant differences between epileptic and non-epileptic rats were found in a population of glial cells (Table I). In the SmI, WAG/Rij rats showed a lower density of glial cells as compared to control ACI rats, and differences were significant in layers V and IV (*post-hoc* test, both *p's*<0.05). 'Glia-neuron' index in WAG/Rij rats was also lower as compared with ACI rats, and this effect was significant in layers V and IV ($p<0.05$).

Similar to the SmI, the cingulate cortex in WAG/Rij rats was characterized by a low number of glial cells as compared to control, whereas significant differences were found in layers V and IV (both *p's*<0.05). 'Glia-neuron' index in WAG/Rij rats was also lower than in ACI rats, and this effect was significant in layers V and VI ($p<0.05$).

In both the SmI and the cingulate cortical areas, the number of neurons in WAG/Rij did not differ from that in ACI rats (Table I).

In the motor cortex, density of neurons at the II/III layer was significantly lower than in control ACI rats. At the same time, WAG/Rij rats demonstrated a higher density of glial cells in these layers versus control.

Our main finding is that absence epilepsy in WAG/Rij rat model could associate with microanatomic changes in epileptic zone (the SmI) and non-epileptic area (the cingulate cortex), such as a deficit of glial cells in deep layers and a lower glia-neuron index in these areas. These results are in line with our preliminary data (Kulikova et al. 2009). Taken together, these findings suggest that a reduced or defective glial supply to neuronal cells may promote epileptic processes and lead to absence epilepsy. One of the possible mechanisms, by which glial cells could effectively control seizure activity, might involve glia's ability to buffer extracellular potassium ions. K^+ currents are known to play an essential role in controlling neuronal excitability, and too high extracellular $[K^+]$ lead to epileptiform activity (Fröhlich et al. 2008). Deficiency of glial cells may disturb buffering of extracellular potassium and this may result in an increase K^+ concentration, therefore neurons may easily become hyperexcitable and produce epileptic discharges (Roitbak 1999).

Microstructural changes that were found in the SmI in WAG/Rij might correlate with the primary role of the SmI in initiating of absence seizures (Meeren et al. 2002). More specifically, epileptic discharges are known to originate from layers V/IV[3] of the SmI (D'Antuono et al. 2006). According to our data, epileptic rats are distinguished from control by the low number of glial cells and low glia-neuron index in layers V/VI of the SmI. This allows us to assume that a deficit of glial cells and an impairment of neuroglial interactions in deep layers may contribute to the state of neuronal hyperexcitability in the SmI (D'Antuono et al. 2006). It remains to be claified whether the cellular changes in neocortex are primary to epileptogenic processes or secondary to recurrent seizure activity (so-called maladaptive response). It is possible that neuronal and glial content of neocortex changes during development in parallel with age-dependent increase of seizure activity. This problem deserves special considerations and can be investigated in the future.

In the motor cortex, WAG/Rij rats displayed a lower density of neurons in layer II/III and an increase of glial cells. It is known that in the motor cortex spike-and-wave discharges reach the highest amplitude and show the most regular waveform in comparison to the other cortical areas (van Luijtelaar and Sitnikova 2006). Perhaps, too strong epileptic activity in motor cortex could *per se* cause neuronal death due to excitotoxicity.

Similar to the SmI, the cingulate cortex in WAG/Rij rats was distinguished from control by a lower density of glial cells and a lower glia-neuron index. It is known that the cingulate cortex is merely involved in epileptic activity, but it is responsible for cognitive functions, e.g., learning and memory. Most likely, microstructural disturbances in the cingulate cortex could underlie cognitive, linguistic, and psychiatric comorbidities in patients with absence epilepsy (e.g., Caplan et al. 2008) and similar syndromes in animal models (Sarkisova et al. 2005).

Interestingly, the SmI and the cingulate cortical areas display similar microstructural changes in WAG/Rij rats as compared to ACI (Table I), and these changes are likely to be genetically predetermined. Considering the fact that the SmI and cingulate cortex are involved in different neuronal networks and have different functions, similar microstructural findings in these two areas may have different consequences. In the SmI, a deficit of glia may prerequisite epilepsy, and in the cingulate cortex it may lead to comorbidities.

[3] This *in vitro* study in brain slices demonstrates that neurons in deep layers in the SmI show an increased NMDA-related synaptic excitability and could play a primary role in initiating of epileptic discharges.

In conclusion, microstructure of neocortex in epileptic WAG/Rij rats differed from non-epileptic ACI rats by lower density of glial cells in the somatosensory and cingulate cortical areas (the number of neurons appeared to be the same). In the motor cortex (layer II/III), epileptic rats revealed one-third reduction in number of neurons at the expense of the higher number of glial cells. Absence epilepsy might be closely associated with a reduction of the number of glial cells in deep layers (V and IV) in epileptic focus in the SmI. A reduced number of neurons in the motor cortex (superficial layers) might be secondary to epileptic seizures.

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