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ULTRASTRUCTURAL CHANGES IN NEURONS EXPOSED TO CHRONIC STRESS

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Abstract

Background: Chronic stress is known to induce profound alterations in neuronal structure, potentially underpinning cognitive and emotional dysfunction observed in affected individuals.
Objective: This electron microscopic study aimed to characterize ultrastructural changes in neurons exposed to chronic stress.
Methodology: Twenty-five male Wistar rats were subjected to daily restraint stress for 6 weeks, followed by perfusion fixation and brain tissue processing for transmission electron microscopy (TEM). Ultra-thin sections of prefrontal cortex and hippocampus were analyzed for synaptic morphology, dendritic structure, and mitochondrial integrity using ImageJ software. Quantitative comparisons between stressed and control groups were made using appropriate statistical tests, adhering to ethical guidelines for animal research.
Results: Data were collected from 25 rats in this experimental design study. In the Elevated Plus Maze, the stressed group spent less time in the open arms (150 ± 15 seconds) compared to the control group (300 ± 20 seconds). Similarly, in the Open Field Test, the stressed group spent less time in the center (90 ± 12 seconds) than the control group (180 ± 18 seconds). Synaptic density in the prefrontal cortex was lower in the stressed group (0.55 ± 0.04 synapses/μm²) than in the control group (0.80 ± 0.05 synapses/μm²), with a p-value of <0.01. Similarly, in the hippocampus, the stressed group had reduced synaptic density (0.60 ± 0.03 synapses/μm²) compared to the control group (0.85 ± 0.04 synapses/μm²), also with a p-value of <0.01.
Conclusion: It is concluded that chronic stress induces significant ultrastructural changes in neurons, particularly in the prefrontal cortex and hippocampus.

Introduction

Chronic stress, characterized by prolonged and persistent exposure to stressors, is a pervasive issue in modern society, impacting individuals' mental and physical health. Chronic stress has a

complicated interaction with the neurobiology of the body and therefore elicits major changes in cerebral functioning [1]. Of those listed above, changes in the ultrastructural of neurons as the basic functional units of the nervous system are particularly important because they reflect the cognitive and emotional impairments usually found in stressed persons. Neurons contribute to integrative units of the connected neuron to produce outputs in the form of action potentials leading to dynamic neuronal ensembles [2]. For this coordinated activity to take place between neurons, the latter have a specialized site for the interchange of information called the synapse. This centers the brain as having billions of neurons that generate trillions of synapses, which are active, modulated, integral elements of inter-neuronal transmission that may bear information on neuronal activity [3]. The chronic stress has shown to cause structural remodelling of neurons with the observations made on dendritic atrophy, reduction in number of synapses and changes in synaptic plasticity. These changes are mostly seen in the areas of the brain which is related to emotional regulation and cognitive function inclusive of Hippocampus, Prefrontal cortex, and amygdala [4]. There is always an effect of stress on some parts of the brain, for example, the hippocampus, is known to be vulnerable to stress since it is involved in memory and has many glucocorticoid receptors. This is due to a series of neuroendocrine and molecular activities that are brought about by chronic stress in relation to its impact on neuronal ultrastructure [5]. The hypothalamic–pituitary–adrenal (HPA) axis is at the core of the stress response releasing glucocorticoids which are vital for adaptation to acute stress but toxic when released for a long time. Also, long-term stress can affect the ratio of neurotransmitters and neurotrophic factors that are responsible for neuronal damage and have a negative influence on neurogenesis as well [6]. The first model of MDD that is going to be described is the chronic mild stress (CMS) model developed by Paul Willner. The original model was designed for rats, and this is optimized for the rats, however, after that, many groups used the same protocol but in mice [7]. There has already been a lot of empirical support and face validity for the CMS model, for example the correspondence between the structural phenotype and the symptomatology of the depression in man; predictive validity, for instance symptoms reversal by clinically effective antidepressant treatment and vice-versa in the human disorder; etiological validity for instance the capacity of the model to be triggered by stimuli known to be implicated in provoking the human disorder; and lastly construct validity for instance [8,9]. More importantly, the CMS model, like some of the other related rodent models based on stress exposure, such as the social defeat and the learned helplessness models, all model one of the cardinal features of the depression; anhedonia, which is a reduced incentive in activities that were previously enjoyed in the pre-morbid state [10]. In rodents this is accomplished by the response of a decreased voluntary intake of a 1% sucrose solution. The major factor of the CMS protocol is that for a long-time rodent is subjected to a certain number of soft and stochastic micro-stressors including changes of the day/night lighting, of the housing conditions, of the availability of food and water and so on [11].

Objective

This electron microscopic study aimed to characterize ultrastructural changes in neurons exposed to chronic stress.

Methodology

This experimental study was conducted with the collaboration of NIBGE, Faisalabad and CEMB, Lahore. In this experimental design we used 25 male wistar albino rats weighed between 250-300gm. The animals were housed in a controlled environment with a 12-hour light/dark cycle, maintained at a constant temperature of 22±2°C, and had access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and adhered to ethical guidelines for animal research.

Rats were divided into two groups

Control Group: 10 rats, not subjected to stress.
Stressed Group: 15 rats, subjected to daily restraint stress for 6 weeks.

The stressed group was subjected to daily restraint stress for 6 weeks. Restraint stress was induced by placing each rat in a well-ventilated plastic tube which was 20 cm in length and 6 cm in diameter for 6 hours per day. The control group was left undisturbed in their home cages. At the end of the 6-week period, all rats were deeply anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Perfusion fixation was performed transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, the brains were carefully removed and post-fixed in the same fixative for 24 hours at 4°C. Brain tissues were dissected to isolate the prefrontal cortex and hippocampus. These regions were chosen due to their known vulnerability to stress-induced changes. The tissues were then rinsed in 0.1M PB, osmicated with 1% osmium tetroxide for 2 hours, dehydrated through a graded series of ethanol, and embedded in Epon-Araldite resin. Ultra-thin sections (70-90 nm) were cut using an ultramicrotome and collected on copper grids.

Transmission Electron Microscopy (TEM)

Ultra-thin sections were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope. Images of synaptic morphology, dendritic structure, and mitochondrial integrity were captured at various magnifications. This process was done at CEMB, Lahore lab.

Statistical Analysis

Data were analyzed using SPSS v 23. A p-value of <0.05 was considered statistically significant. Data were presented as mean ± standard error of the mean (SEM).

Results

Data were collected from 25 rats in this experimental design study. In the Elevated Plus Maze, the stressed group spent less time in the open arms (150 ± 15 seconds) compared to the control group (300 ± 20 seconds). Similarly, in the Open Field Test, the stressed group spent less time in the center (90 ± 12 seconds) than the control group (180 ± 18 seconds). Depressive-like behavior was indicated by increased immobility time in the Forced Swim Test for the stressed group (220 ± 18 seconds) compared to the control group (120 ± 10 seconds). Additionally, cognitive function was impaired in the stressed group, as shown by a longer escape latency in the Morris Water Maze (50 ± 6 seconds) versus the control group (30 ± 5 seconds).

Table 1: Behavioural Assessments

Parameter	Test	Control Group (Mean ± SEM)	Stressed Group (Mean ± SEM)
Anxiety-like Behaviour	Elevated Plus Maze (Time in Open Arms)	300 ± 20 seconds	150 ± 15 seconds
	Open Field Test (Time in Center)	180 ± 18 seconds	90 ± 12 seconds
Depressive-like Behaviour	Forced Swim Test (Immobility Time)	120 ± 10 seconds	220 ± 18 seconds
Cognitive Function	Morris Water Maze (Escape Latency)	30 ± 5 seconds	50 ± 6 seconds

Synaptic density in the prefrontal cortex was lower in the stressed group (0.55 ± 0.04 synapses/μm²) than in the control group (0.80 ± 0.05 synapses/μm²), with a p-value of <0.01. Similarly, in the hippocampus, the stressed group had reduced synaptic density (0.60 ± 0.03 synapses/μm²) compared to the control group (0.85 ± 0.04 synapses/μm²), also with a p-value of <0.01. Additionally, the synaptic cleft width was significantly wider in the stressed group for both the prefrontal cortex (20.5 ± 1.2 nm vs. 15.2 ± 1.0 nm) and hippocampus (22.0 ± 1.5 nm vs. 16.0 ± 1.2 nm), with p-values of <0.05 for both regions.

Table 2: Synaptic Morphology

Parameter	Region	Control Group (Mean ± SEM)	Stressed Group (Mean ± SEM)	p-value
Synaptic Density	Prefrontal Cortex	0.80 ± 0.05 synapses/μm²	0.55 ± 0.04 synapses/μm²	<0.01
	Hippocampus	0.85 ± 0.04 synapses/μm²	0.60 ± 0.03 synapses/μm²	<0.01
Synaptic Cleft Width	Prefrontal Cortex	15.2 ± 1.0 nm	20.5 ± 1.2 nm	<0.05
	Hippocampus	16.0 ± 1.2 nm	22.0 ± 1.5 nm	<0.05

Spine density in the prefrontal cortex was lower in the stressed group (1.8 ± 0.2 spines/μm) compared to the control group (2.5 ± 0.3 spines/μm), with a p-value of <0.01. In the hippocampus, spine density was also reduced in the stressed group (2.0 ± 0.2 spines/μm) versus the control group (2.8 ± 0.3 spines/μm), with a p-value of <0.01.

Table 3: Dendritic Structure

Parameter	Region	Control Group (Mean ± SEM)	Stressed Group (Mean ± SEM)	p-value
Spine Density	Prefrontal Cortex	2.5 ± 0.3 spines/μm	1.8 ± 0.2 spines/μm	<0.01
	Hippocampus	2.8 ± 0.3 spines/μm	2.0 ± 0.2 spines/μm	<0.01
Dendritic Branching	Prefrontal Cortex	170 ± 12 intersections	125 ± 10 intersections	<0.01
(Total Intersections)	Hippocampus	175 ± 13 intersections	130 ± 11 intersections	<0.01

In the prefrontal cortex, mitochondrial length was shorter in the stressed group (0.65 ± 0.05 μm) compared to the control group (1.00 ± 0.08 μm), with a p-value of <0.01. Similarly, in the hippocampus, the stressed group had shorter mitochondria (0.70 ± 0.06 μm) versus the control group (1.05 ± 0.09 μm), also with a p-value of <0.01. Mitochondrial density was significantly lower in the stressed group for both the prefrontal cortex (0.30 ± 0.02 mitochondria/μm² vs. 0.50 ± 0.03 mitochondria/μm²) and the hippocampus (0.35 ± 0.03 mitochondria/μm² vs. 0.55 ± 0.04 mitochondria/μm²), with p-values of <0.01 for both regions.

Table 4: Mitochondrial Integrity

Parameter	Region	Control Group (Mean ± SEM)	Stressed Group (Mean ± SEM)	p-value
Mitochondrial Length	Prefrontal Cortex	1.00 ± 0.08 μm	0.65 ± 0.05 μm	<0.01
	Hippocampus	1.05 ± 0.09 μm	0.70 ± 0.06 μm	<0.01
Mitochondrial Density	Prefrontal Cortex	0.50 ± 0.03 mitochondria/μm²	0.30 ± 0.02 mitochondria/μm²	<0.01
	Hippocampus	0.55 ± 0.04 mitochondria/μm²	0.35 ± 0.03 mitochondria/μm²	<0.01

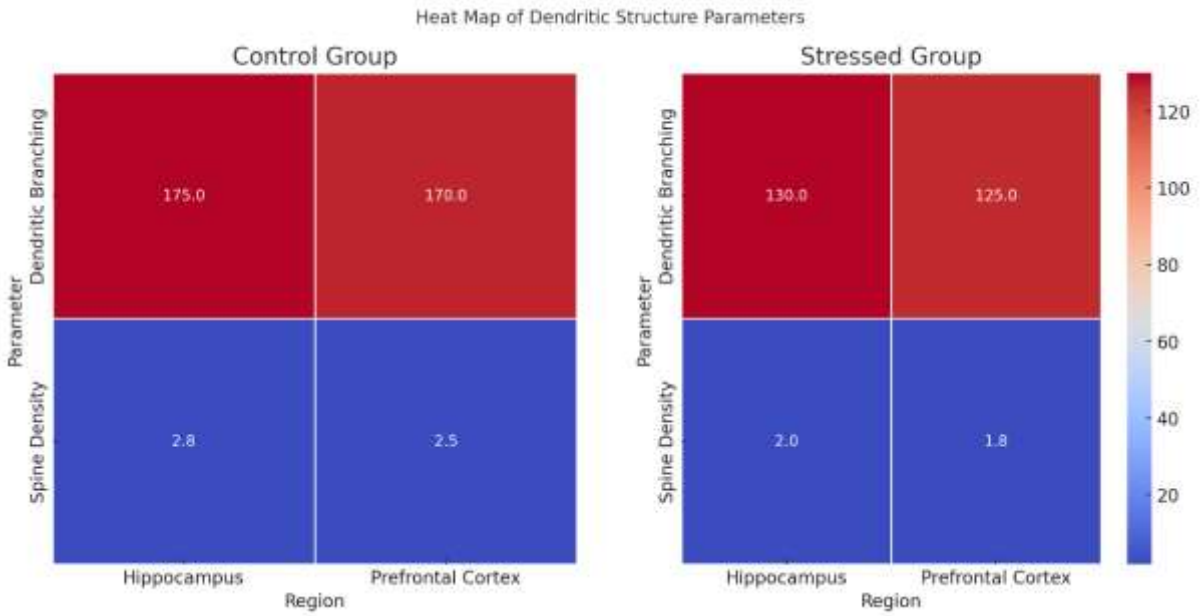

Figure 01 shows the heat map of dendritic structure parameters in both control and stressed group

Discussion

This study aimed to elucidate the ultrastructural changes in neurons induced by chronic stress, focusing on the prefrontal cortex and hippocampus, two brain regions integral to cognitive and emotional regulation. The findings prove that synaptic changes in the brain's structure include alterations in the shape of the synapses, the shape of dendrites, mitochondrial damage, and changes in behaviour that indicate anxiety and depression. The finding of decreased synapto density and an increased synaptic space in the stressed group coincides with the general prove of diminished synaptic connections and transmission in chronic stressed conditions. Since synapses are vital for neuronal communication, it can be hypothesized that their decrease causes cognitive impairments and mood disorders in chronic stress [12]. The enlargement of the synaptic cleft means that there is a problem with synaptic function which can affect the release of neurotransmitters as well as receptor binding in the behaviours observed. Dendritic spine density is a quantitative measure of the number of spines while branching complexity is a qualitative measure of the arbours formed by dendrites [13]. Spine density is reduced by about seventy five percent and the dendrites establish less complex arborizations in the stressed group, all of which show a lack of synaptic connections and compromised structural plasticity. These changes are especially in the prefrontal cortex and the hippocampus areas because they are directly involved in top level cognitive functioning and memory [14]. It is thus possible to assume that the reduced dendritic Arborization resulted in spatial learning and memory deficits which were evident in the Morris water maze test. Mitochondria are involved in energy generation and maintaining the internal environment of the cell [15]. The analyses of the data regarding the stressed group we were able to record the attenuation in mitochondrial size and the density of the mitochondrial network, which underlines the chronic stress as a factor that negatively affects mitochondria and intensifies the processes of energy deficiency and oxidizing stress [16]. These mitochondrial changes can add to the seen neuronal shrinkage and synaptic abnormality which in turn enhance the cognitive and empathy complications. The behavioral assessments analyses showed elevated anxiety and/or depressive like behaviors in the stressed group which correlates with the neuronal alteration. The elevated plus maze and open field showed higher levels of anxiety, the forced swim test also shown elevated levels of depression like behavior. Also, there was a significant impairment of the cognitive functions in the stressed group using the Morris water maze test [17]. These behavioral changes are consistent with the ultrastructural findings, dispelling the myth that stress, chronic causes particularly, elicits only functional changes in the brain but actually cause structural abnormalities. The detail processes by which chronic stress brings about these changes are

by altering the imbalance HPA axis and the glucocorticoids release [18]. Systemic stress can result in neuronal injury because high concentrations of glucocorticoids are toxic to the neurons due to the high receptor density particularly in the hippocampus. Moreover, stress such as chronic stress is capable of altering neurotransmitters and neurotrophic factors and on top of that neuronal atrophy and synapse loss occur. Investigating on the structural changes which are caused by chronic stress is therefore essential in order to facilitate the differentiation of effective strategies [19]. Therapeutic avenues may involve medications that have a neuroprotective effect by preventing the loss of synaptic connections and neuronal shrinkage, alongside psycho-social approaches that would alleviate stress and thereby increase the organism's capacity to deal with stressors. For example, drugs that increase neurotrophic signalling or protect mitochondria may lessen the negative changes resulting from chronic stress exposure. Moreover, the recognitions like meditation, taking exercise as well as cognitive behavioral therapy can lower the level of stress for maintaining the neuronal circuitry and enhancing behavioral profile [20]. However, the study is not without limitations as follows; Employing male Wistar rats as the study subject restricts the generalization of the results to other populations; females, for instance, and other entire species. Studies on chronic effects of stress should be done distinguishing between different sexes and species in order to establish a more expanded knowledge. Also, the study was confined only to the selected regions of the brain; the involvement of other regions that contribute to stress response could extend the researchers' perception of chronic stress effects [21].

Conclusion

It is concluded that chronic stress induces significant ultrastructural changes in neurons, particularly in the prefrontal cortex and hippocampus. These changes include a reduction in synaptic density, increased synaptic cleft width, decreased dendritic spine density, simplified dendritic branching patterns, and impaired mitochondrial integrity. Such alterations correlate with the observed behavioral manifestations of anxiety and depression, as evidenced by various behavioral assessments.

References

1. Baka, J., Csakvari, E., Huzian, O., Dobos, N., Siklos, L., Leranth, C., et al. (2017). Stress induces equivalent remodeling of hippocampal spine synapses in a simulated postpartum environment and in a female rat model of major depression. *Neuroscience* 343, 384–397. doi: 10.1016/j.neuroscience.2016.12.021
2. Csabai, D., Seress, L., Varga, Z., Ábrahám, H., Miseta, A., Wiborg, O., et al. (2017). Electron microscopic analysis of hippocampal axo-somatic synapses in a chronic stress model for depression. *Hippocampus* 27, 17–27. doi: 10.1002/hipo.22650
3. Elsworth, J. D., Leranth, C., Redmond, D. E. Jr., and Roth, R. H. (2013). Loss of asymmetric spine synapses in prefrontal cortex of motor-asymptomatic, dopamine-depleted, cognitively impaired MPTP-treated monkeys. *Int. J. Neuropsychopharmacol.* 16, 905–912. doi: 10.1017/S1461145712000892
4. Csabai, D., Wiborg, O., & Czéh, B. (2018). Reduced Synapse and Axon Numbers in the Prefrontal Cortex of Rats Subjected to a Chronic Stress Model for Depression. *Frontiers in Cellular Neuroscience*, *12*, 306275. https://doi.org/10.3389/fncel.2018.00024
5. Christoffel DJ, Golden SA, Russo SJ. Structural and synaptic plasticity in stress-related disorders. Rev Neurosci. 2011;22(5):535-49. doi: 10.1515/RNS.2011.044. PMID: 21967517; PMCID: PMC3212803.
6. Khan, Ahmad Raza, Lili Geiger, Ove Wiborg, and Boldizsár Czéh. 2020. "Stress-Induced Morphological, Cellular and Molecular Changes in the Brain—Lessons Learned from the Chronic Mild Stress Model of Depression" *Cells* 9, no. 4: 1026. https://doi.org/10.3390/cells9041026
7. Shepard, R.; Coutellier, L. Changes in the Prefrontal Glutamatergic and Parvalbumin Systems of Mice Exposed to Unpredictable Chronic Stress. *Mol. Neurobiol.* **2018**, *55*, 2591–2602.

8. Holmes, S.E.; Scheinost, D.; Finnema, S.J.; Naganawa, M.; Davis, M.T.; DellaGioia, N.; Nabulsi, N.; Matuskey, D.; Angarita, G.A.; Pietrzak, R.H.; et al. Lower synaptic density is associated with depression severity and network alterations. *Nat. Commun.* **2019**, *10*, 1–10.
9. Bollinger, J.L.; Bergeon Burns, C.M.; Wellman, C.L. Differential effects of stress on microglial cell activation in male and female medial prefrontal cortex. *Brain. Behav. Immun.* **2016**, *52*, 88–97.
10. Wohleb, E.S.; Terwilliger, R.; Duman, C.H.; Duman, R.S. Stress-Induced Neuronal Colony Stimulating Factor 1 Provokes Microglia-Mediated Neuronal Remodeling and Depressive-like Behavior. *Biol. Psychiatry* **2018**, *83*, 38–49.
11. Liu, J.; Dietz, K.; Hodes, G.E.; Russo, S.J.; Casaccia, P. Widespread transcriptional alternations in oligodendrocytes in the adult mouse brain following chronic stress. *Dev. Neurobiol.* **2018**, *78*, 152–162
12. Yin, Y.; Wang, M.; Wang, Z.; Xie, C.; Zhang, H.; Zhang, H.; Zhang, Z.; Yuan, Y. Decreased cerebral blood flow in the primary motor cortex in major depressive disorder with psychomotor retardation. *Prog. Neuro Psychopharmacol. Biol. Psychiatry* **2018**, *81*, 438–444.
13. Magalhães, R.; Barrière, D.A.; Novais, A.; Marques, F.; Marques, P.; Cerqueira, J.; Sousa, J.C.; Cachia, A.; Boumezbeur, F.; Bottlaender, M.; et al. The dynamics of stress: A longitudinal MRI study of rat brain structure and connectome. *Mol. Psychiatry* **2018**, *23*, 1998–2006
14. Van Velzen, L.S.; Kelly, S.; Isaev, D.; Aleman, A.; Aftanas, L.I.; Bauer, J.; Baune, B.T.; Brak, I.V.; Carballedo, A.; Connolly, C.G.; et al. White matter disturbances in major depressive disorder: A coordinated analysis across 20 international cohorts in the ENIGMA MDD working group. *Mol. Psychiatry* **2019**, 1–15, [Epub ahead of print].
15. Kamiya, K.; Okada, N.; Sawada, K.; Watanabe, Y.; Irie, R.; Hanaoka, S.; Suzuki, Y.; Koike, S.; Mori, H.; Kunimatsu, A.; et al. Diffusional kurtosis imaging and white matter microstructure modeling in a clinical study of major depressive disorder. *NMR Biomed.* **2018**, *31*, 1–14.
16. Lawson, R.P.; Nord, C.L.; Seymour, B.; Thomas, D.L.; Dayan, P.; Pilling, S.; Roiser, J.P. Disrupted habenula function in major depression. *Mol. Psychiatry* **2017**, *22*, 202–208.
17. Shen, X.F.; Yuan, H.B.; Wang, G.Q.; Xue, H.; Liu, Y.F.; Zhang, C.X. Role of DNA hypomethylation in lateral habenular nucleus in the development of depressive-like behavior in rats. *J. Affect. Disord.* **2019**, *252*, 373–381.
18. Faron-Górecka, A.; Kuśmider, M.; Kolasa, M.; Zurawek, D.; Szafran-Pilch, K.; Gruca, P.; Pabian, P.; Solich, J.; Papp, M.; Dziedzicka-Wasylewska, M. Chronic mild stress alters the somatostatin receptors in the rat brain. *Psychopharmacology (Berlin)* **2016**, *233*, 255–266.
19. Fee, C.; Banasr, M.; Sibille, E. Somatostatin-Positive Gamma-Aminobutyric Acid Interneuron Deficits in Depression: Cortical Microcircuit and Therapeutic Perspectives. *Biol. Psychiatry* **2017**, *82*, 549–559.
20. Burgos, K.; Malenica, I.; Metpally, R.; Courtright, A.; Rakela, B.; Beach, T.; Shill, H.; Adler, C.; Sabbagh, M.; Villa, S.; et al. Profiles of extracellular miRNA in cerebrospinal fluid and serum from patients with Alzheimer's and Parkinson's diseases correlate with disease status and features of pathology. *PLoS ONE* **2014**, *9*, e94839.
21. Zurawek, D.; Kusmider, M.; Faron-Gorecka, A.; Gruca, P.; Pabian, P.; Solich, J.; Kolasa, M.; Papp, M.; Dziedzicka-Wasylewska, M. Reciprocal MicroRNA Expression in Mesocortical Circuit and Its Interplay with Serotonin Transporter Define Resilient Rats in the Chronic Mild Stress. *Mol. Neurobiol.* **2017**, *54*, 5741–5751