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Daily rhythm of synapse turnover in mouse somatosensory cortex

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The whisker representations in the somatosensory barrel cortex of mice are modulated by sensory inputs associated with animal motor behavior which shows circadian rhythmicity. In a C57/BL mouse strain kept under a light/dark (LD 12:12) regime, we observed daily structural changes in the barrel cortex, correlated with the locomotor activity level. Stereological analysis of serial electron microscopic sections of the barrel cortex of mice sacrificed during their active or rest period, revealed an increase in the total numerical density of synapses and in the density of excitatory synapses located on dendritic spines during the rest, as well as an increase in the density of inhibitory synapses located on double-synapse spines during the active period. This is the first report demonstrating a daily rhythm in remodeling of the mammalian somatosensory cortex, manifested by changes in the density of synapses and dendritic spines. Moreover, we have found that the excitatory and inhibitory synapses are differently regulated during the day/night cycle.

Key words: daily rhythm, locomotor activity, synaptic plasticity, somatosensory cortex, electron microscopy

Synaptic plasticity in the central nervous system can be induced by injury, external sensory stimulation and by learning and memory processes. However, it also occurs spontaneously, for example generated by a circadian clock, and under the influence of cyclic changes of environmental cues, especially the daily changes of light and darkness (Pyza 2002).

The neuronal plasticity generated by a circadian clock has been extensively studied in the visual system of flies (Pyza and Gorska-Andrzejak 2008, Pyza 2010). The rhythmic, daily and circadian changes include the number of synapses (Pyza and Meinertzhagen 1993), the size and morphology of neurons and glial cells (Pyza and Meinertzhagen 1999, Pyza and Gorska-Andrzejak 2004), as well as morphology of dendritic trees and axons (Pyza and Meinertzhagen 1995, Weber et al. 2009).

Neuronal plasticity in mammals can be modulated by the light/dark cycle, as demonstrated in the retina of rats and mice (Behrens et al. 1998, Balkema et al. 2001) and in rat visual and prefrontal cortex (Tsanov et al. 2007, Perez-Cruz et al. 2009). In turn, the rhythmic changes of neuronal morphology or synaptic protein expression have been found to be correlated with locomotor activity (Behrens et al. 1998, Nelson et al. 2004, Perez-Cruz et al. 2009) which shows a species-specific circadian rhythm.

Mice are nocturnal animals; they spend most of their time exploring the environment during the night and sleeping during the day. Therefore, their locomotor and exploring activities are higher during the dark phase of the day/night cycle. During locomotor activity, sensory information flows from the whisker follicles to the barrels in the 4th layer of somatosensory cortex and, as a result, the whisker representations in the somatosensory cortex receive specific information. It induces plastic changes evoked by various environmental stimuli manifested by the alterations of syn-

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apse number, as well as dendritic spine number and morphology (Lendvai et al. 2000, Knott et al. 2002, Spires et al. 2005, Knott and Holtmaat 2008, Jasinska et al. 2006, 2010) . It has been found that even a brief stimulation, transmitted through the whisker-barrel afferent pathway, evokes significant changes at the synaptic level in the somatosensory cortex (Jasinska et al. 2010, 2013).

In the present study we demonstrate for the first time that in the mammalian somatosensory cortex the number of excitatory and inhibitory synapses shows a daily rhythm.

The study was performed on 12 male C57Bl/6 mice aged 4–5 weeks. The experiments were compliant with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Animal Care and Use Committees of the Polish Academy of Sciences and the Jagiellonian University.

The animals were habituated for two weeks under light/dark (LD12:12) regime (12 h of light and 12 h of darkness, light 60 lx, the beginning of light phase was at 08:00 AM and the beginning of darkness at 08:00 PM), at 25°C and 50% humidity. After habituation, all animals were transferred for 10–14 days to a sound-proof insulated recording room and kept under the same temperature, humidity and LD regime in individual metal (wire netting) cages with free access to a running wheel (diameter 20.5 cm) coupled with an IBM PC/AT computer. The running activity of each mouse was continuously recorded using Chronos processing package (Domoslawski 1993). The animals were fed a standard diet and water *ad libitum*.

On the basis of the obtained actograms, only animals showing a distinct daily rhythm of locomotor activity were selected for further experiments. They were sacrificed 1–2 h after the beginning of dark phase in which the mice were highly active (n=5, group ACTIVE) or 1–2 h after the beginning of light phase in which they were resting (n=5, group REST).

The mice were anesthetized with Morbital (25–30 mg/kg b.w.; Biowet, Pulawy, Poland) and perfused through the heart with 20 ml of rinse buffer (0.2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) followed by 100–150 ml of fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brains were removed immediately after perfusion and left in the same fixative for 24 h at 4°C.

After wash in 0.1 M phosphate buffer (pH 7.4), 60 µm tangential vibratome sections of the barrel cortex region were cut and examined under a stereomicroscope (Nikon Optihot, Japan). Sections containing layer 4 of the barrel field cortex were collected for further processing. They were washed again in 0.1 M cacodylate buffer (pH 7.4), postfixed twice with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4 (the first change containing 1.5% potassium ferrocyanide), washed in 70% ethanol containing 1% uranyl acetate, and after dehydration in graded series of ethanol, embedded in Epon (Polysciences, USA) between two silicone-coated glass slides. The embedded slices were photographed at ×5 magnification under Nikon Optiphot equipped with computer-assisted Nikon DXM 1200

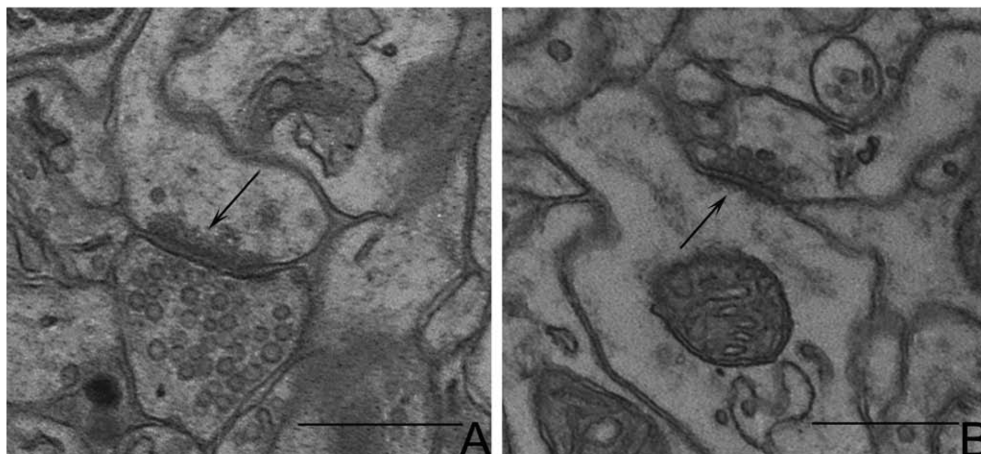

Fig. 1. Representative electron micrographs showing (A) excitatory synapse (arrow) and (B) inhibitory synapse (arrow) in B2 barrel hollow. Scale bars are 0.5 µm.

F digital camera. The images were stacked with the use of the Adobe Photoshop CS (Adobe Systems), and the barrel field was reconstructed. The region of B2 barrel was identified according to the procedure described previously (Jasinska et al. 2010). The embedded slices containing the B2 barrel were then trimmed into blocks.

Series of 3–6 successive sections (65–75 nm thick) were cut from each sample. The sections were collected on formvar-coated copper-palladium slot grids and contrasted with 1% lead citrate. For examination of synapse density, 9–10 series of electron micrographs (3 serial micrographs each) of the B2 barrel central area in which cell bodies are sparse, were taken at 7K under a JEOL 100SX transmission electron microscope at accelerating voltage of 80 kV (JEOL, Japan). The micrographs were aligned using Adobe Photoshop CS software, and stacks of serial images were prepared at a final digital magnification of 30 K.

Synapses were defined according to Knott and colleagues (2002). The assessment of synapse type was based on the symmetry of synaptic membranes (asymmetric/symmetric) and on the appearance of synaptic vesicles (round/ovoidal) (Fig. 1). The accordance of excitatory (asymmetric) and inhibitory (symmetric) synapse morphology with glutamate and GABA synaptic markers was previously verified by immunocytochemistry (Jasińska et al. 2010).

Quantitative analysis of synapses was carried out by employing the stereological dissector method (Sterio 1984). A grid of a two-dimensional sampling frame was placed over the stack of serial sections, using NIH Image J Cell Counter software (http://rsb.info.nih.gov/ij/). The synapses were counted per volume unit (μm^3). Nine to ten dissectors were randomly chosen per barrel, yielding a sampling volume of approx. 100 μm^3 per mouse. Each structure was counted only once in the stack and only structures located fully within the frame or intersecting the left and the upper borderlines of the frame were included (DeFelipe et al. 1999, Tang et al. 2001, 2003). Axo-somatic synapses were omitted from the analysis. Only synaptically connected spines were included. The density of excitatory and inhibitory synapses was calculated according to the stereological formula $N_V = \Sigma Q^- / V$, where ΣQ^- is the number of synapses found in the entire volume V (Fiala and Harris 2001). The counting was done blind – the observer did not know whether the micrographs were taken from the ACTIVE or REST group.

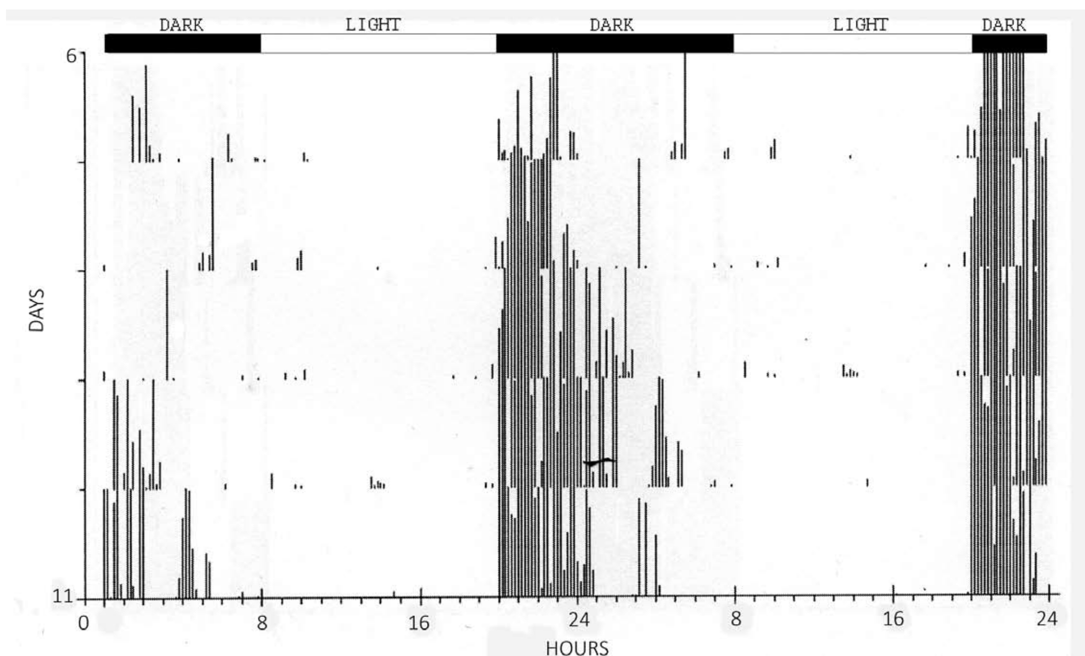

Fig. 2. Double-plotted actogram from day 6 to 11 showing the locomotor activity of a single mouse in LD 12:12 conditions (lights on and off at 08:00 AM and 08:00 PM, respectively). Each long vertical bar on the actogram represents at least one full cycle of the running wheel. The x axis shows the consecutive hours of recording, where 8 – the beginning of the light phase, 20 – the beginning of the dark phase.

All data were analysed using GraphPad Prism 4.0 software (GraphPad Software Inc., USA). To compare synapse density between the groups, unpaired Student's t-test was used after pretesting for normality and homogeneity. If variances were significantly different (Bartlett's test for equal variances), data were analyzed by unpaired Student's *t*-test with Welch's correction.

The synapses were counted in the following total tissue volumes: ACTIVE group – 499.14±14.21 μm^3 (mean volume per animal 99.83±5.86 μm^3); REST group – 527.69±16.03 μm^3 (mean volume per animal 105.54±6.46 μm^3). The sampling volumes were not significantly different across the groups (two-tailed unpaired *t* test, P=0.1815, t_8=1.464).

As revealed by actograms obtained after recording the running-wheel activity, among 12 animals studied 10 showed a distinct daily rhythm of locomotor activity. The pattern of locomotor activity was similar to that already observed in this strain by other authors (Tankersley et al. 2002). The mice were not running inside the running-wheels during the light phase. Sometimes they entered and slightly moved the wheels (small vertical bars on actograms) but their running activity (long, densely distributed vertical bars on actograms) started after the light was switched off (Fig. 2).

The total density of synapses (both excitatory and inhibitory) was by approx. 21% higher in the group REST compared with the group ACTIVE (0.87±0.06/μm^3 vs. 0.71±0.05/μm^3, respectively; unpaired *t*-test, P<0.01, t_8=4.819) (Fig. 3A). The group REST also showed higher density of excitatory synapses (by about 40%) than the group ACTIVE (0.73±0.03/μm^3 vs. 0.52±0.03/μm^3, respectively; two-tailed unpaired *t* test, P<0.0001, t_8=11.56) (Fig. 3B). In contrast, the density of inhibitory synapses was by about 26% higher in the group ACTIVE (0.19±0.02/μm^3 vs. 0.14±0.03/μm^3; two-tailed unpaired *t* test, P<0.05, t_8=2.982, n=10) (Fig. 3C).

The obtained results show that within the B2 barrel of mouse somatosensory cortex, the density of both inhibitory and excitatory synapses located on dendritic spines undergoes cyclic changes under light/dark (LD 12:12) conditions. It has already been demonstrated that the neuronal circuits exhibit structural synaptic plasticity over the course of the day and night in other regions of the mammalian brain: in the retina of rats and mice (Behrens et al. 1998, Balkema et al. 2001), in the suprachiasmatic nucleus (Girardet et al. 2010) and in the infralimbic cortex of rats (Perez-Cruz et al. 2009). This study, however, pro-

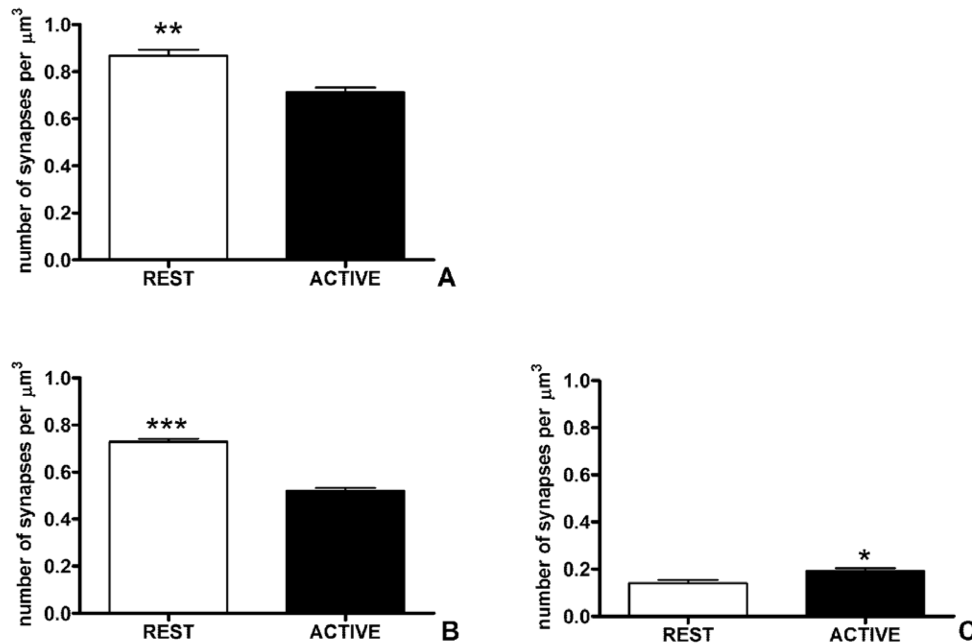

Fig. 3. Numerical density of synapses in B2 barrel: (A) total density of synapses; (B) density of excitatory synapses and (C) density of inhibitory synapses in groups ACTIVE and REST. All graphs show means ± SD (two-tailed unpaired *t*-test, *P<0.05, ** P<0.01, ***P<0.001).

vides the first strong evidence that circadian effects also include a change in the density of synapses in the mammalian somatosensory cortex. Moreover, we have found that the two types of synapses, excitatory and inhibitory, are differently regulated during the day/night cycle.

In the present study, an increase in the total synapse density as well as higher density of excitatory synapses was recorded 1–2 hours after the beginning of the light phase, in which the animals are mostly sleeping and display little or no locomotor activity, whereas higher density of inhibitory synapses was recorded 1–2 hours after the beginning of the dark phase, in which they are highly active.

Our results seem to confirm homeostatic synaptic plasticity hypothesis (Turrigiano and Nelson 2000, Pozo and Goda 2010) which postulates the existence of adaptive compensatory mechanisms regulating the neuronal activity at the synaptic level. Some observations in line with the results of the present study were reported in the barrel cortex of mice. Persistent stimulation of whiskers for 24 h induced a nearly 3-fold increase in the number of inhibitory synapses in layer IV of the corresponding barrel and a decreased responsiveness of its neurons to whisker deflection (Knott et al. 2002). Conversely, tactile sensory deprivation caused by whisker trimming lasting one week resulted in upregulation of the excitatory synapses and downregulation of the inhibitory ones as well as in an increase in the excitatory synaptic transmission, enhancing the sensitivity of the barrel cortex to sensory inputs (Zhang et al. 2013).

Although such compensatory synaptic remodeling was observed after sensory input changes lasting 24 hours or one week, we cannot exclude that it might represent common plastic responses of the somatosensory cortex to an increase or decrease in the sensory inputs and that such responses could also occur in the activity/rest phases of the diurnal cycle. Upregulation of inhibitory synapses during the activity phase may represent a compensatory reaction to excessive sensory stimulation in the running wheel. In turn, upregulation of excitatory synapses increases the sensitivity of the barrel cortex during sleep and promotes animal's reaction to very weak stimuli. It can be speculated that such increased sensitivity to weak stimuli facilitates reaction of a sleeping animal to possible danger.

It is not clear whether the observed synaptic plasticity in the barrel cortex directly reflects the rhythmic changes of the sensory input associated with the rhythm of locomotor activity or whether it is also influenced by other factors such as light or the endogenous circadian clock. Rhythmic neuronal plasticity can also be controlled by a sleep-dependent mechanism. However, an increase in total synapse density in the rest phase and a decrease in the activity phase observed in this study is not consistent with the synaptic homeostasis hypothesis of sleep, which postulates synaptic upscaling during wake period and downscaling during sleep, as demonstrated in several cortical circuits (Tononi and Cirelli 2006).

It should be, however, emphasized that the balance between the circadian and homeostatic regulation of rhythmic structural synaptic plasticity significantly varies among the circuits, depending on their functions (Elbaz et al. 2013) and the studies showing circadian or wake/sleep-dependent neuronal plasticity in mammals did not include the quantification of synapse density. Only one study on the mammalian somatosensory cortex (Yang and Gan 2012) could be regarded as relevant to our approach in terms of dendritic spine density assessment: the authors demonstrated higher elimination rate of dendritic spines during sleep. However, they investigated layer 5 pyramidal neurons, a selected population of barrel cortex cells and assessed the number of all spines, including those not associated with the synapses, whereas our study demonstrates total synapse and synapse-associated spine density in layer 4. Moreover, in contrast to mice used in the present study (8–9 weeks old at the end of experiment), the mice studied by Yang and Gan (2012) were only 3 weeks old, an age characterized by a progressive elimination of dendritic spines in the cerebral cortex (Zuo et al. 2005). Hence, the results reported by Yang and Gan (2012) cannot be directly compared with those of the present study.

In conclusion, this study has demonstrated that the daily changes in the locomotor activity are associated with rhythmic modifications of the excitatory and inhibitory synapse density in the mammalian somatosensory cortex.

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