The impact of chronic stress on limbic brain regions in rats: experimental quantitative electron microscopic investigations

Theses for Doctoral (PhD) Dissertation

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Pécs
2023.

1. Introduction

The main purpose of stress response to environmental and internal stimuli is to maintain homeostasis, protect the body from negative stimuli and to avoid and compensate similar future exposures more effectively. Chronic stress can cause abnormal physiological changes in various organ systems, including the central nervous system, which may play a role in the development of a variety of mental disorders.

Through several genetic factors, chronic stress contributes to the development of Major Depressive Disorder (MDD), a major social, economic and health burden globally (Flint & Kendler, 2014; Shadrina et al., 2018). Alterations in certain brain regions, including the prefrontal cortex (PFC) and hippocampus (HC), are known to be involved in the development of MDD (Duman & Aghajanian, 2012b; Phillips et al., 2015). Chronic stress causes disturbances in the HPA axis (hypothalamic-pituitary-adrenocortical axis), which may also contribute to the development of MDD (Jacobson, 2014; Ross et al., 2017).

Certain brain regions have a more pronounced role in stress and emotion regulation - including the prefrontal cortex, amygdala and hippocampus, in which stress causes functional and morphological changes (Leuner & Shors, 2013). In chronic mild stress (CMS), the cytoarchitecture of the PFC is altered, dendritic complexity is reduced, and thus synaptic connectivity is decreased (Kolb & Gibb, 2015). The density of certain hippocampal GABAergic perisomatic inhibitory interneuron populations (parvalbumin positive basket cells, PV+) is also lowered after CMS (Czéh et al., 2005, 2015).

CMS, one of the best validated animal models of MDD, induces a systemic stress response in the HPA axis. The severity of anhedonia - the reduced response to reward and pleasure, also a major symptom in MDD - is easily measured in sensitive experimental animals exposed to stress (Willner, 2017). During CMS, the activity of the HPA axis and the intensity of the stress response can be maintained by continuously varying microstressors (Radley & Sawchenko, 2015).

The regions that regulate physiological and cognitive functions in coordination with the environment, and orchestrate visceral processes with behavioural mechanisms, are collectively referred to as the limbic system (Mesulam, 2000). The

main targets of limbic system projections are the prefrontal cortex, hypothalamus, hippocampus and cortical regions involved in the control of behavioural and motor responses (Herman et al., 2005).

The PFC is also connected to other brain centres that control emotions, memory and cognitive processes. One of the most important functions of the infralimbic cortex (mPFC IL) is to coordinate responses to chronic stress (Flak et al., 2012). The arborization of mPFC neurons is reduced following stress (Cook & Wellman, 2004; Liston et al., 2006; Liu & Aghajanian, 2008; Radley et al., 2004, 2006), and thus based on the dendritic tree rearrangement, an alteration in the number of synapses in the region might be expected.

The hippocampus is an essential brain region for spatial orientation, learning and cognitive function (Sigurdsson & Duvarci, 2016) which is negatively affected by stress (Lucassen et al., 2014; McEwen et al., 2015). CMS causes malformation of neuronal function and structure, mainly manifested by shrinkage of the dendritic tree of HC neurons, reduction of neuronal complexity and reduction of total HC volume (Bessa et al., 2009). It is known from immunohistochemical studies that synaptic connections on the soma of HC pyramidal cells are exclusively project GABAergic axosomatic inhibition (Megías et al., 2001). GABAergic inhibition plays a critical role in regulating the firing pattern of HC pyramidal cells (Buzsáki & Wang, 2012; Wang, 2010), with a significant proportion of this firing pattern being provided by the parvalbumin positive (PV+) GABAergic interneurons of the HC (Freund & Buzsáki, 1996; Markram et al., 2004; Ribak et al., 1990). Chronic stress can affect the function of GABAergic interneurons and their neuronal inhibition, thus altering the systemic function of the limbic system, especially the hippocampus.

The dedicated zone of communication between neurons is the synapse. Reduced synaptic plasticity can contribute to functional impairment and loss of normal control of mood and emotion in depression (Liang et al., 2019). Electron microscopic studies have shown that chronic stress can lead to loss, rearrangement and structural abnormalities of synapses in the hippocampus (Magariños et al., 1997).

Recent theories have suggested that mitochondria (MIT) represent a key role in the stress response, and both their structure and network are damaged by chronic stress (Allen et al., 2021; Picard et al., 2018). Mitochondrial dysfunction may adversely affect synaptic communication, plasticity and survival and may be involved in neurodegenerative processes (Fischer et al., 2018). One can hypothesize that

ultrastructural alterations in MIT may also play a role in the appearance of behavioural changes in chronic stress and MDD.

2. Aims and goals

In our research we performed electron microscopic investigations to confirm changes in brain regions sensitive to CMS (HC CA1, mPFC IL). We examined putative changes in the density and structure of synaptic connections and compared our results with observations from previous studies. We also aimed to analyse the number and morphology of mitochondria in neurons of the mPFC IL after CMS.

In addition to comparing the control and treated groups, we also intended to report control values that may provide new information for a more detailed understanding of the anatomy of the healthy rat brain.

Our specific objectives were the following:

1. **We determined whether CMS-induced alterations in the IL area of the mPFC is also present in the number of synapses.**
2. **Following CMS treatment, we investigated the presumed difference in morphology of mPFC IL neuropil axo-spinous synapses between control and anhedonic groups.**
3. **We investigated whether CMS causes alterations in mitochondrial numbers and morphology in the neuropil of mPFC IL cortex.**
4. **We examined whether the reduction in PV+ neuron number induced by CMS is accompanied by a decrease in the number of perisomatic inhibitory synapses formed on HC CA1 pyramidal cells.**
5. **We determined whether the reduction in the number of HC CA1 PV+ cells in the CMS-exposed group is accompanied by morphological alterations of the terminals and synapses.**

3. Materials and methods

3.1 Animals

The CMS paradigm was carried out and the brains of the animals were extracted by Professor Ove Wiborg's lab at Aarhus University in Denmark. Adult male Wistar rats (Taconic, Denmark) were used for our studies. The control group included n = 4 rats, while the stressed group included n = 4 animals. Animals were given *ad libitum*

access to food and water. Diet and circadian rhythm were varied only for the stressed group according to the CMS protocol.

3.2 Animal model for depression

The CMS protocol induces an anhedonic state in stress-sensitive treated rats. Animals were divided into two homologous groups based on their baseline sucrose intake, and then treated rats were exposed to microstressors for 9 weeks. The control group was kept undisturbed under standard conditions. The stress procedure consisted of seven different stressors: one period of alternating lighting, strobe, paired housing, food or water deprivation, two periods of damp bedding and three periods of 45º tilted cage.

The behaviour of the animals was categorised by the sucrose consumption test (SCT). Susceptible animals to CMS showed a decrease in sucrose intake compared to baseline, indicating anhedonic behaviour similar to depression. For our study, we used highly anhedonic animals along with the controls.

3.3 Perfusion, Brain Tissue Preparation and Immunohistochemistry

Animals were anesthetized with sodium pentobarbital (200 mg/ml dissolved in 10% ethanol), followed by transcardiac perfusion with ice-cold 0.9% saline and 4% paraformaldehyde (PFA) containing 0.2% glutaraldehyde dissolved in 0.1 M phosphate buffer (PB, pH 7.4). Brains were postfixed overnight at 4°C in PFA without glutaraldehyde. Along the septo-temporal axis, 80 μm thick coronal serial sections of the whole PFC and hippocampal formation were obtained using a vibratome. Two IL-containing sections from each animal were selected from Bregma 3.20 to 2.20 and dehydrated in a series of rising alcohols (70% ethanol containing 1% uranyl acetate) after osmification (1% OsO4 dissolved in PB for 30 min). After complete dehydration, the sections were incubated in propylene oxide followed by incubation in a mixture of propylene oxide and Durcupan resin, and the selected sections were flat-embedded in Durcupan resin (Fluka-Sigma-Aldrich, Hungary). The polymerized (56°C, 48 h) samples were verified under light microscopy and the region of interest (ROI) was selected for re-embedding and subsequent ultrathin sectioning. Identification of IL regions was performed on toluidine stained semi-thin (500 nm) sections. Ultrathin (60 nm) sections were prepared with a Leica Ultracut UCT microtome and collected on Formvar-coated single-slot copper grids. The samples were contrasted with uranyl

acetate (Electron Microscopy Sciences, USA) and lead citrate (Fluka-Sigma-Aldrich, Hungary).

Anti-PV immunohistochemical labelling was applied on HC sections (Czéh et al., 2015). To summarize the protocol: endogenous peroxidase inhibition with 1% H2O2 pre-treatment; aspecific binding site blocking with 5% normal goat serum (Vector Laboratories); overnight incubation with monoclonal anti-parvalbumin antibody (1:10 000, Swant, Cat #: 235). The antigen-antibody reaction was performed by incubation with biotinylated anti-mouse secondary antibody (1:200; Vector Laboratories) followed by chromogenesis with avidin-biotin-horse-radish peroxidase (1:200; Vectastaine Elite ABC Kit, Vector) and DAB (1:200; DAB Peroxidase Substrate Kit, Vector). Sections from the anhedonic and control groups were incubated simultaneously, eliminating the possibility of false deviations during the procedure and visualization.

For electron microscopy samples, the Triton X-100 was replaced by the freeze-thaw technique, as the first one would lead to a strong degradation of the ultrastructure. After immunolabelling, the relevant CA1 regions were selected following the general EM sample preparation and embedding described above, then sections were cut and contrasted as explained. Imaging was performed using a JEOL 1200 EX-II transmission electron microscope (TEM).

3.4 Quantification of Neurons and Neuronal Structures

Samples were given a random code to avoid examiner bias. For the mPFC IL and HC CA1, a total of almost 8000 high-resolution images, 1000 pyramidal cells, 25000 synaptic terminals and 65000 mitochondria were analysed for quantification and morphological analysis.

Density of PV+ interneurons in the HC was determined by random systemic light microscopy. Sections selected from a series of 80 μm thick sections covering the entire dorsal hippocampus spaced ~400 μm apart (n = 8-10) were labelled with anti-PV immunohistochemistry. PV+ interneurons were counted separately for each HC subregion (gyrus dentatus, CA1-3). Cells were examined and quantified at 200× magnification after manual outlining of subregion contours and layers.

We investigated perisomatic inhibitory synapses in the dorsal region of HC CA1, exclusively in the *stratum pyramidale*. At approximately 20000× magnification, we identified axosomatic synapses of the region. For quantification, images were

evaluated at magnification 5000×-50000×. We determined the perimeter of the somata, synaptic membrane length, number of active zones, and the length of axosomatic terminals contacting the cell membranes.

Random systemic sampling was carried out for the mPFC IL region during brain sectioning and EM imaging. From the ultrathin sections, at least 5-5 evenly spaced sections at 5-6 μm apart were selected for further analysis for each animal. 10 ultrathin sections were analysed by TEM with at least 10 non-overlapping EM images for each cortical layer in every animal. Thus, a total of 400-500 EM images were analysed. The cortical layers (I-VI) of the study region were defined based on the anatomical study of Gabbott et al (1997).

Synapses and mitochondria were counted and measured at 40000× magnification using an "unbiased" counting frame of ~15 μm2 (3.87 ×3.87 μm). In the IL region, symmetric (type I) synapses were counted and measured separately from asymmetric (type II) synapses.

All synapses were identified and typed based on their synaptic density, synaptic vesicle morphology and synaptic gap. We also determined the length of the synaptic densities formed by the interconnected membrane densities and the length of the associated terminal membrane.

Morphological examination of the mitochondria was performed in every fifth EM image. Several morphological parameters were examined from the contours of the MIT cross sections using the evaluation software.

We also determined the total number and density of myelinated axons in different cortical layers. The values obtained in this study were expressed in density and the number of synapses and mitochondria (volume × density = total number of structures) for the total volume of the region.

In our studies, we searched for signs of neuronal degeneration or cell death, such as condensation of chromatin and fragmentation of the nucleus, or swelling and disintegration of mitochondria, in both the hippocampus and prefrontal regions of CMS-treated animals.

3.5 Volumetric measurements

The volume of infralimbic regions was evaluated according to the Cavalieri principle (Gundersen et al., 1988). The entire IL cortex was coronally sliced into 80 μm thick sections. In every third (n=5-6) section, the entire IL cortex was contoured

with software and the cross-sectional areas of cortical layers I, II and III-VI were separated based on the publication of Gabbott et al (1997). Since deeper (III-VI) layers could not be separated macroscopically with sufficient precision, they were grouped together for volumetric measurements. The volume was determined by multiplying the cross-sectional area of the measured regions by the section thickness.

3.6 Statistical analysis, software applications

HC CA1 pyramidal cell perimeters and morphological characteristics of the synapses were obtained by manual measurement using the Olympus iTEM software.

Numerical and morphological measurements of the mPFC IL region were also performed manually using the Neurolucida software (Version 11.08.2, MicroBrightField, Williston, VT, USA).

Statistical analysis of the data and graphical presentation of the results were performed using GraphPad Prism v7.00 and Microsoft® 365 Word and Excel software. The sucrose consumption data were analysed and evaluated by two-way analysis of variance (ANOVA, stress × time) followed by Bonferroni *post hoc* test. Two-tailed unpaired *t*-test was used for group-level comparisons of the measurements. Group-level comparisons of synaptic profiles and mitochondrial parameters were performed using a two-tailed unpaired Student's *t*-test, and the distribution of structures across cortical layers was analysed by two-way analysis of variance (ANOVA, stress × cortical layer) followed by Sidak's *post hoc* test. The results are presented as mean ± S.E.M. (standard error of the mean). Significant differences are indicated by * for probability $p \leq 0.05$ and ** for probability $p \leq 0.01$

4. Results

4.1 Effects of Chronic Mild Stress on the behaviour of experimental rats

For quantifying the anhedonia induced by CMS, the sucrose consumption test was used. Nine weeks of CMS significantly reduced the animals' sucrose consumption, even in the weeks prior to the termination of the full protocol.

4.2 Quantitative EM determination of IL cortical synapses and cortical distribution of synapses

The average values for the two groups and results of the comparisons are shown in Table 1.

Table 1: Group averages for mPFC IL quantitative EM analysis

	Control group (n = 4)	Stress group (n = 4)	Results of statistics
Total number of areas examined	1859	1672	-
Total number of axon terminals analysed	11755	8897	-
Total synaptic density (n/μm^3)	5.97 ± 0.27	5.91 ± 0.26	n.s.
Total number of asymmetric synapses	(1.15 ± 0.04) × 10^9	(0.98 ± 0.04) × 10^9	***t* = 2.84, *P* < 0.05**
Asymmetric synaptic density (n/μm^3)	5.46 ± 0.22	5.44 ± 0.25	n.s.
Total number of symmetric synapses	(1.06 ± 0.11) × 10^8	(0.83 ± 0.06) × 10^8	***t* = 1.77, *P* = 0.06**
Symmetric synaptic density (n/μm^3)	0.50 ± 0.06	0.46 ± 0.04	n.s.
Terminal membrane length (nm)	488.68 ± 11.20	482.07 ± 12.55	n.s.
Synaptic membrane length (nm)	207.17 ± 1.74	212.21 ± 3.40	n.s.
Total number of myelinated axons	(1.00 ± 0.10) × 10^8	(0.68 ± 0.04) × 10^8	***t* = 2.63, *P* < 0.05**
Density of myelinated axons (n/μm^3)	0.47 ± 0.05	0.38 ± 0.03	n.s.

Data represent the mean ± S.E.M.
n.s.: non-significant difference

The density of type I synapses in the cortical layers decreased towards the deeper layers, but there was no significant difference between the groups (Figure 1.).

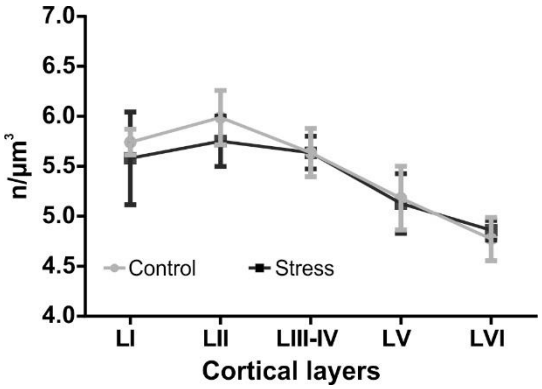

Figure 1: synapses by cortical layers. Both groups show a significant decrease of 10-20% towards the deeper layers (two-way ANOVA, $F_{(4,30)} = 5.29$, $P < 0.005$).

In contrast, the density value of inhibitory synapses showed a homogeneous distribution in each layer (Figure 2).

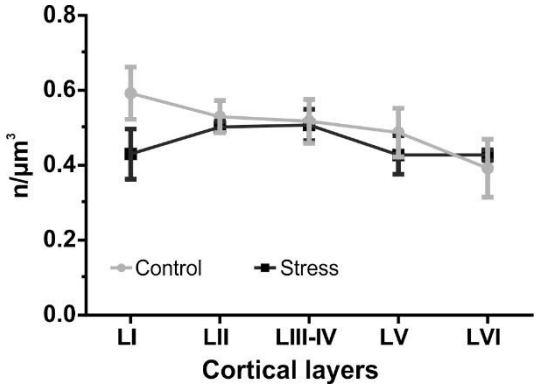

Figure 2: . No significant difference is found between layers and groups according to the two-way ANOVA.

The average membrane length of cortical synaptic terminals in the mPFC IL of control rats was 488.7 ± 13.5 nm (Figure 3.), and the length of synaptic contacts was 207.2 ± 1.8 nm. In stressed animals, the synaptic densities formed on the terminals were larger for all layers (Figure 4.).

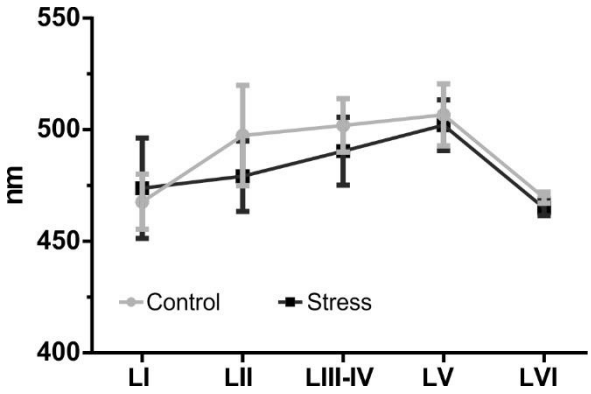

Figure 3: of axon terminals is homogeneous in each cortical layer. Two-way ANOVA found no difference between cortical layers and the two groups.

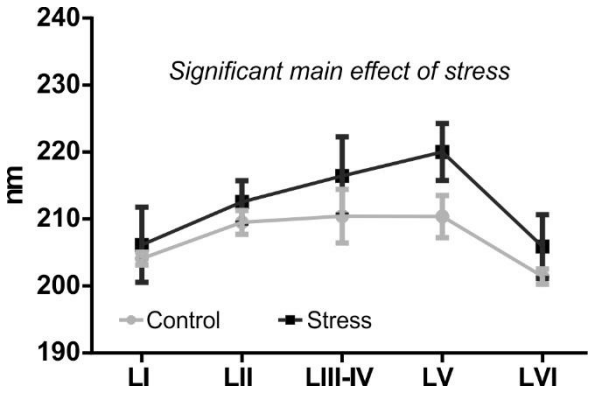

Figure 4: The membrane length distribution of synaptic densities differs between cortical layers (two-way ANOVA, $F_{(4,30)} = 3.51$, $P < 0.05$) and there is a significant difference between groups (two-way ANOVA, $F_{(1,30)} = 4.32$, $P < 0.05$).

The ratio of synaptic densities to membrane lengths was 52.7 ± 0.9 %, and an average of 116.3 ± 2.0 synapses per 100 axon terminals was counted. The values obtained did not differ between the groups.

Myelinated axon density was highest in the deeper layers, especially in layer VI, where it was three times the average value of 0.47 ± 0.05 / µm^3 (Figure 5).

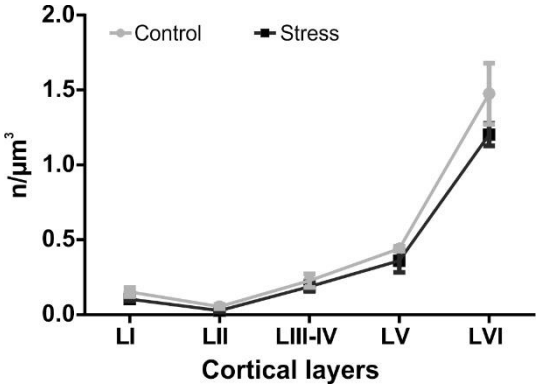

Figure 5: The distribution of myelinated axon densities in each cortical layer was significantly different according to two-way ANOVA ($F_{(4,30)}$ = 97,1, $P < 0.0001$), whereas no difference was found between groups.

4.3 Impact of CMS on IL volume

The volume of the infralimbic region was significantly reduced in anhedonic animals (Figure 6).

Figure 6: Group values for total mPFC IL region volume. (*t* test, $t = 3.57$, ** $P < 0.01$).

The volume of cortical layers was altered in case of both stress and cortical layers. The deeper layers (III-VI) were the most affected by stress (Figure 7).

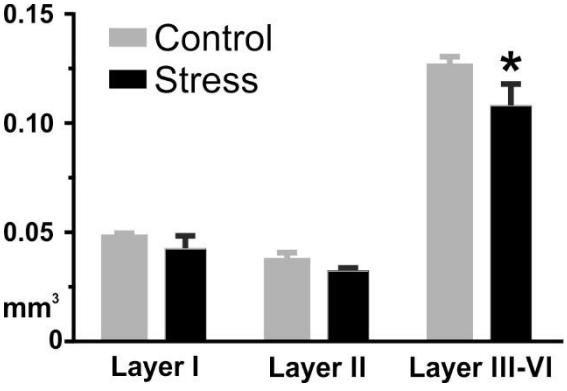

Figure 7: Group values of mPFC IL volume by cortical layers. Two-way ANOVA showed a significant difference between groups ($F_{(1,18)}$ = 17.75, $P < 0.001$; *post hoc* analysis, * $P < 0.001$) and layers ($F_{(2,18)}$ = 575.6, $P < 0.001$).

4.4 Effect of stress on the number of IL cortical synapses

We determined the total number of synapses in the region, which was significantly reduced by chronic stress (Figure 8).

Figure 8: Estimated number of type I excitatory synapses in IL cortex. (*t* test*:* $t = 2.84$, * $P < 0.05$).

In the case of symmetric synapses, the statistical analysis indicates no significant reduction (Figure 9).

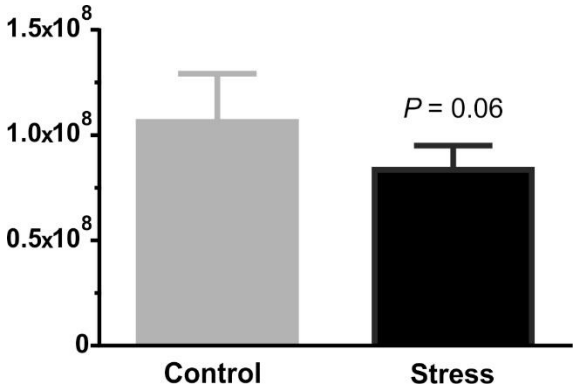

Figure 9: Estimated total number of type II inhibitory contacts in IL cortex. (*t* test: $t = 1.77$, $P = 0.06$).

The cortical distribution of type I synapses varied for both stress and layers. The decrease was most significant in cortical layers III-VI (Figure 10).

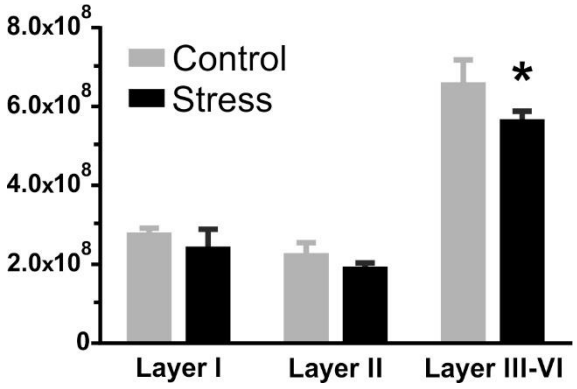

Figure 10: The number of asymmetric synapses differs between groups (two-way ANOVA, $F_{(1,18)} = 11.63$, $P < 0.01$; *post hoc* analysis, * $P < 0.05$) and in each cortical layer (two-way ANOVA, $F_{(2,18)} = 252.6$, $P < 0.0001$).

The number of type II synapses varied for both stress and cortical layers (Figure 11).

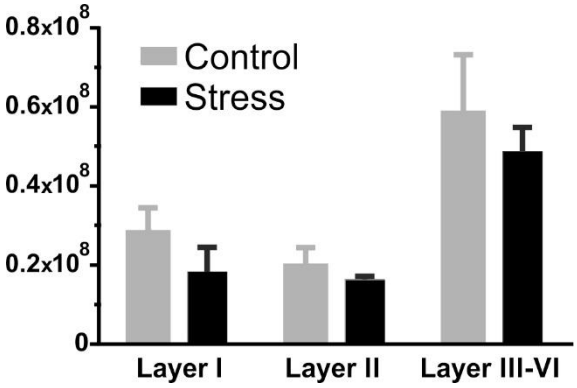

Figure 11: The number of type II inhibitory synapses differs between the two groups (two-way ANOVA, $F_{(1,18)} = 5.82$, $P < 0.05$) and in each cortical layer (two-way ANOVA, ($F_{(2,18)} = 49.5$, $P < 0.0001$).

4.5 Effect of CMS on mitochondria of the IL cortex

The numerical and morphological properties of mPFC IL mitochondria are shown in Table 2. The total number of mitochondria was significantly reduced by stress (Figure 12).

Table 2: Group results of mPFC IL EM analysis of mitochondria

	Control group (n = 4)	Stress group (n = 4)	Results of statistics
Density (n/μm^3)	16.43 ± 0.89	14.31 ± 1.55	n.s.
Total number (#)	(3.47 ± 0.19) × 10^9	(2.70 ± 0.24) × 10^9	***t* = 2.50, *P* < 0.05**
Perimeter (nm)	929.70 ± 31.17	977.90 ± 31.49	n.s.
Area (μm^3)	0.06 ± 0.01	0.07 ± 0.01	n.s.
Feret Min	186.30 ± 8.21	203.80 ± 17.90	n.s.
Feret Max	352.80 ± 10.03	365.00 ± 15.39	n.s.
Flatness	1.89 ± 0.02	1.83 ± 0.07	n.s.
Compactness	0.75	0.76 ± 0.01	n.s.
Form factor	0.83	0.85 ± 0.01	n.s.
Convexity	0.998	0.999	n.s.

The values represent the mean ± S.E.M.
n.s.: non-significant difference

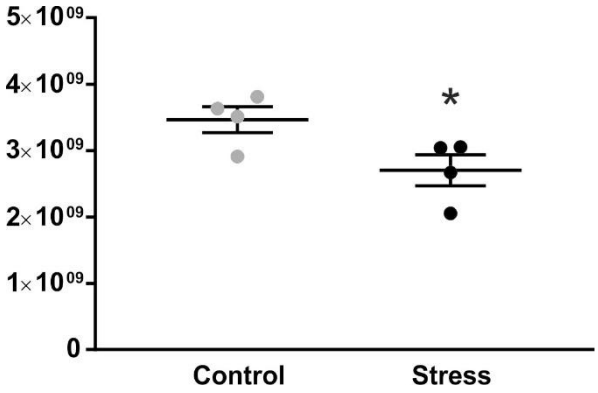

Figure 12: Mitochondrial numbers in the IL region were significantly reduced by CMS compared to the control group (*t* test $t = 2.49$, * $P < 0.05$).

The density of mitochondria is homogeneous between the layers, with only a slight trend towards a decrease (Figure 13). However, when comparing the total mitochondrial counts, a clear difference was observed (Figure 14).

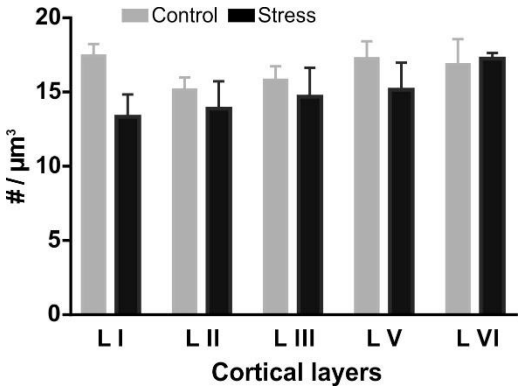

Figure 13: Values of mitochondrial density per cortical layer. Only a trend-like, negligible decrease is seen for the stressed group.

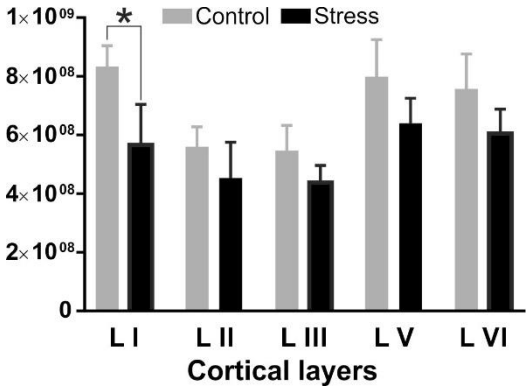

Figure 14: Total number of mitochondria in the IL region of the mPFC in separate cortical layers. There are differences between strata (two-way ANOVA, $F_{(4,30)} = 40.23$, $P < 0.0001$) and between groups (two-way ANOVA, $F_{(1,30)} = 25.59$, $P < 0.0001$; *post hoc* analysis, * $P < 0{,}005$).

4.6 Effect of CMS on myelinated axons in the IL cortex

The number of myelinated axons was significantly reduced by CMS (Figure 15).

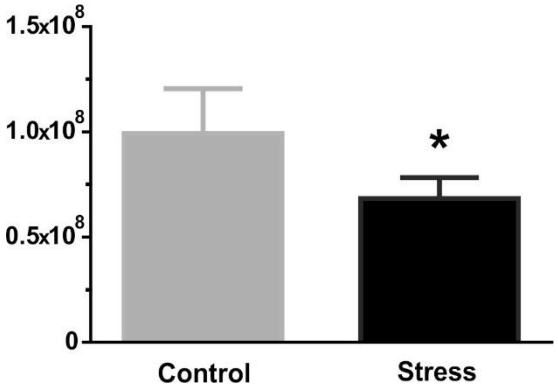

Figure 15: Stress significantly reduced the number of myelinated axons in IL cortex by 30% (*t* test, $t = 2.63$ * $P < 0.05$).

The number of myelinated axons per different cortical layers differed between the two groups for both stress and layers, decreasing mainly in the deeper (layers III-VI) layers (Figure 16).

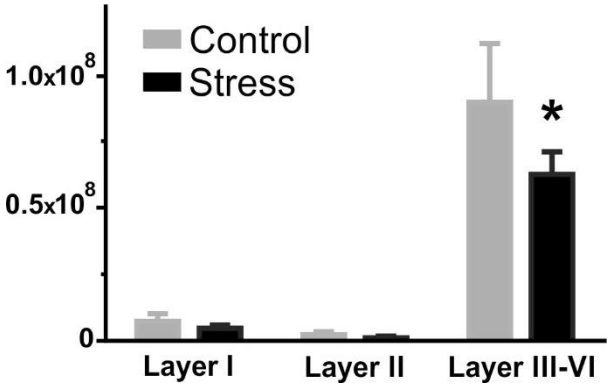

Figure 16: The number of myelinated axons differs between groups (two-way ANOVA, $F_{(1,18)} = 6.71$, $P = 0.01$) and between cortical layers (two-way ANOVA, $F_{(2,18)} = 147.6$, $P < 0.0001$; *post hoc* analysis * $P < 0.05$).

4.7 Light microscopic analysis of hippocampal CA1 parvalbumin-positive cells

We confirmed that CMS significantly reduced the number of PV+ interneurons in each subregion of the dorsal HC (Figure 17).

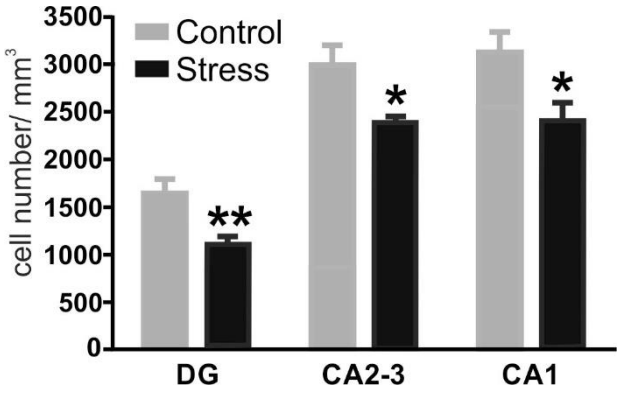

Figure 17: Changes in the number of PV+ interneurons in each hippocampal subregion. (*t* test, * $P < 0.05$, ** $P < 0.01$).

4.8 EM analysis of hippocampal CA1 axosomatic synapses

The CMS protocol had no significant effect on the synaptic parameters of the

treated group. Control values and data for the stressed group are shown in Table 3.

Table 3: Group results of quantitative EM analysis of HC CA1

	Control group (n = 5)	Stress group (n = 4)
Number of pyramidal cells analysed	118 ± 14.74 (Σ 591)	128 ± 11.17 (Σ 510)
Number of terminals analysed	507 ± 94.87 (Σ 2536)	577 ± 72.22 (Σ 2307)
Terminal / some cross-section (#)	4.19 ± 0.21	4.49 ± 0.20
Terminal / 100 µm cell membrane (#)	10.12 ± 0.51	10.54 ± 0.29
Terminal / soma surface (#)	56.61 ± 4.31	62.29 ± 6.84
Average soma perimeter (µm)	41.76 ± 1.86	42.80 ± 2.88
Synapse / terminal ratio (%)	32.13 ± 0.71	33.56 ± 1.68
Synapse per 100 terminals (#)	119.57 ± 2.16	119.48 ± 2.82
Average terminal membrane length (nm)	920.13 ± 24.50	898.35 ± 33.59

Data represent the mean ± S.E. M. values.

4.9 Neuronal degeneration and cell death

During electron microscopic analysis, we looked for tissue lesions indicative of possible neuronal degeneration and/or cell death, all of which could be easily identified even on low magnification EM images. The neural tissue examined in the IL region of the mPFC and in the CA1 pyramidal cell layer of the HC appeared healthy, with no evidence of neuronal degeneration or cell death.

5. Discussion

In summary, we are the first to demonstrate CMS-induced reductions in synapse and mitochondrial number in the medial prefrontal cortex of rats. Consistent with other studies, we found indirect evidence for stress-induced dendritic atrophy of pyramidal cells in layers II-III of IL cortex (Goldwater et al., 2009; Izquierdo et al., 2006; Martin & Wellman, 2011; Perez-Cruz et al., 2007, 2009; Radley et al., 2004). A reduction in synapse number in the neuropil may also indicate axon reduction and functional dysfunction may be induced via this degeneration (Menon, 2011).

To understand the pathomechanisms of the healthy and dysfunctional brain, it is necessary to assess the morphology and number of synapses. In the hippocampus of stressed animals, the connectivity of excitatory synapses is reorganized (Donohue et al., 2006; Hajszan et al., 2009; Magariños et al., 1997; Sandi et al., 2003; Stewart et al., 2005) and a similar change is hypothesized in the prefrontal cortex (Musazzi et al.,

2015).

The morphological alterations we found may be due to molecular processes that cause changes in signal transduction in the prefrontal cortex of stressed animals. Down-regulation of synaptic signalling mediated by AMPA, and NMDA receptors may also lead to a decrease in glutamate receptor expression in the mPFC of stressed animals, thus causing cognitive impairment (Yuen et al., 2012). Among the factors involved in the pathophysiology of MDD, one of the most significant, according to the neuroplasticity-based theory of MDD, is the structural and functional alteration of excitatory synapses (Duman, 2014; Duman et al., 2016; Duman & Aghajanian, 2012a; Licznerski & Duman, 2013; Popoli et al., 2011; Thompson et al., 2015; Timmermans et al., 2013). Thus, it is hypothesized that alterations in the synaptic system negatively affect the limbic system and lead to cognitive impairment characteristic of depression.

Total synapse number was significantly reduced in the IL region of the mPFC of anhedonic rats. Significant increases were also found in the synaptic membrane length of animals subjected to CMS. The observed alteration is presumably a compensatory mechanism counteracting the effects of the reduction in synapse number.

The deep layers occupied 65% of the total cortical volume, so although all cortical layers shrank under stress, this was most pronounced in the combined group of layers III-VI. CMS caused a 15-20% reduction in the number of IL cortical synapses in anhedonic animals, mostly in the asymmetric synapses of layers III-VI. The number of myelinated axons also decreased in the deeper layers. Our observations of a CMS-induced reduction in synapse number in the deep layers of the mPFC are in agreement with the results of previous studies that also demonstrated atrophy of the dendritic tree of neurons in this region (Perez-Cruz et al., 2007; Shansky et al., 2009).

For mPFC IL mitochondria, we also found that the total number of organelles was significantly reduced by CMS (Figure 12). Although the MIT density of anhedonic animals was homogeneous (Figure 13), the stress-induced decline of number was most pronounced in layer I (Figure 14). There was no difference in MIT morphology between the groups. One of the explanations for the observed differences may be the volume reduction resulting from neuronal death, which ultimately induces a reduction in the number of mitochondria. Glucocorticoid hormones, which are also involved in the stress response, can also induce apoptosis by binding to MIT glucocorticoid receptors (Sionov et al., 2006). Li and his team found that stress exposure causes morphological changes in mitochondria (Li et al., 2010). Another

theory is that neuronal dendrites shrink, and their complexity is reduced, ultimately leading to neuropil shrinkage. The significant reduction in mitochondrial number in cortical layer I may be a consequence of stress-induced shrinkage of the apical dendritic tree and loss of axons (Perez-Cruz et al., 2009; Wellman et al., 2020).

Thus, we can say that CMS-induced reduction of the dendritic tree and degeneration of axons together cause a volume shrinkage in the cortical layers, and presumably result in a loss of asymmetric synapses, myelinated axons and mitochondria in the IL region. Thus, the alteration may lead to a variety of cognitive dysfunctions without apoptotic processes in the neurons of the frontal cortex.

No differences were found between groups in the number and morphology of perisomatic synapses projecting to the pyramidal cells of the HC CA1 *stratum pyramidale*, even though CMS reduced the density of PV+ interneurons in the hippocampus of stressed animals (Figure 17). One possible explanation for our results is that axo-somatic synapses are more conserved structures compared to the more plastic axo-spinous synapses, and thus their rearrangement does not occur or occurs more slowly.

The reduction in the number of HC PV+ interneurons in stressed rats may be explained by a stress-induced neurotoxic environment or by a reduction in the expression of intracellular PV by neurons below detectable levels. Since the parameters tested were similar in both the control and stressed groups, we can conclude that instead of the destruction of PV+ interneurons, the concentration of proteins expressed by them, including parvalbumin, is altered in the treated group. A decrease in PV content has also been described in other diseases affecting the central nervous system- e.g. in animal models of epilepsy, PV positivity of basket cells may disappear completely in HC CA1 (Scotti et al., 1997a, 1997b). It is known that during chronic stress, excessive excitation of PV+ interneurons disrupts their normal calcium buffer system (Hu et al., 2010). It is plausible that the persistent elevation of Ca^{2+}-concentration during stress disrupts PV+ neurons, thereby altering their integrity, structure and function. Disruption of PV+ basket cells may also negatively affect GABA-regulated rhythmic oscillations in the hippocampus.

We have also documented the number and morphological parameters of axosomatic terminals and synapses in the CA1 region of HC in control rats. Our findings are consistent with studies quantifying inhibitory synapses in the rodent hippocampus (Megías et al., 2001; Takács et al., 2015).

In our research, we did not find any evidence of neuronal degeneration and neuronal death in either the IL cortex of the mPFC or the CA1 region of the HC in stressed animals. However, we should also highlight that the histopathological evaluation was performed after sacrificing the animals, so that any cell death that might have occurred during the chronic stress protocol could have been without any trace, since apoptotic events are rapid processes.

6. Summary of the new findings

New findings from our research on which this dissertation is based include:

1. We have shown that CMS reduces the number of axo-spinous synapses in the infralimbic region of the medial prefrontal cortex in rats.
2. We proved that the length of the active zone of axo-spinous synapses increases in the treated group after CMS.
3. We documented that CMS exposure causes a decrease in the number of mitochondria in the infralimbic region, whose morphology, in contrast, remains unchanged.
4. We have described that after CMS exposure, the density of perisomatic inhibitory synapses formed on pyramidal cells of the CA1 region in the hippocampus of anhedonic rats is not altered.
5. We have shown that the changes observed in the hippocampal CA1 region of the treated group are not reflected in the morphology of the terminals and synaptic connections.
6. Finally, we have documented ultrastructural synaptic and mitochondrial control data for the mPFC IL and HC CA1 that may help guide future translational research.

7. Acknowledgements

I am grateful to my supervisor, research group leader, mentor, professional and personal example Prof. Dr. Boldizsár Czéh for his tireless, altruistic and always wise guidance and exceptional support during my PhD and the years before.

I would like to thank Prof. Dr. László Seress for his inspiring help, for sharing his professional skills and for his inspiring ideas and thoughts.

I am grateful to Prof. Dr. Hajnalka Ábrahám, Head of the Institute, for the pleasant, welcoming and motivating working environment and for teaching me how to

use the electron microscope.

I am grateful to the former and current staff members of the Central Electron Microscopy Laboratory of the PTE-ÁOK, Emese Papp, Dr. Judit Lórándné Misley, Gáborné Domján and Tünde Faragó for their altruistic sharing of their knowledge and expertise, which is indispensable for sample preparation and manual work.

Special appreciation goes to Professor Ove Wiborg and his team for the CMS paradigm treatment, phenotyping and brain extraction of the animals used in the studies.

My dissertation would not have been possible without my friends and colleagues, who have helped my professional development by inspiring me with professional discussions and constructive criticism, and by keeping my enthusiasm alive.

I would like to thank my family, especially my wife Éva Bakonyi for their loving support, for their moral and emotional stability and calm patience and perhaps most importantly - I can never thank my son enough for the hours of playtime he sacrificed on my thesis writing.

The research for this thesis and the author were funded by:

- Nemzeti Agykutatási Program - KTIA_NAP_13-2014-0019 és 20017-1.2.1-NKP -2017- 00002 számú projektjei
- Lundbeck Alapítvány R83-A7631-számú projektje
- Nemzeti Kiválóság Program (TÁMOP 4.2.4. A/2-11-1-2012-0001)
- Tématerületi Kiválósági Program (TKP2021-EGA-16) projektje
- Nemzeti Tehetség Program (NTP-NFTÖ-18-B-0313)
- Nemzeti Tehetség Program (NTP-NFTÖ-22-B-0005)

8. List of publications

8.1 List of publications on which the thesis is based

1. **Csabai D**, Sebők-Tornai A, Wiborg O, Czéh B
   A Preliminary Quantitative Electron Microscopic Analysis Reveals Reduced Number of Mitochondria in the Infralimbic Cortex of Rats Exposed to Chronic Mild Stress. FRONTIERS IN BEHAVIORAL NEUROSCIENCE. *16*(May), 1–10. (2022)
   Q1; IF= 3.558, Independent citations: 1

2. **Csabai D**, Wiborg O, Czéh B
   Reduced synapse and axon numbers in the prefrontal cortex of rats subjected to a chronic stress model for depression. FRONTIERS IN CELLULAR NEUROSCIENCE 12: Paper 24. 15 P. (2018)
   Q1; IF= 3.9, Independent citations: 45

3. **Csabai D**, Seress L, Varga Z, Ábrahám H, Miseta A, Wiborg O, Czéh B
   Electron microscopic analysis of hippocampal axo-somatic synapses in a chronic stress model for depression. HIPPOCAMPUS 27:(1) pp. 17-27. (2017)
   Q1; IF= 3,966, Independent citations: 19

- Cumulative IF of the publications on which the thesis is based: 11.424
  Total number of independent citations: 65

8.2 Additional publications

2021

1. Gombos K, Földi M, Kiss Sz, Herczeg R, Gyenesei A, Geiger L, Csabai D, Futács K, Nagy T, Miseta A, Somogyi B, Hegyi P, Szentesi A
   Analyses of 5463 COVID-19 test results in Hungary: epidemiology, diagnostics and clinical outcome. FRONTIERS IN MEDICINE 2020; 7: 625673. (2021)
   Q1; IF= 5.091, Independent citations: 5

2020

2. Nagy Sz, Vranesics A, Varga Zs, **Csabai D**, Bruszt N, Bali Zs, Perlaki G, Hernádi I, Berente Z, Miseta A, Dóczi T, Czéh B
   Stress-induced microstructural alterations correlate with the cognitive performance of rats: A longitudinal in vivo diffusion tensor imaging study. FRONTIERS IN NEUROSCIENCE 14 Paper: 474, 20 p. (2020)
   Q2; IF= 4.677, Independent citations: 4

2018

3. Czéh B, Vardya I, Varga Z, Febbraro F, **Csabai D**, Martis L-S, Højgaard K, Henningsen K, Bouzinova EV, Miseta A, Jensen K, Wiborg O
   Long-Term Stress Disrupts the Structural and Functional Integrity of GABAergic Neuronal Networks in the Medial Prefrontal Cortex of Rats. FRONTIERS IN CELLULAR NEUROSCIENCE 12: Paper 148. 21 p. (2018)
   Q1; IF= 3.9, Independent citations: 60

4. Rusznák K, Csekő K, Varga Z, **Csabai D**, Bóna Á, Mayer M, Kozma Z, Helyes Z, Czéh B
   Long-Term Stress and Concomitant Marijuana Smoke Exposure Affect Physiology, Behavior and Adult Hippocampal Neurogenesis. FRONTIERS IN PHARMACOLOGY 9: Paper 786. 20 p. (2018)
   Q1; IF= 3.845, Independent citations: 17

2017

5. Varga Z, **Csabai D**, Miseta A, Wiborg O, Czéh B
   Chronic stress affects the number of GABAergic neurons in the orbitofrontal cortex of rats. BEHAVIOURAL BRAIN RESEARCH 316: pp. 104-114. (2017)
   Q1; IF= 3,173, Independent citations: 22

2016

6. **Csabai D**, Csekő K, Szaiff L, Varga Z, Miseta A, Helyes Z, Czéh B
   Low intensity, long term exposure to tobacco smoke inhibits hippocampal neurogenesis in adult mice. BEHAVIOURAL BRAIN RESEARCH 302: pp. 44-52. (2016)
   Q1; IF= 3,002, Independent citations: 18

- Cumulative IF of the additional publications: 23.688
  Total number of independent citations: 126

8.3 Other publications, conference presentations

2022

1. Csabai D, Sebők-Tornai A, Wiborg O, Stockmeier C A, Czéh B
   Ultrastructural mitochondrial alterations in rodent model of depression and potential clinical relevance /POSTER/
   FENS Forum 2022, Paris, France (2022)

2. Csabai D, Sebők-Tornai A, Wiborg O, Stockmeier C A, Czéh B
   Ultrastructural mitochondrial alterations in rodent model of depression and potential clinical relevance /SPEECH/
   Second Symposium on Super-resolution and Advanced Fluorescence Microscopy and István Ábrahám Memorial Workshop, Pécs, Hungary (2022)

2019

3. Csabai D, Wiborg O, Czéh B
   Quantitative ultrastructural analysis of mitochondria in the neuropil of medial prefrontal cortex of rats exposed to chronic stress /POSTER/
   48th Meeting of the European Brain and Behaviour Society, Prague, Czech Republic (2019)

4. Csabai D, Czéh B
   STRESS IN ANIMALS: AN ECOLOGICAL VIEW /ELŐADÁS/
   Summer School on Stress: From Hans Selye's original concept to recent advances, Saint Petersburg, Russia (2019)

5. Csabai D, Wiborg O, Czéh B
   Identifying quantitative parameters of neuronal mitochondria in the medial prefrontal cortex of chronically stressed rats /POSTER/
   VIII. Interdisciplinary Doctoral Conference, Pécs, Hungary (2019)

6. Csabai D, Wiborg O, Czéh B
   Quantitative ultrastructural analysis of neuronal mitochondria in the medial prefrontal cortex of chronically stressed rats /POSTER/
   2nd Munich Winter Conference on Stress, Garmisch-Partenkirchen, Germany (2019)

7. Csabai D, Wiborg O, Czéh B
   Quantitative ultrastructural analysis of neuronal mitochondria in the medial prefrontal cortex of chronically stressed rats /POSTER/
   16th Biannual Conference of the Hungarian Neuroscience Society, Debrecen, Hungary (2019)

8. Rusznák K, Csekő K, Varga Zs, Csabai D, Mayer M, Kozma Zs, Helyes Zs, Czéh B
   Long-Term Stress and Concomitant Marijuana Smoke Exposure Affect Physiology, Behavior and Adult Hippocampal Neurogenesis /POSTER/
   48th Meeting of the European Brain and Behaviour Society, Prague, Czech Republic (2019)

9. Rusznák K, Csekő K, Varga Zs, Csabai D, Bóna Á, Mayer M, Kozma Zs, Helyes Zs, Czéh B
   Long-Term Stress and Concomitant Marijuana Smoke Exposure Affect Physiology, Behavior and Adult Hippocampal Neurogenesis /POSTER/
   VIII. Interdisciplinary Doctoral Conference, Pécs, Hungary (2019)

10. Vranesics A, Nagy Sz A, Berente Z, Perlaki G, Orsi G, Varga Zs, Csabai D, Dóczi T, Czéh B
    In vivo diffusion tensor imaging of the brains of stressed rats /POSTER/
    16th Biannual Conference of the Hungarian Neuroscience Society, Debrecen, Hungary (2019)

2018

11. Rusznák K, Csekő K, Varga Zs, Csabai D, Bóna Á, Mayer M, Kozma Zs, Helyes Zs, Czéh B
    The effects of long-term cannabis smoke exposure in mice /POSTER/
    59th General Assembly of the Hungarian Society for Laboratory Diagnostics, Pécs, Hungary (2018)

12. Rusznák K, Csekő K, Varga Zs, Csabai D, Mayer M, Kozma Zs, Helyes Zs, Czéh B
    A stressz és marihuána füst hatása kísérleti állatokban /POSTER/
    IX National Congress of the Hungarian Psychiatric Society, Debrecen, Hungary (2018)

2017

13. Csabai D, Wiborg O, Czéh B
    Synapse numbers in the medial prefrontal cortex of rats subjected to chronic stress /POSTER/
    FENS Regional Meeting, Pécs, Hungary (2017)

14. Rusznák K, Csekő K, Varga Zs, Csabai D, Helyes Zs, Czéh B
    The Effect of Chronic Stress and Concomitant Cannabis Exposure on Behavior and Adult Hippocampal Neurogenesis in Mice /POSTER/
    FENS Regional Meeting, Pécs, Hungary (2017)

15. Csabai D, Wiborg O, Czéh B
    Synapse numbers in the infralimbic cortex of rats subjected to long-term stress /POSTER/
    30th ECNP Congress, Paris, France (2017)

16. Csabai D, Wiborg O, Czéh B
    Quantitative analysis of synaptic contacts in the ventral medial prefrontal cortex of rats. /POSTER/
    Spring Brain Conference 2017, Copenhagen, Denmark (2017)

2016

17. Csabai D, Seress L, Varga Z, Tényi T, Miseta A, Wiborg O, Czéh B, Simon M
    Quantitative electron microscopic analysis of hippocampal inhibitory synapses in an animal model for depression /POSTER/
    29th ECNP Congress, Vienna, Austria (2016)

18. Csabai D, Seress L, Varga Z, Miseta A, Wiborg O, Czéh B
    A krónikus stressz hatása a gátló, periszomatikus szinapszisokra a hippokampusz CA1 régiójában. Egy elektronmikroszkópos vizsgálat /POSTER/
    FAMÉ Pécs, Hungary (2016)

19. Varga Z, Csabai D, Miseta A, Wiborg O, Czéh Boldizsár
    A krónikus stressz hatása a fronto-kortikális GABAerg neuronok számára /POSTER/
    FAMÉ Pécs, Hungary (2016)

20. Csabai D, Seress L, Ábrahám H, Wiborg O, Czéh B
    Stress-induced changes of axo-somatic synapses of hippocampal CA1 pyramidal neurons. An electron microscopic study. /POSTER/
    IBRO Workshop 2016, Budapest, Hungary (2016)

21. Varga Z, Csabai D, Miseta A, Wiborg O, Czéh B
    Stress-induced changes in GABAergic neuron numbers in the medial prefrontal cortex of rats /POSTER/
    IBRO Workshop 2016, Budapest, Hungary (2016)

22. Mezriczky Z, Csabai D, Varga Z, Wiborg O, Czéh B
    Chronic stress affects the number of parvalbumin-positive inhibitory neurons in the rat hippocampus /POSTER/
    IBRO Workshop 2016, Budapest, Hungary (2016)

23. Szaiff L, Csekő K, Csabai D, Varga Z, Miseta A, Helyes Z, Czéh B
    Low intensity, long term exposure to tobacco smoke inhibits hippocampal neurogenesis in adult mice /POSTER/
    IBRO Workshop 2016, Budapest, Hungary (2016)

2015

24. Varga Z, Csabai D, Wiborg O, Czéh B
    Reduced number of hippocampal GABAergic neurons in an animal model for depression /POSTER/
    IX Symposium of the Experimental Pharmacology Section of the Hungarian Society of Experimental and Clinical Pharmacology. Velence, Hungary (2015)

25. Varga Z, Csabai D, Wiborg O, Czéh B
Reduced number of hippocampal GABAergic neurons in an animal model for depression /POSTER/
15TH ANNUAL CONFERENCE OF THE HUNGARIAN NEUROSCIENCE SOCIETY. Budapest, Hungary (2015)

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