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Running head: Astrocytes as a basis for depression

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Abstract

The contribution of glial cells to the pathophysiology of depression is an emerging research purpose. This study investigated the deficits in glial cells, specifically astrocytes in various brain regions, after the development of depression and then after voluntary running in depressed rats. Forty-five adult male Wistar rats aged 8-10 weeks were used in the study. A depression model was generated through a forced swimming Programe; voluntary running was allowed on rat running wheels; and brain sections were taken from

the hippocampus, dentate gyrus (DG), medial prefrontal cortex (mPFC) and cerebellar cortex. After immunostaining with specific antibodies immuno-stain, the astrocytes, oligodendroglia and microglial cells were counted, and certain indices relating astrocytes to other glial cells were calculated. Astrocytic morphology was studied, and the optical density (OD) of glial fibrillary protein (GFAP) immuno-expression was measured in different groups. Compared to the control group, animals in the depression group exhibited significant decreases in the mean astrocyte count in all studied brain areas, significant decreases in GFAP OD values in all areas and significantly reduced values for all glial astrocyte indices in the hippocampus, DG and mPFC. Compared to the rats in the depression group, those in the depression/exercise group exhibited significantly elevated mean astrocyte and oligodendroglia counts in all studied areas, significantly elevated GFAP OD values in all studied areas, and non-significant differences in glial astrocyte indices in the hippocampus, mPFC and cerebellar cortex. Astrocytes, rather than other glia, constitute a basis for the development and/or relief of depression.

Key words: stress, dentate gyrus, hippocampus, cerebellum, frontal cortex, glial cells, voluntary running

INTRODUCTION

Depression is a neuropsychiatric ailment that has a major impact on socio-economic status. This disorder results from maladaptive neuroplastic processes in the cellular structures concerned with emotional processing, such as the prefrontal cortex (PFC), hippocampus and amygdala [1]. Neurotrophins and neuroplasticity are generally accepted to play an essential role in the pathophysiology of depression. However, the disturbance of synaptic plasticity due to glial cell pathology, particularly in the frontolimbic areas of the brain, is an emerging proposed mechanism that may substantially contribute to the pathophysiology of mood disorders [2].

Physical exercise can provide relief for depressed mood, but the underlying mechanisms remain uncertain. Studies designed to identify these mechanisms have discussed the changes in neurons with slight attention paid to changes in glial cells [3].

The term neuroglia describes all supporting non-neuronal cells in the central nervous system (CNS) that represent the homeostatic and defensive arm of nervous system[4]. Recently, a system of complex crosstalk between neurons and glia has been proposed, and the active role for glial cells in central homeostatic processes has been presented [5].

Astrocytes are a heterogeneous class of glial cells that preserve the neural microenvironment [6]. Their dynamic role in shaping synaptic function and their impact on behavioural expression are noteworthy [2]. Microglia are specific immune cells of the CNS that are highly dynamic, surveying the brain parenchyma and contributing to neuroprotection. During neurogenesis, microglia have a role in determining the ultimate patterns of the neural circuits important for behaviour and disease development [7]. These glia also have a critical role in the synaptic carving needed for adaptive brain function [8]. Oligodendroglia are myelin-forming cells that are generated continuously in healthy adult brains for regulation of the axonal function[9].

Experimental studies on the pathophysiology of depression usually focus on neuronal changes, with little attention has paid to changes in the neuroglia. This study aimed to consider changes in astrocytic counts and morphology in the hippocampus, dentate gyrus (DG), medial prefrontal cortex (mPFC) and cerebellar cortex of Wistar rats in an experimental depressive state and after voluntary running.

MATERIALS AND METHODS

This experimental study was conducted in the behaviour laboratory of the College of Medicine, Taibah University, Saudi Arabia. Cell counting and analysis were performed in the Department of Histology at the Bogomolets National Medical University, Ukraine. The animal handling was conducted in accordance with EC Directive 86/609/EEC for animal studies and the National Committee guidelines of Saudi Arabia.

Experimental animals

Fifty-five adult male Wistar rats aged 8-10 weeks and weighing 200-250 g were obtained from the animal house of the behaviour laboratory, housed in the laboratory for 7

days for acclimatization, and observed for their movements and food consumption as markers of their ordinary behaviour. A 6-minute forced swimming test (FST) was performed for choosing rats with extreme test behaviour (less immobility duration and high distance moved)[10]. Selected animals (n=45 rats) were housed in ventilated cages (5 animals/cage) with a standard chow diet and an adjusted laboratory environment (12/12-hour light/dark cycle, 22-24°C, 50-60% humidity) [3,11].

Selected rats were randomly divided into 3 equal groups as follows:

- Control group: Rats were fed a standard chow diet for a period of 5 weeks
- Depression group: Rats were subjected to the forced swimming Programe for two weeks and then fed the standard chow diet for the following 3 weeks
- Depression/exercise group: Rats were subjected to the forced swimming Programe for two weeks and then allowed to voluntary wheel running for the following 3 weeks. For all groups, the locomotor activities of the rats were assessed by the 6-minute FST before being sacrificed.

Induction of depression

Depression was induced by forced swimming inside an upright glass cylinder inclosing water (water height 45 cm and temperature at 23-25°C) for 15 min daily for 14 days. Every day after swimming, the rats were allowed to dry (32°C) for 15 min before being returned to their cages [3].

Accessing voluntary running

the rats were allowed to run voluntarily for 3 weeks in rat running wheels supplied with counters (Lafayette Instrument Company, Inc. Lafayette, IN, USA).

Confirmation of the development and/or the relief of the depressive state

Two means were used to confirm the development or relief of a depressive state just before the rats were euthanized:

- The 6-minute FST, through automatic tracing via a software (Ethovision XT version 8.0, Noldus Information Technology, Wageningen, The Netherlands), was conducted to

assess the locomotor behaviour of the rats in terms of a) immobility duration (in seconds) and b) the distance the rats moved (in centimetres) [12].

▪ Quantification of serum corticosterone levels; in the morning, after anaesthetising the rats with ether, blood was collected from the retro-orbital veins and put into serum separation tubes for centrifugation. The sera were reserved in −80°C until the levels of corticosterone (ALPCO Diagnostics, Orangeburg, NY, USA) were measured using ELISA kits rendering the manufacturers' instructions [13,14].

Tissue preparation and quantification of glial cells

At the quantified time, the rats were sacrificed by cervical dislocation and perfused with formaldehyde (4%) in phosphate-buffered saline. The brains were removed, coronally sectioned, and fixed in buffered formalin for histological examination of the hippocampus, DG, mPFC and cerebellar cortex. Brain tissue thin sections (4-5 μm) were immunostained with polyclonal anti-rat antibodies (Dako Cytomation, USA) against a. glial fibrillary acidic protein (GFAP); b. myelin basic protein (MBP); and c. cluster of differentiation 68 (CD68). The staining procedure was performed as previously described [15] with an immunoperoxidase technique and a Bench-Mark instrument (Ventana Company, Oro Valley, Arizona, USA) briefly; The paraffin sections were deparaffinised, rehydrated, treated with 3% H2O2 for 5 min and washed with phosphate-buffered saline (PBS) for 15 min. The sections were blocked with 1.5% normal goat serum in PBS to control background staining and then incubated for 45 min at room temperature with the primary antibody. The sections were subsequently incubated with a second-stage biotinylated antibody at room temperature. After rinsing with PBS, the reaction products were visualised by immersing the sections in diamnobenzidine. Finally, the sections were counter- stained with haematoxylin, dehydrated and cover-slipped using Protex mounting media (DAB- Stock Stain box; Boster Biotechnology, Pleasanton, CA, USA)[16].

Sections were examined with a bright field automated microscope (Olympus BX 36 provided with a true-colour image analysis software package), photographs were captured from different brain sections to represent all strata of the hippocampus (alveus, oriens and radiatum and pyramidal), all layers of the DG (molecular, granular and polymorphic), all

cell layers of the cerebellar cortex (molecular, Purkinje and granular) and all layers of the mPFC (molecular, outer granular, pyramidal, inner granular layer, ganglionic and multiform). The images were digitized by a DP27 digital video camera (2448×1920-pixel matrix) to identify different types of glial cells; GFAP+ve astroglia, MBP+ve oligodendroglia, and CD68+ve microglia[15] (Fig. 1). Cell counting was performed in the Department of Histology at the Bogomolets National Medical University, Ukraine. For each group, the stained astrocytes, oligodendroglia and microglia were counted at 400X in 15 fields from the selected sections of the four brain regions.

The glial astrocyte indices

Three glial astrocyte indices (GAI) were calculated as a simple way to express the ratio of astrocytic count to the counts of other types of glia and to explore the impact of altered astrocytic numbers on glial structural homeostasis in the context of depression and also after voluntary running of depressed rats. They were expressed as follows: a) GAI1 (A/M) = number of astrocytes/the number microglia; b) GAI2= number of astrocytes/ number of oligodendroglia; and c) GAI3 (A/T) = number of astrocytes/total number of counted glial cells.

The measurement of optical densities (ODs) of the GFAP

The optical densities (ODs) of the GFAP-stained sections were measured as described by Varghese et al[17] in five illustrative non-overlapping fields (×400). The images were deconvoluted via the DAB vector, the brown DAB images were calibrated and the mean grey value. The OD was calculated using the equation: [OD=log (max intensity/mean intensity)], where max intensity=250 and mean intensity=mean grey value.

Statistical analysis

Statistical analyses were done with the IBM SPSS statistical package (version 21). The data were stated as the mean ± standard error (SE). The means of the studied groups were compared using one-way ANOVA with the least significant different (LSD) test for

analysis of the locomotor parameters, corticosterone levels, glial cell counts and glial astrocyte indices. Statistical significance was set at *P<0.05*.

RESULTS

The development and relief of the depressive state

The mean immobility duration recorded for the depression group was significantly higher than that for the control group. Further, the mean immobility duration recorded for the depression/exercise group was significantly lower than that for the depression group (Fig. 2).

The mean distance moved by the rats in the depression group was significantly lower than that moved by the rats in the control group. Also, the mean distance moved by the rats in the depression/exercise group was significantly higher than that recorded for the depression group (Fig. 2).

The mean serum corticosterone level was significantly higher in the depression group than in the control group. Furthermore, it was significantly lower in the depression/exercise group than in the depression group (Fig. 3).

Glial cell counts and glial astrocyte indices

The mean numbers and significance of the glial cells and glial astrocyte indices are presented in table1 and table 2. Changes in the mean numbers of astrocytes in different regions for all groups are shown in Fig. 4.

The mean number of astrocytes was significantly lower in the depression group than in the control group in all studied brain areas (hippocampus, DG, mPFC and cerebellar cortex). However, no significant differences were detected for either oligodendroglia or microglia in the hippocampus, DG and mPFC between the depression group and the control group. Only oligodendroglia was significantly decreased in number in the cerebellar cortex.

Total glial cell counts were significantly lower in the depression group than in the control group in all studied brain areas except in the mPFC, where the decrease was not significant.

Excitingly, all glial astrocyte indices (GAI1, GAI2 and GAI3) were significantly lower in the depression groups than in the control group in all studied brain areas, except for a non-significant decrease in GAI1 and GAI3 in the cerebellar cortex.

The mean numbers of astrocytes and oligodendroglia were significantly higher in the depression/exercise group than in the depression group in all studied brain areas (hippocampus, DG, mPFC and cerebellar cortex). The mean number of microglia was different in different regions (increased significantly in the DG and mPFC, decreased significantly in the hippocampus, and no significant increase in the cerebellar cortex).

Total glial cell counts were significantly higher in the depression/exercise group than in the depression group in all studied brain areas except in the hippocampus, where the increase was not significant.

Notably, no significant differences were found in any of the glial astrocyte indices in the hippocampus, mPFC or cerebellar cortex. In the DG, significant decreases in GAI1 and GAI3 were detected, but these decreases were accompanied by highly significant increases in the numbers of microglia and oligodendroglia.

GFAP immunohistochemical study

An examination of the hippocampus, DG, mPFC and cerebellar cortex in the control group revealed star-shaped astrocytes with dense, distinct, dark cell bodies and complex and "bushy", densely packed processes (Fig. 5; A1, 2, 3, 4). In depressed rats, astrocytes were dispersed in all examined brain regions and displayed relatively indistinct cell bodies and distorted processes (Fig. 5; B1, 2, 3, 4). In the depression/exercise group, astrocytes in all regions appeared to regain their immune reactivity, exhibiting denser cell bodies and complex, denser process extensions than the depression group (Fig. 5; C1, 2, 3, 4).

Compared to the control group, depression caused a significant decrease in GFAP OD in the hippocampus (P=0.013), DG ($P<0.001$), mPFC ($P<0.001$), and cerebellar cortex ($P<0.001$) (Fig. 6).

Compared to the depression group, running caused a significant increase in GFAP OD in the hippocampus ($P=0.042$), DG ($P=0.027$), mPFC ($P=0.037$), and cerebellar cortex ($P=0.026$) (Fig. 6).

DISCUSSION

The development of depression and/or its relief were assessed both mechanically by evaluating locomotor behaviours (through FST) or biochemically by evaluating the serum corticosterone levels in all groups. The significant decrease of the distance moved, the significant increase in the immobility duration and the increased corticosterone levels after the forced swimming protocol confirmed the development of depression. Similarly, a significant increase in the mean distance moved, a significant decrease in the mean immobility duration and a significant decrease in serum cortisone levels occurred after a voluntary running protocol confirmed the relief of depressive behaviour [13,18].

Depression was primarily proposed to develop due to increased levels of corticosteroids [19]; later, neurotrophic factors and disordered neuroplastic processes in the frontal cortex and limbic system were proposed as underlying mechanisms [3,20]. Based upon the emerging concept that glial cells, specifically astrocytes, are critical factors affecting brain plasticity [2], this study evaluated the changes in glial cells (specifically astrocytes) in the hippocampus, DG, mPFC, and cerebellar cortex as a key mechanism triggering the pathophysiology of depression.

The significant decrease in the mean total glial cell counts in the hippocampus, DG, and cerebellar cortex reflected the contribution of glial cells to the pathophysiology of depression. This finding was consistent with a recorded reduction in glia during depression that was proven to precede the decreased neuronal density, which appears to occur later after longer periods of depression [21].

The significant decrease in the mean number of astrocytes, along with non-significant differences in the mean count of oligodendroglia or microglia, in most of the examined brain areas, can reflect the major role of astrocytes in the pathology of depression. Additionally, the decreased glial astrocyte indices can outline the role of astrocytes over other glia in the development of depression. These findings are in

consistent with the reported decreased density of astrocytes associated with synaptic dysfunction in the frontolimbic regions during depression [2] and in the hippocampus during chronic stress [22].

It is imperative to consider that the contribution of glial cells to the pathogenesis of depression is not contradicted by the old theories of depression that attributed the development of depression to the raised corticosterone or deficits in neurotrophic factors [1,20] as these factors have an impact on glial function [2], however, the role of astrocytes and glial-glial interactions in controlling depressive behaviour constitutes a new era of neuroscience research. These cells play major roles in synaptic function in specified brain regions [23,24], and have tough interactions with the synapses that make it difficult to distinguish whether the dysfunction resulted from neuronal or astrocyte modifications [25]

In accordance with our results, some evidence supports the idea that astrocyte modification in some brain areas leads to depression [26]. In humans, post-mortem brain samples from adults suffering from major depressive disorder showed a decreased number of astrocytes in the frontolimbic structures [27].

The significant decrease in the OD of GFAP immune-expression in the brain areas in the depression group was consistent with other studies that described a modification in the density of different astrocytic markers in the frontolimbic regions in major depressive disorder [28]. Moreover, GFAP-expressing cells are decreased in the PFC after 5 weeks of chronic stress [22] and in genetically stressed Wistar Kyoto rats [29]. Furthermore, postmortem brain samples showed decreased GFAP expression in the hippocampus and PFC, with no neuronal effects, although defective neuronal metabolism or connections have been suggested [30].

Regarding the cerebellum, the significant reduction in the number of astrocytes and in the OD of GFAP in the cerebellar cortex of rats in the depression group could represent an indication for the involvement of the cerebellar cortex in the pathophysiology of depression, as previously suggested [31]. One possible explanation is the known anatomical and functional connections of the cerebellum to the PFC and the subcortical limbic structures [32]. Nonetheless, in the cerebellum, oligodendroglia are involved in cerebellar pathology, with a significant decrease in their mean count in the depression

group, which could explain the non-significant decreases in GAI1 and GAI3 that were specifically observed in the cerebellar cortex.

Practising voluntary running by depressed rats improved the depressive locomotor behaviour and produced a significant increase in the total glial cell counts and significant increase of the OD of the GFAP-stained sections in the brain areas. This can indicate the concomitant improving effect on glial cell counts with the improvement of the depressive behaviour after voluntary running. This was consistent with the observed alleviation of depression-like symptoms and the enhancement of synaptic, and glial measures in several brain regions upon exercise [4].

The increased number of astrocytes in the mPFC, hippocampus and DG, along with improved depressive behaviour after voluntary running, are in line with the suggested physical activity-induced plasticity of astrocytes and inhanced synaptic markers in the brain regions for cognition [33]. The improved behaviour might be due to the trophic support and regulation of neuronal blood flow by astrocytes, which influence neuronal function and synaptic growth[31,34].

Also, the improved astrocytic counts and increased GFAP expression after running are consistent with the suggested important glioprotective effects and the prevention of stress-induced decreases in GFAP expression in the hippocampus after treatment with antidepressants [35].

Notably, although astrocyte counts increased in all brain areas after running, there were non-significant increases in the proposed glial astrocyte indices in the hippocampus, mPFC and cerebellar cortex. This finding showed that voluntary running increased the astrocyte count to levels capable of preserving neurons and oligodendroglia, limiting brain tissue degeneration and preserving the function without reaching the levels of astrogliosis, which can lead to neurotoxicity, potential neural dysfunction and the development of neurodegeneration [36]. The significant decreases in GAI1 and GAI3 in the DG can be explained by the specifically significant increase in microglia to this region, which might be enhanced to help shaping the final patterns of neural connections during neurogenesis [7,37].

CONCLUSIONS

These collected data provide evidence for a prominent role of glial cells in the pathology of depression. Furthermore, the study presents significant changes in astrocyte count and/or morphology in the hippocampus, DG, mPFC and cerebellar cortex in depression. The study reveals the noteworthy ability of voluntary running to reverse the effects of depression on the abundance of astrocytes and on their specific marker GFAP in the brain regions associated with depression. Additionally, this work provides evidence that both astrocytes and oligodendroglia are implicated in the mitigating effect of voluntary running on depression in the hippocampus, DG, mPFC and cerebellar cortex.

Conflict of interest

The authors declare that no conflicts of interest for this work.

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Fig. 1: Photographs of sections of the control medial frontal cortex stained for a) glial fibrillary acidic protein (GFAP) to detect astrocytes; b) myelin basic protein (MBP) to detect oligodendroglia; c) cluster of differentiation 68 (CD68) to detect microglia (GFAP, MBP and CD68, 400× magnification).
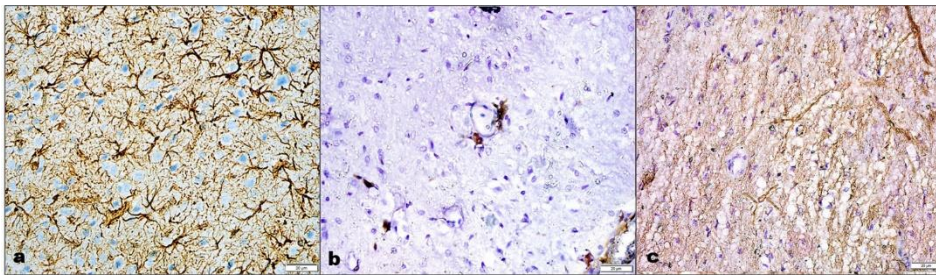

Fig. 2: Graphs showing the results from behaviour tracings during the 6-minute FST for all groups: (a) immobility duration and (b) distance moved.
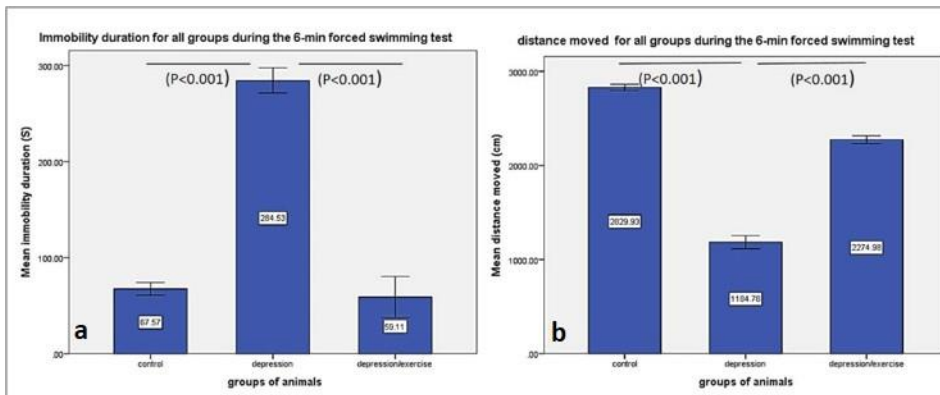

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Fig. 3: Graph showing the changes in mean serum corticosterone levels in different groups.
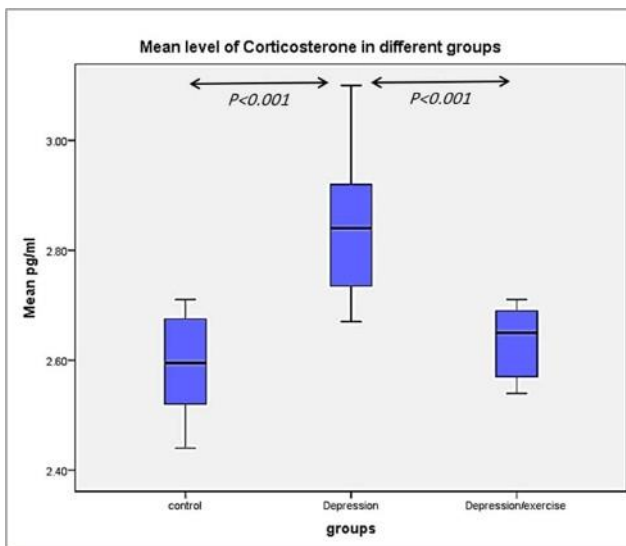

Fig. 4: Graph showing the changes in mean astrocytic counts in different brain areas.
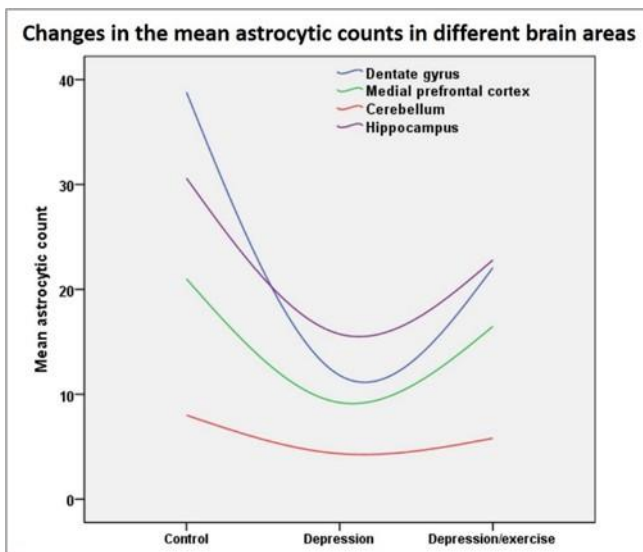
Fig. 5: Plate showing immunohistochemistry for GFAP-positive cells (arrows) in the brain areas of different groups. In the control group (a), astrocytes display dense, distinct, dark cell bodies and "bushy", densely packed processes in the hippocampus (a1), DG (a2), mPFC (a3) and cerebellar cortex (a4). In the depression group (b), the astrocytes appear dispersed and display relatively indistinct cell bodies and distorted processes in the hippocampus (b1), DG (b2), mPFC (b3) and cerebellar cortex (b4). In the depression/exercise group (c), the astrocytes appeared to regain their immunoreactivity, exhibiting dense cell bodies and complex, dense process extensions in the hippocampus (c1), DG (c2), mPFC (c3) and cerebellar cortex (c4) (GFAP, 1000× magnification).
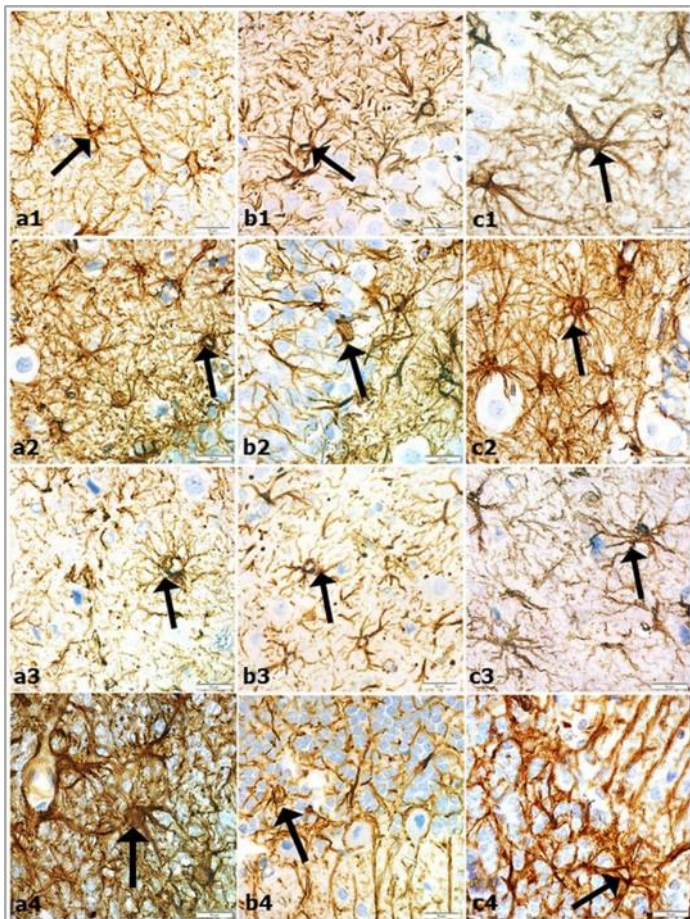
Fig. 6: Graph showing the mean OD values of GFAP immunostaining in different brain areas of different groups.
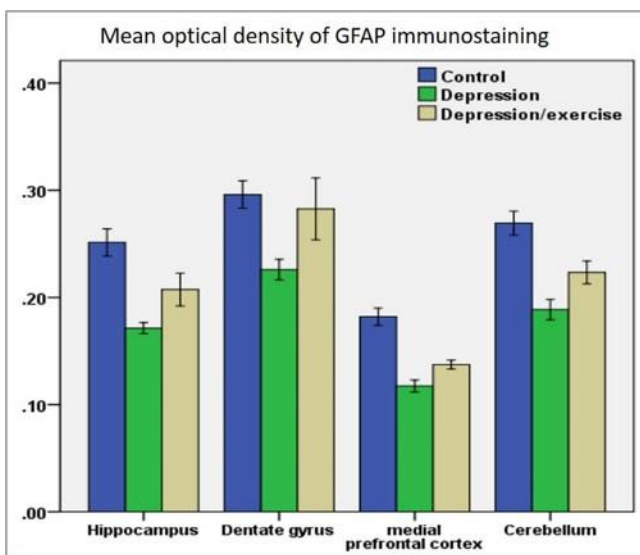

Table 1. Means and significance of different glia cells' numbers and the total glia cells' counts in different groups in all examined brain areas.

Regions	Variables	Control (n=15)	Depression (n=15)	Depression/Exercise (n=15)	*P* value
Dentate gyrus	**Astrocytes**	38.8±5.6	11.8±3.1	22.07±1	*P*1<0.001* *P*2=0.004*
	Oligodendroglia	11.47±2.1	9.2±0.8	98±10.5	*P*1=0.314 *P*2<0.001*
	Microgliocytes	4.9±0.3	5.7±0.7	16±1.9	*P*1=0.255 *P*2<0.001*
	Total glia cells	55.1±6.3	26.7±3.2	136.1±10.1	*P*1=0.007* *P*2<0.001*
Medial frontal cortex	**Astrocytes**	21±4	9.2±3	16.5±1.1	*P*1=0.027* *P*2=0.033*
	Oligodendroglia	7.7±1.2	11.1±1.7	30.5±5.6	*P*1=0.11 *P*2=0.003*
	Microgliocytes	11.3±1.5	8.9±0.8	13.9±1.3	*P*1=0.196 *P*2=0.004*
	Total glia cells	39.9±4.5	29.2±3.7	60.8±5.1	*P*1=0.098 *P*2<0.001*

Region	Cell type	Control	Depression	Depression/Exercise	P value
Hippocampus	**Astrocytes**	30.6±2.6	15.7±2.8	22.8±0.8	P1<0.001* P2=0.024*
	Oligodendroglia	21.7±2.6	18.5±2.2	26.7±0.8	P1=0.368 P2=0.002*
	Microgliocytes	10.9±1.6	10.7±1	7.7±0.7	P1=0.918 P2=0.018*
	Total glia cells	63.2±3.8	45±4.1	57.3±1.6	P1<0.001* P2=0.871
Cerebellar cortex	**Astrocytes**	8±1.2	4.33±0.4	5.8±0.3	P1=0.007* P2=0.01*
	Oligodendroglia	238.3±14	180±10.3	353.3±15.2	P1=0.002* P2<0.001*
	Microgliocytes	4.2±0.4	4.3±0.3	4.7±0.3	P1=0.777 P2=0.301
	Total glia cells	250.5±14	188±10.4	363.8±15.2	P1=0.002* P2<0.001*

*P*1 significance of the depression group compared to the control group. Significance at *p*< 0.05

*P*2 significance of the depression/exercise group compared to the depression group. Significance at *p*< 0.05

Table 2. Astrocytic indices and significance in different groups in all examined brain areas.

Regions	Glial Glial Astrocyte Indices	Control (n=15)	Depression (n=15)	Depression/Exercise (n=15)	*P* value
Dentate gyrus	**GAI1 (A/M)**	4.1±0.5	1.6±0.5	0.3±0.1	P1=<0.001* P2=0.029*
	GAI2 (A/O)	8.4±1.3	2.3±0.7	1.6±0.1	P1<0.001* P2=0.534
	GAI3 (A/Total)	0.7±0.04	0.4±0.1	0.2±0.03	P1<0.001* P2=0.022*

Medial frontal cortex	**GAI1 (A/M)**	3.8±0.8	1±0.3	0.8±0.1	*P*1<0.001* *P*2=0.805
	GAI2 (A/O)	2.7±0.8	1±0.4	1.4±0.2	*P*1=0.023* *P*2=0.640
	GAI3 (A/Total)	0.5±0.1	0.2±0.1	0.3±0.02	*P*1<0.001* *P*2=0.269
Hippocampus	**GAI1 (A/M)**	2.6±0.8	0.9±0.2	0.7±0.1	*P*1=0.015* *P*2=0.872
	GAI2 (A/O)	7.5±1.6	2.7±0.5	2.5±0.2	*P*1=0.001* *P*2=0.899
	GAI3 (A/Total)	0.5±0.3	0.3±0.05	0.4±0.01	*P*1<0.001* *P*2=0.052
Cerebellar cortex	**GAI1 (A/M)**	0.04±0.007	0.03±0.003	0.02±0.002	*P*1=0.072 *P*2=0.165
	GAI2 (A/O)	1.9±0.3	1.02±0.1	1.3±0.1	*P*1<0.001* *P*2=0.291
	GAI3 (A/Total)	0.03±0.006	0.02±0.003	0.02±0.002	*P*1=0.061 *P*2=0.192

GAI1 (A/M) = number of astrocytes/ number of microglia; GAI2 (A/O) = number of astrocytes/ number of oligodendroglia; and GAI3 (A/T) = number of astrocytes/total number of counted glial cells. *P*1 significance of the depression group compared to the control group. *P*2 significance of the depression/exercise group compared to the depression group. Significance at *p*< 0.05