Neonatal Tactile Stimulation Modulates Dendritic Spine Densities in Somatosensory Cortex of Adult WAG/Rij rats

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Abstract

Objective: The aim of our study is to examine the effects of neonatal tactile stimulatons on the brain structures that previously defined as the focus of epilepsy in the Wistar-Albino-Glaxo from Rijswijk (WAG/Rij) rat brain with genetic absence epilepsy.

Methods: In the present research, morphology and density of dendritic spines were analyzed in the somatosensory cortex (SoCx) of WAG/Rij rats (non stimulated control, tactile-stimulated and maternal separated rats) and healthy Wistar (non-epileptic) rats. To achieve this, a Golgi-Cox method was used.

Results: Dendritic spine number in layer V of the SoCx has been detected significantly higher in adult WAG/Rij rats at post natal day 150 in comparison to non-epileptic adult control Wistar rats ($p<0,001$). Moreover, quantitative analyses of dendrite structure in adult WAG/Rij rats showed a decrease in dendrite spine density of pyramidal neurons of SoCx which occurred in early neonatal exposure to maternal separation (MS) and tactile stimulation (TS) ($p<0,001$).

Conclusions: Our findings provide the first evidence that tactile stimulations during the early postnatal period have a long-term impact on dendrite structure in WAG/Rij rat's brain and suggest a reduction in dendrite spine density is linked to absence seizure reduction.

Keywords: Absence Epilepsy; Dendrites; Golgi-Cox Staining; Maternal Separation; Somatosensory Cortex; Tactile Stimulation; WAG/Rij; Rat

Introduction

The Wistar-Albino-Glaxo from Rijswijk (WAG/Rij) rat is an extensively utilized genetic model for generalized absence epilepsy with comorbid depression, in which cognitive impairment and poor maternal behavior has also been recently reported **[1,2,3].** Additionally, they have been considered as an absence epileptogenesis animal model **[1].**

2–3 months-old WAG/Rij rats begin to manifest the spontaneously occuring 7–10 Hz spike-wave discharges (SWDs) in the cortical electroencephalogram (EEG) alongside decreased consciousness and immobility as in human absence epilepsy. All rats of this strain, at six months of age, show about 16-20 SWDs an hour **[4]**. WAG/Rij rats of the same age show depression-like behaviour symptoms, which tend to aggravate alongside with SWD increase **[5].**

It is broadly accepted that SWDs occur in an interconnected intact cortico-thalamo-cortical network. This network that comprises of cerebral cortex, the thalamic relay nuclei, the intralaminar thalamic nuclei, and the reticular thalamic nucleus, has a role in the appearance of synchronized and generalized SWDs in absence epilepsy **[6].** SWD generation and occurance are not completely understood. However, it appears that SWDs in genetic absence models have a cortical focal origin in the deep layers (layer V- VI) of the perioral region of the SoCx and that the thalamus also has the function of a resonator for SWD maintenance **[1,7].**

Manipulations in maternal environment are well-known to affect epileptic activity in adult WAG/Rij rats that are genetically predisposed to absence epilepsy. Sitnikova et al. (2011) demonstated that whisker trimming during early life in WAG/Rij rats resulted in seizure activity increase in adulthood **[8]**. WAG/Rij rat pups that were handled for 15 mins during postnatal days 1-22 demonstrated a decrease in SWD at adult ages. During the same postnatal period, the reduction in SWD number was observed in pups that were exposed to maternal deprivation for 180 minutes **[9].** It should be mentioned that these two procedures commonly increase maternal care.

It has recently been shown that maternal care decreases the number and mean duration of SWDs and delays the appearance and progression of absence epilepsy. Increased early maternal care not only exerts antiepileptogenic effects but also counteracts the development of comorbid depression in adult WAG/Rij rats **[10].** A previous study by our group have shown that tactile stimulation (TS) during postnatal period in WAG/Rij rats genetically predisposed to epilepsy reduced epileptic activity and comorbid depression in adulthood **[11].**

The outcomes of these studies show that an increase in tactile stimulations in initial development periods forms absence seizure-modifying effects in WAG/Rij rats. Application of TS mimics maternal licking and grooming behavior in rats, which is a sensory stimulation method to the skin **[12].** Studies show that TS therapy stimulates maturation in rat pups and in human infants **[13].** Evidence has shown that TS during initial periods of development enables to reorganize dendritic organization in various brain regions and induces behavioural benefits in adult age **[14].** Given during early developmental periods, TS improves anxiety-like behaviors, prevents preference to addictive drugs and depression-like behaviors **[12].** When given in adult rats, however, TS shows beneficial influence on the brain function, preventing cortical lesion and increasing neurotrophin and dendritic length **[15].**

Based on this background, we hypothesized that the effects of neonatal tactile stimulations on absence seizure might be associated with changes in neuronal organization. Therefore, in this study, we examined the effects of the neonatal TS on dendritic morphology in layer 5 pyramidal neurons of SoCx in genetically predisposed WAG/Rij rat's brain in adulthood. To achive this, a Golgi-Cox method was used. SoCx was chosen for analysis because in WAG/Rij rats it has been shown that spontaneous SWDs have their onset in the deep layers of this region.

Material and Methods

Animals and study design

Each pregnant female Wistar and WAG/Rij rat from the breeding facility of University of Kocaeli was housed in a plexiglas cage and had free access to food and water under controlled temperature of 22-23 °C and on a 12:12 h light:dark cycle.

The births were monitored and at postnatal day one (P1) male pups of litters were allocated at random to four groups: Control Wistar (not touched) rats, control WAG/Rij (not touched) rats, maternally separated WAG/Rij rats and, tactile-stimulated WAG/Rij rats. For randomization, male WAG/Rij pups of six litters were placed together in a warm plastic box and then were returned to the lactating dams. Wistar control pups collected from two different litters randomly.

From postnatal day 3 to day 21, TS was applied three times per day (9:00 AM, 1:00 PM, and 4:00 PM). At each session, mothers stayed in a different cage with food and water. A soft baby brush was used to brush each pup in the TS group during 15 minutes **[16].**

In maternal separation (MS), pups remained in a warm plastic box without their mother during 15 minutes three times per day (9:00 AM, 1:00 PM, and 4:00 PM) between P3-P21. By the end of procedure, rat pups were placed back with their mothers. The control groups stayed in their cage without receiving any stimulation and were only handled during the home cage regular cleaning, two times a week. Following TS and MS on P21, pups were weaned and housed in groups. Body weight of rat pups was monitored each week as weight loss or decreased food intake were the study criteria for exclusion. All attempts were made to use the least number of animals in the experiments and minimize their suffering from experimental procedures**.**The experiment was approved by Ethical Committee on Animal Experimentation of the University of Kocaeli, Turkey (KOÜ-HADYEK 6/5-2016).

Golgi-Cox Staining Procedure

At PND 150, total of 30 rats (6 rats in each group) were deeply anesthetized with 100 mg/kg intraperitoneal injections of sodium pentobarbital and sacrificed by decapitation using a rodent guillotine. The corneal reflex was used to determine the deep level of anesthesia. After decapitation, the brains were removed. We used Hito Golgi-Cox OptimStain Kit (Hitobiotec

Inc., Wilmington, DE, USA) for tissue preparation and staining method. The staining method was carried out according to the manufacturer's user manual. A series of 50-80μm coronal sections was obtained from the SoCx that contains the epileptic zone **[17].** In quantification of dendritic spine density, coordinates were provided from Paxinos and Wattson atlas (2007): for the first point to bregma AP 0.0 ML 8.0; for the second point from IL from bregma AP −2.0 ML 7.0. (Fig. 1) **[18].** Three images that cover the whole thickness of every dendrite were provided by making use of a 100× oil immersion objective on a bright-field microscope (Leica DM2500). Fragments of individual randomly selected dendrites were quantified in order to measure spine density. A region at least 30 μm from the soma was made use of and, in this way, any confounds of decreased spine density in promixal dendrites were avoided. 3 well-identified dendrites in the deep layer (V) pyramidal neurons of the SoCx were used per section (54 dendrites per group). Spine densities of the SoCx were analyzed by making use of the Image J software (National Institutes of Health, Bethesda, MD, USA). Quantification was carried out blindly and for each animal, the average spine density values (number of spines/μm) were measured. Morphometric analysis was conducted for each spine, and measurements categorized spines into stubby, thin, and mushroom (Fig.2) subtypes.

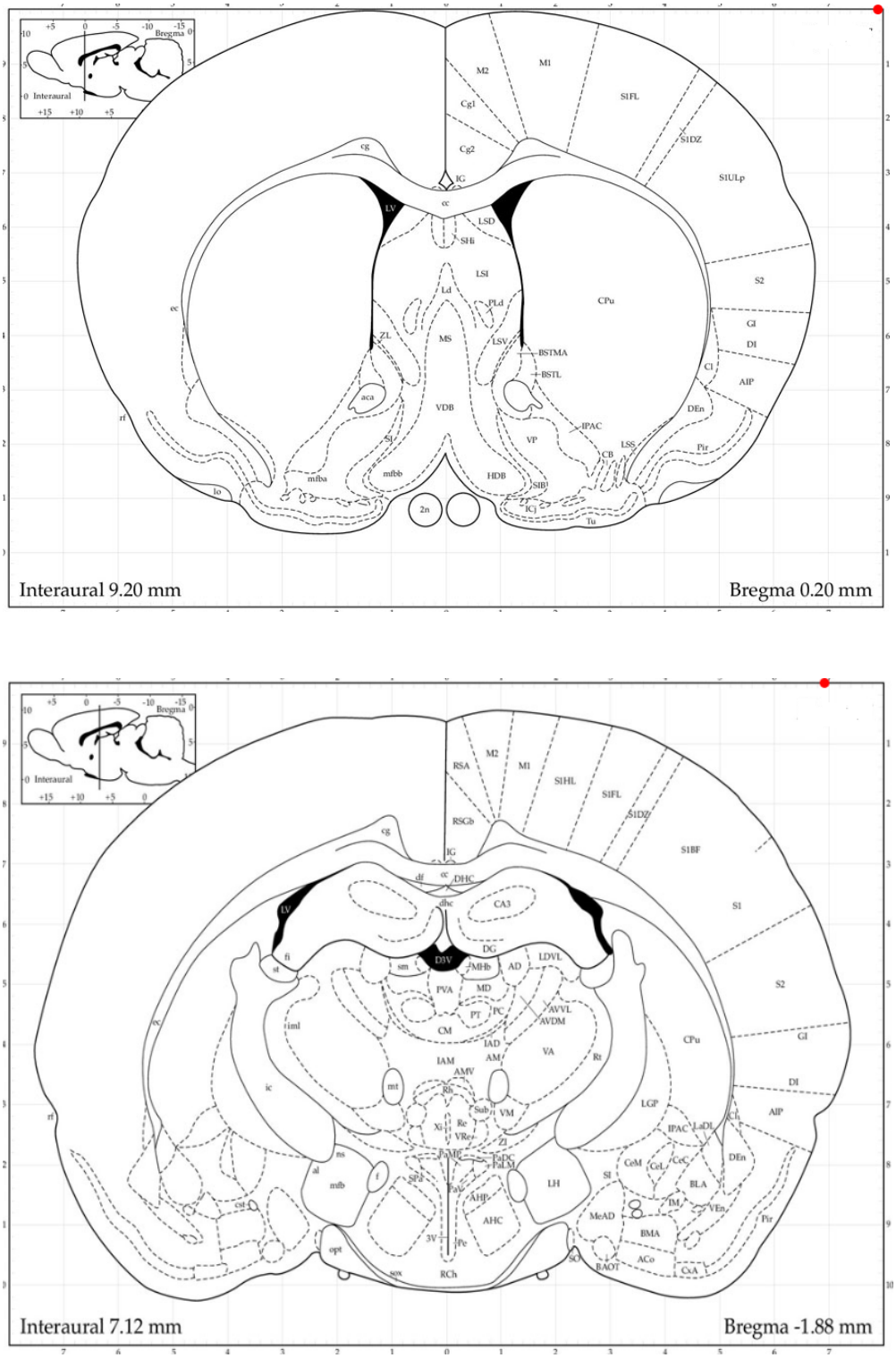

Fig. 1. Investigated two areas in the somatosensory cortex. The secondary somatosensory (S2) area (AP 0.0 ML 8.0); the edge of the secondary somatosensory area and vibrissae (AP-2.0 ML 7.0) (Paxinos and Watson, 2007).

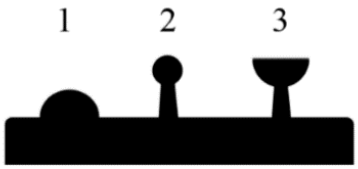

Fig.2. Dendiritc spine morphology: Stubby (1), thin (2), and mushroom (3) spine subtypes

Statistical analysis

Average values for each animal and experimental group were used for the statistical analyses. Data are presented as mean ± Std Error. Data for the density of dendritic spine and the number of each spine type per group were compared by a one- way ANOVA test followed by the Tukey test. The statistical significance level was set as $p<0.05$. We used the GraphPad InStat version 7.03 (GraphPad Software, USA).

Results

The dendritic shafts and spines of deep layer pyramidal neurons of the SoCx were distinctly observed by the Golgi-Cox staining procedure (Fig. 3).

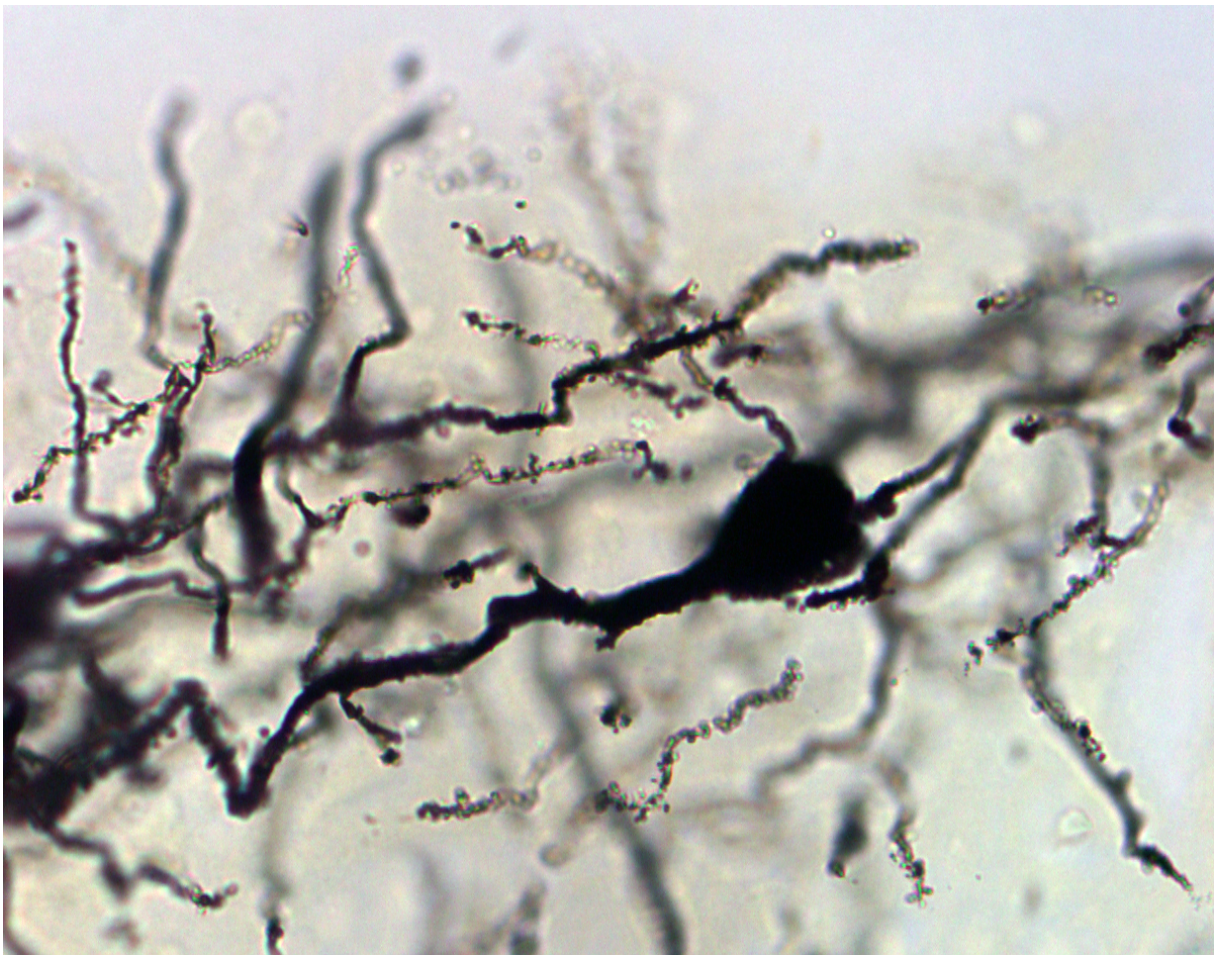

Fig. 3. Photomicrograph demonstrating Golgi-Cox impregnated pyramidal neurons of the somatosensory cortex from control adult WAG/Rij rats.

The dendritic spine density of the SoCx pyramidal cells was different between WAG/Rij control and nonepileptic Wistar rats. The SoCx of WAG/Rij control displayed significantly higher mean spine density compared with control Wistar rats ($p<0.001$) (Fig. 4). Repeated exposure to TS and MS reduced dendiritic spine density in WAG/Rij rats ($p<0.001$). TS and MS in WAG/Rij rat pups caused dendritic spine density of the SoCx to decrease to the levels of Wistar rats ($p>0.05$).

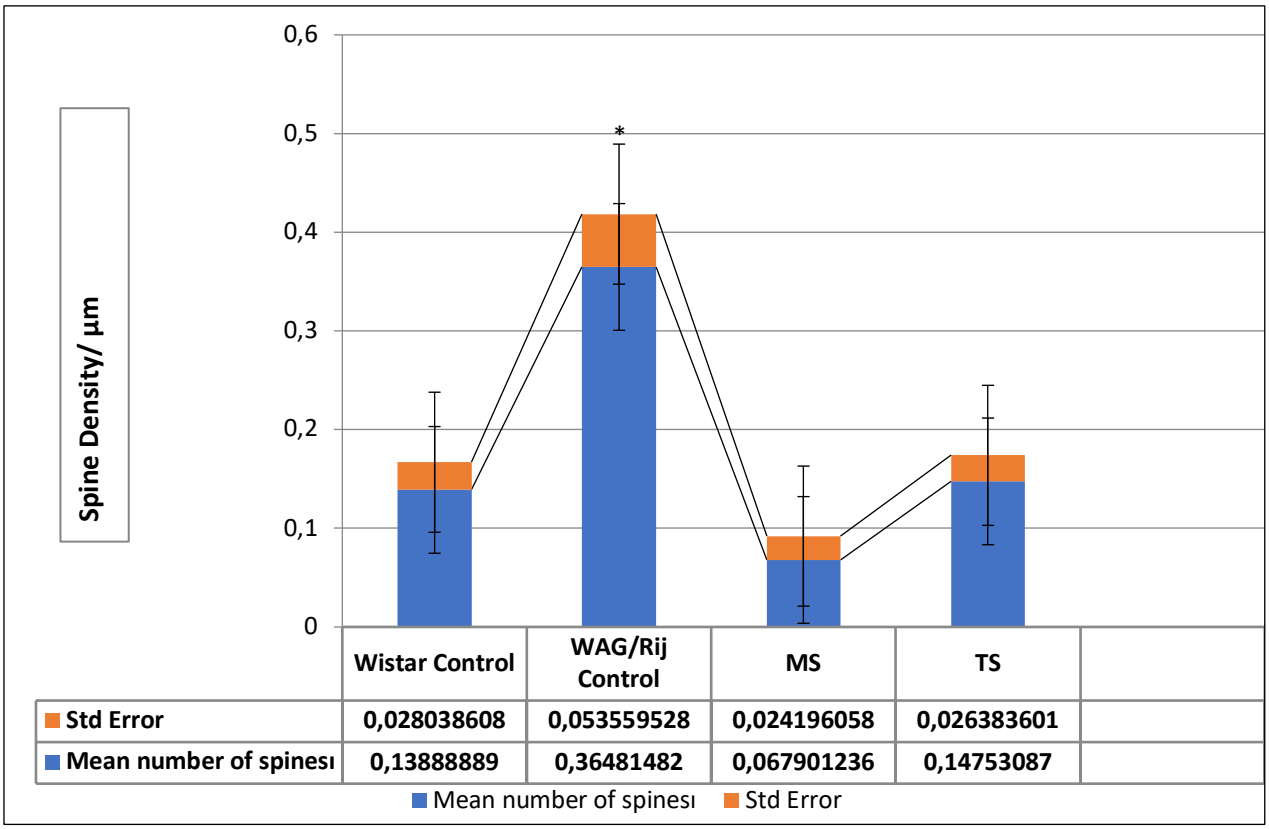

Fig. 4. Mean number of spines (± Std Error) in layer V pyramidal neurones of control Wistar, control WAG/Rij, maternally separated WAG/Rij and tactile-stimulated WAG/Rij rats. *$p<0.001$ WAG/Rij control compared to other groups (one-way ANOVA).

Stubby, thin and mushroom spines were the most common types found in deep layer (V) of the rat SoCx of control Wistar and control WAG/Rij rats (Fig.5, Fig. 6). There were marked differences in the number of each type of dendritic spine between control Wistar and control WAG/Rij rats. Stubby, thin and mushroom spines were higher in the SoCx of control WAG/Rij compared to Wistar control rats ($p<0.0001$). Stubby, thin and mushroom spines were reduced

significantly in TS and MS compared to WAG/Rij control brains ($p<0.0001$) (Fig. 5). TS and MS reduced the number of all spine subtypes to Wistar control values (Fig. 5). As shown in figure 5, no significant differences were observed in MS, TS compared to Wistar control rats ($p>0.05$). However, in tactile-stimulated rats, the number of thin spines were significantly higher from that of MS group ($p<0.001$).

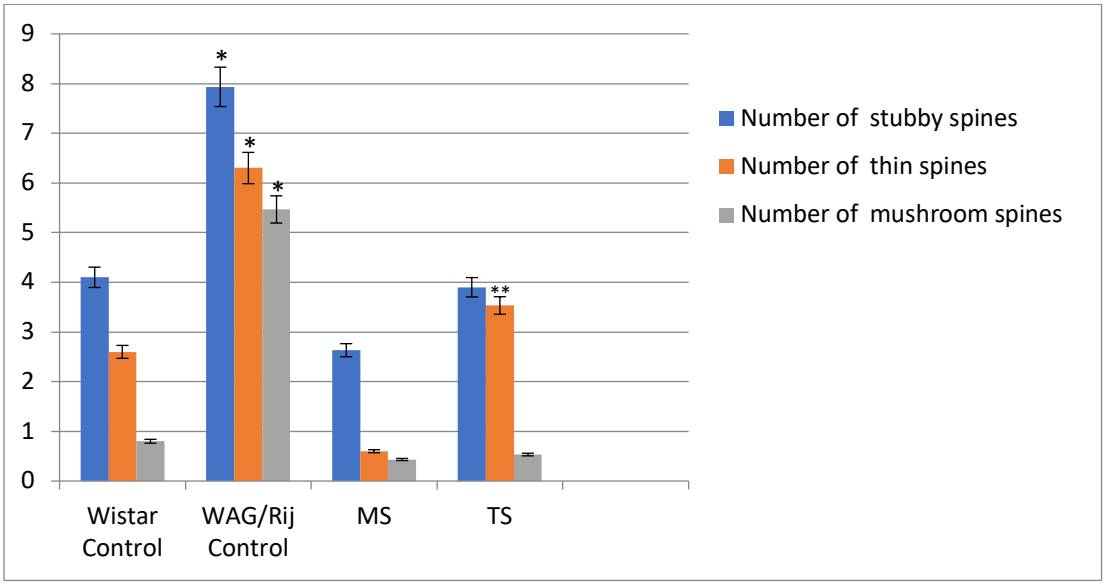

Fig. 5. Mean number of spines subtypes (± Std Error) in layer V pyramidal neurons of control Wistar, control WAG/Rij, maternally separated WAG/Rij, and tactile-stimulated WAG/Rij rats . $^{*}p<0.001$ WAG/Rij control compared to other groups, $^{**}p<0.001$ TS compared to number of thin spines at MS (one-way ANOVA).

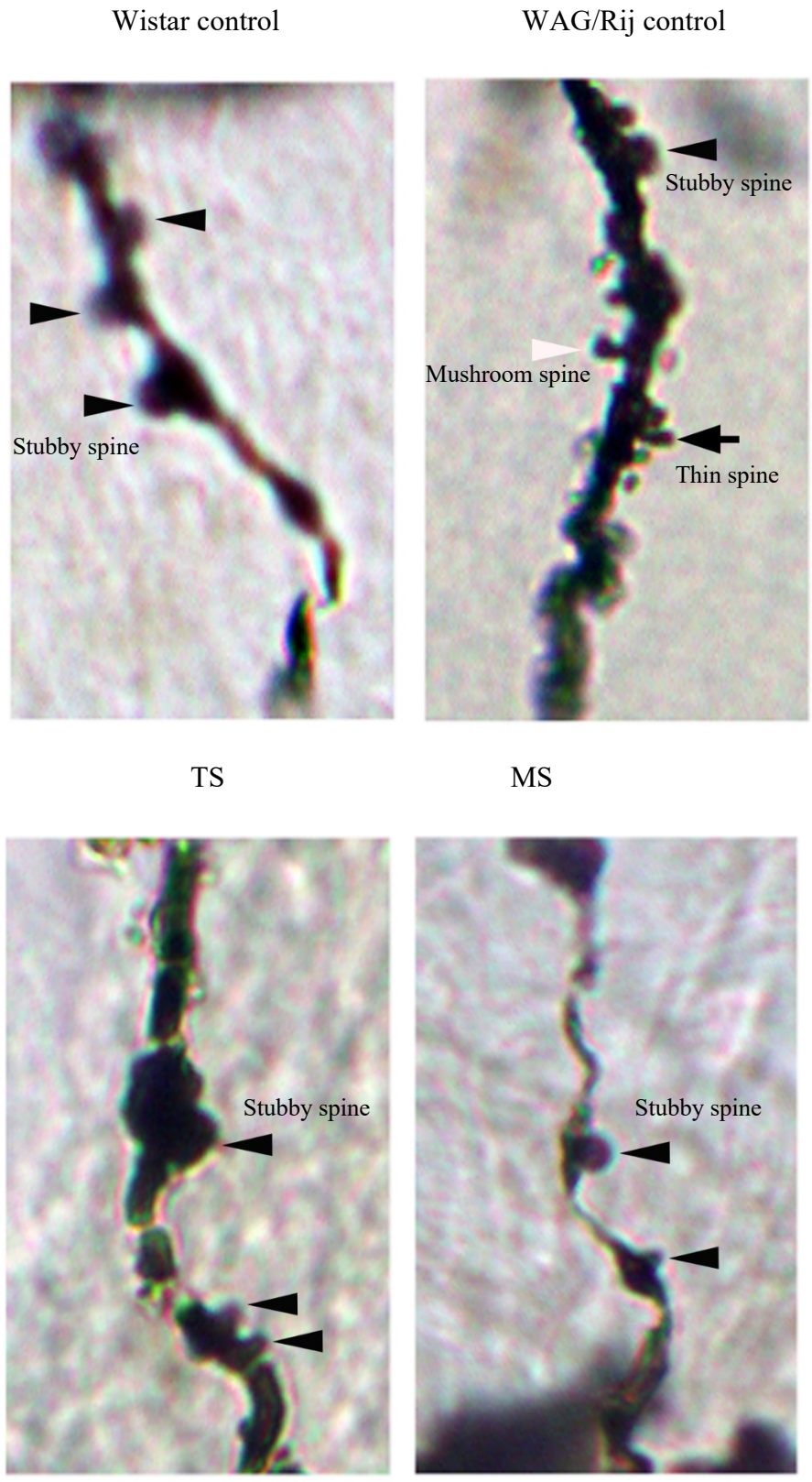

Fig. 6. Photomicrographic (2000×) examples of dendritic spines in the four groups.

Discussion

Our present results showed that, in SoCx, WAG/Rij rats had a higher density of dendritic spine when compared to healthy Wistar rats. Furthermore, our results showed for the first time that sensory experiences in early developmental period can cause permanent changes in dendritic spine density in WAG/Rij rats. A decrease occurred in the density of dendritic spines in the deep layer of SoCx in adult WAG/Rij rats when subjected to TS and MS during neonatal period. Additionally, sensory experiences induced changes in spine morphology within the SoCx. In the deep pyramidal cells of maternally separated animals there were less thin spines than tactile-stimulated animals.

The SoCx in rats that are genetically predisposed to absence epilepsy is regarded to trigger epileptic discharges. In general, it was concluded that the spike-wave discharges firstly appear at SoCx and then rapidly spreads to the remaining parts of the cortex and cortico-thalamic network. Seizure activity initially takes place in the deep-pyramidal cells, afterwards in the superficial layer pyramidal neurons and spreads towards ipsilateral cortical areas **[19].**

The SoCx in WAG/Rij rats is characterized by synaptic hyperexcitability **[8].** In absence seizures, electrophysiological intracellular recordings demonstrated that the pyramidal cells in the deep layers of SoCx show fast activation, hyperexcitability and hypersynchronizing characteristics **[19].** Karpova et al. (2005) demonstrated significant structural changes in dendritic and axonal arborization in pyramidal neurons SoCx of WAG/Rij rats. In WAG/Rij rats, it was found that there were longer dendrites and had less branching in pyramidal neurons of SoCx compared with non-epileptic control rats **[20].** According to these data that were collected in the upper layers, it is suggested that epileptic rats might have atypically characterized neurons in the SWD generation site with greater arborizations and more synaptic connections between neurons. These features might facilitate the initiation and spreading of SWD **[1].**

In our experiment, different from previous study (Karpova et al., 2005), the neuronal organization of deep layer of SoCx was investigated by Golgi-Cox staining in WAG/Rij rats with genetic epilepsy and in non-epileptic control rats with the same age. Similarly, we found quantitative differences in the density of dendritic spines between WAG/Rij rats and non-epileptic control rats in the deep layers of SoCx. It is likely that dendritic abnormalities are both the cause and result of seizures in this genetic model.

Dentritic spines are thin protrusions that emerge from the surface of various neurons, specifically postsynaptic structures, which play mostly an excitatory role in synaptic communications **[21,22].** The morphology and density of dentritic spine is crucial for synaptic plasticity. Physiological and pathological conditions are reported to be associated with the spine

morphology and density **[23,22].** Dendritic spine abnormalities are generally reported in the brain specimens of epileptic patients in hippocampal tissue of epilepsy patients with temporal lobe, decrease in dendritic spine density is frequently reported. However, alterations in dendritic length, shape, and branching patterns and focal increase in dendritic spines are less frequently reported in cortical and hippocampal tissues. In various animal models that involve acute seizures or chronic epilepsy, similar dendritic changes have been observed, which are primarily loss of dendritic spines and less frequently increase in dendritic spines **[24].**

Generally, the majority of excitatory synaptic inputs are received and integrated by dendritic spines in the central nervous system, and therefore have influence on neuronal excitability. In certain kinds of epilepsy, hyperexcitable circuits and seizures might result from dendritic spine abnormalities. Therefore, it is safe to assume that high dendritic spine number and associated excitatory synaptic input disturb the balance between excitation and inhibition in the WAG/Rij rat brain, causing seizure. In addition, types of spine may have distinct functions and alterations in the spine type ratio may cause a significant effect on neuronal excitability and function **[25, 26].**

In our study, all types of spines were reduced in epileptic rats exposed to TS and MS. However, MS in WAG/Rij rats decreased the most in thin spines. It is known that mushroom spines are more stable whereas thin spines are newly formed **[27, 28]**. Therefore, our findings indicate that tactile stimulations not only modulate structural plasticity in the SoCx by decreasing spine numbers, but also by changing the ratio of new/mature spines.

It is not surprising that tactile stimulations led to change spine density in the SoCx, where tactile sensation gets processed **[17].**Various data confirm the beneficial effects of enriched environment on synaptic plasticity in different animal models **[29,30].**

Studies showed that TS, which is an enriching positive experience that mimics maternal licking and grooming, has the potential to affect the neuroanatomic organization of the brain **[12, 16, 31, 32, 33].**

Richards et al., (2012) showed that TS early in life increased spine density, dendritic branching, and dendritic length in prefrontal cortex and amigdala of rats **[12]**. In another study, TS treatment, by increasing dendritic complexity, length and synaptic contact in all cortical areas and amygdala, reversed neuroanatomical alterations caused by prenatal valproic acid exposure in rats **[31].** However**,** Kolb and Gibb (2010) demonstrated that TS may differently alter synaptic organisation in different brain areas **[32]** . According to their study, TS treatment early in life decreases spine density and dendritic length in the parietal cortex. The same

environmental exposure can alter spine density differently in accordance with manipulation age and manipulations early in life lead to decrease in spine density while those given in adulthood cause it to increase **[34].**

Interestingly, in SoCx, maternally separated animals showed dendritic density similar to that of tactile-stimulated animals. This result seems to be related to short MS sessions (non-stresful) and the increased compensatory licking and grooming behaviors (tactile stimulations) by the dams to the pups after the separation period **[35].**

It has been suggested that possible mechanisms that underlie the neural and behavioural effects of TS include endocrine function alterations, increased production of neurotrophic factors (insulin-like growth factor, brain-derived neurotrophic factor, and fibroblast growth factor-2) and altered gene methylation **[12].**

Further studies are required to clarify mechanisms underlying the seen effects of TS on the morphology and density of dendritic spines in the deep layer of SoCx in epileptic rat's brain.

Conclusions

Our study provides additional evidence for the structural changes of SoCx pyramidal neurons in genetically absence epileptic rat's brain. We found that the dendritic spine density in the layer V of SoCx the WAG/Rij rat strain was higher than Wistar rats (non-epileptic). Tactile stimulations decreased dendritic spine density and changed the morphology of the spines in WAG/Rij rat's brain. These results indicate that tactile stimulations may exert an important effect on the morphology and density of dendritic spines in the deep layer of SoCx and may affect epileptogenesis through the neuronal plasticity.

Abbreviations

WAG/Rij: Wistar-Albino-Glaxo from Rijswijk
SoCx: Somatosensory cortex
MS: Maternal separation
TS: Tactile stimulation
SWDs: Spike-wave discharges
EEG: Electroencephalogram

Declarations

Ethics Approval

The animal study was reviewed and approved by the Ethics Committee of the University of Kocaeli, Turkey.

Consent for publication

Not applicable; Individual person's data are not presented.

Availability of data and materials

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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