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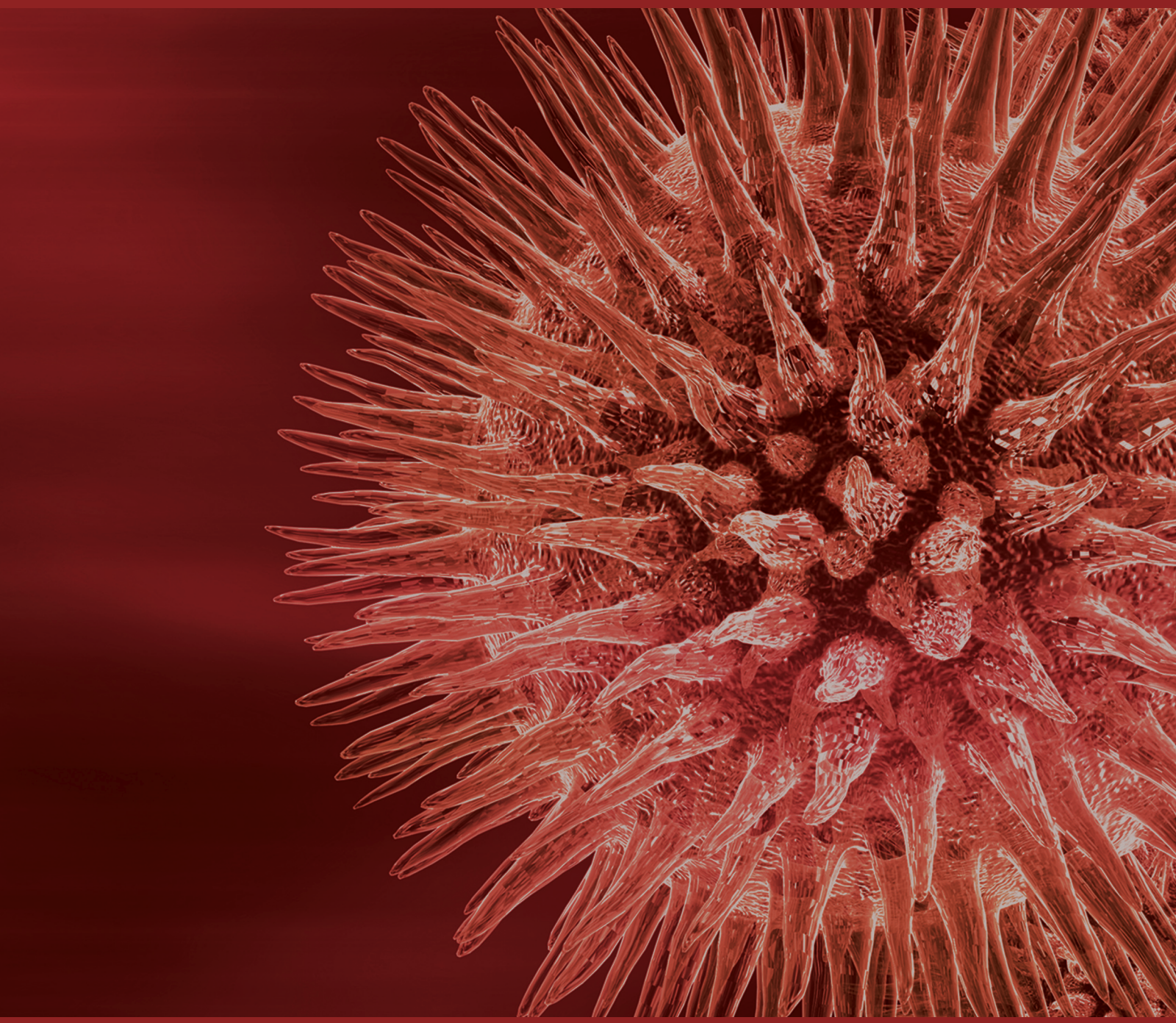
Novelties in the Anatomy of the Central Nervous System and Related Disorders

Guest Editors: Branislav Filipović, Nevena Radonjic, Igor Jakovcevski, and Milos Petrovic



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Editorial

Novelties in the Anatomy of the Central Nervous System and Related Disorders

Branislav Filipović,[1] Nevena Radonjic,[2,3] Igor Jakovcevski,[4] and Milos Petrovic[5]

[1] *Faculty of Medicine, Institute of Anatomy "Niko Miljanic", University of Belgrade, Belgrade, Serbia*
[2] *Faculty of Medicine, Institute of Medical and Clinical Biochemistry, University of Belgrade, Belgrade, Serbia*
[3] *Neuroscience Department, University of Connecticut Health Center, Farmington, CT, USA*
[4] *Center for Molecular Neurobiology, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany*
[5] *Cellular Neurobiology and Neurophysiology Laboratories, Centro de Investigación Médica Aplicada y Universidad de Navarra, Pamplona, Spain*

Correspondence should be addressed to Branislav Filipović; filipovic.branislav@gmail.com

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Anatomy of the central nervous system (CNS) is far beyond the classical approach to anatomy investigations. Studies of the CNS are, nowadays, comprising various fields of medicine, starting from clinical investigations, up to the animal models, so they are useful in studies of different neuropsychiatric diseases and disorders, such as schizophrenia, posttraumatic stress syndrome, and Alzheimer's disease. Serious investigations in the field of the CNS are inseparable from the technological advances in the research, but also in diagnostics. This is the reason why we have gathered, at a glance, the papers that have very little in common. But the *file rouge* is the anatomy of the CNS and its changes illustrated in this unity in differences. Our initial aim was to illustrate the importance of the knowledge of CNS anatomy, for either the investigators in this field or the clinicians, devoted to solving the problem of serious neuropsychiatric diseases.

Two papers are dealing with the effects of maternal deprivation on rats brains. Maternal deprivation represents widely used animal model of schizophrenia and it is based on the 24 hr separation of the 9-day-old offspring from their mother. The rats were sacrificed in early adult period. Dr. M. Aksić and his team revealed that maternally deprived rats had a 28% smaller cell soma area in the prefrontal cortex, (30% in retrosplenial cortex), and 15% in motor cortex compared to the controls. Using the same model, Dr. B. Marković and coauthors outlined the significant decrease in the activity of acetylcholine esterase in the cortex and increase in the hippocampus of maternally deprived rats.

Dr. M. Kutová and coworkers are claiming that gross *postmortem* changes in the planum temporale in Alzheimer's disease may be at least partially attributed to neurohistological changes in the layer III pyramidal neurons.

An interesting study of hypothalamopituitary-adrenocortical role in the hyperthermia has been conducted by Tseng et al. Their results suggest that human umbilical cord blood-derived stem cells therapy may improve outcomes of heatstroke in mice by reducing systemic inflammation as well as hypothalamopituitary-adrenocortical axis impairment.

A clinical study of posttraumatic stress disorder (PTSD), presented by Dr. A. Starcevic et al., demonstrates a significant reduction in the volumes of amygdala in the left hemisphere in the PTSD patients, suggesting the eventual role of amygdaloid complex in this disease.

All presented papers show the interest of the investigators of different kinds in the anatomy of the CNS and its change in different conditions, in the animals in the experimental models, *postmortem* human brains, or PTSD patients. With the advances in knowledge of the CNS anatomy structures,

perhaps their role in the pathogenesis of diseases and disorders of different origin is going to be, even partially, clarified.

Branislav Filipović
Nevena Radonjic
Igor Jakovcevski
Milos Petrovic

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Review Article

Second-Generation Antipsychotics and Extrapyramidal Adverse Effects

Nevena Divac,[1] Milica Prostran,[1] Igor Jakovcevski,[2] and Natasa Cerovac[3]

[1] *Institute of Pharmacology, Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Belgrade, Dr. Subotica 1, 11000 Belgrade, Serbia*
[2] *University Medical Center Hamburg-Eppendorf, Center for Molecular Neurobiology, Falkenried 94, 20251 Hamburg, Germany*
[3] *Clinic for Neurology and Psychiatry for Children and Youth, Faculty of Medicine, University of Belgrade, Dr. Subotica 6a, 11000 Belgrade, Serbia*

Correspondence should be addressed to Nevena Divac; ndivac@med.bg.ac.rs

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Antipsychotic-induced extrapyramidal adverse effects are well recognized in the context of first-generation antipsychotic drugs. However, the introduction of second-generation antipsychotics, with atypical mechanism of action, especially lower dopamine receptors affinity, was met with great expectations among clinicians regarding their potentially lower propensity to cause extrapyramidal syndrome. This review gives a brief summary of the recent literature relevant to second-generation antipsychotics and extrapyramidal syndrome. Numerous studies have examined the incidence and severity of extrapyramidal syndrome with first- and second-generation antipsychotics. The majority of these studies clearly indicate that extrapyramidal syndrome does occur with second-generation agents, though in lower rates in comparison with first generation. Risk factors are the choice of a particular second-generation agent (with clozapine carrying the lowest risk and risperidone the highest), high doses, history of previous extrapyramidal symptoms, and comorbidity. Also, in comparative studies, the choice of a first-generation comparator significantly influences the results. Extrapyramidal syndrome remains clinically important even in the era of second-generation antipsychotics. The incidence and severity of extrapyramidal syndrome differ amongst these antipsychotics, but the fact is that these drugs have not lived up to the expectation regarding their tolerability.

1. Background

Antipsychotic drugs are the cornerstone of the pharmacological treatment of schizophrenia. The introduction of the first antipsychotic chlorpromazine in 1952 marked the new era in psychopharmacology [1]. However, those early antipsychotics, now referred to as first-generation antipsychotics (FGAs), such as chlorpromazine, haloperidol, or fluphenazine, though effective in relieving positive symptoms of the disease, have some serious limitations. Lack of efficacy regarding negative symptoms and the adverse effects, especially extrapyramidal symptoms (EPS), are serious drawbacks of these drugs. The development of newer antipsychotics (risperidone, olanzapine, quetiapine, etc.) since 1990s was met with great expectations. These novel antipsychotics, now referred to as second-generation antipsychotics (SGAs), have been modeled on the prototype drug clozapine [2].

Clozapine was the first antipsychotic that proved to be efficacious in treatment-refractory schizophrenia [3], but it was also the first antipsychotic devoid of EPS. However, the ability of clozapine to cause agranulocytosis as a serious adverse effect led to voluntary withdrawal of the drug by the manufacturer, with subsequent reintroduction in 1989, followed by strict regulation regarding indications and white blood cells count followup [4]. The efficacy of clozapine and its inability to produce EPS were motives for the development of similar antipsychotics, but with the safer profile. Second-generation antipsychotics such as olanzapine, risperidone, quetiapine, and more recently ziprasidone and aripiprazole soon became the mainstay of the treatment of schizophrenia,

despite their higher costs and inconsistency of the data showing their superior efficacy versus FGAs [5, 6].

Clozapine, as the first SGA, actually discredited the theory that EPS are an unavoidable accompaniment of antipsychotic efficacy. Previously, EPS were considered as an essential component of antipsychotic "neuroleptic" effect. The association of antidopaminergic (D2) potency, antipsychotic effect, and EPS (due to loss of dopamine in the extrapyramidal part of the central nervous system) was the foundation for the dopamine hypothesis of schizophrenia [7, 8]. The ability of a substance to induce EPS experimentally was considered as proof of its antipsychotic potential. However, dopamine hypothesis of schizophrenia became obsolete with the introduction of clozapine and other SGAs.

All antipsychotic agents have some degree of antagonistic affinity for dopaminergic D2 receptors. It was shown that first-generation antipsychotics, though known to block other receptors, not only exert their antipsychotic, but also their extrapyramidal effects, primarily by binding to D2 receptors in the central nervous system. First-generation antipsychotics produce their therapeutic (antipsychotic) effect at 60–80% of D2 occupancy, while the 75–80% of D2 receptor occupancy leads to the acute EPS [9–11]. Therefore, the overlap between desired and adverse D2 receptor occupancy is mostly unavoidable with FGAs. On the other hand, the therapeutic effects of SGAs are attributable also to some degree to D2 antagonism, but more to blockade of certain serotonin (mostly 5HT2A) receptors. Surprisingly, clozapine, as the most effective antipsychotic so far, has the lowest D2 affinity (Table 1). It was also suggested and shown in animal models that SGAs actually bind to and dissociate from D2 receptors in an atypical manner (Kapur, 2001). Loose binding to and fast dissociation of SGAs from D2 receptors may be the cause of their lower EPS propensity [12]. The affinity of antipsychotic drugs for D2 receptors is shown in Table 1. While the antipsychotic effect of FGAs correlates with D2 affinity, that is not the case with SGAs.

The efficacy of a pharmacological treatment cannot be interpreted independently from its adverse effects profile. Better tolerability of SGAs was considered as one of their major advantages as a class [7]. The idea of treating schizophrenia without producing EPS was very attractive for psychiatric care professionals, as well as for the patients. However, post-clozapine SGAs have not fully lived up to these expectations and intolerability due to the fact that EPS remain a considerable problem in the treatment of schizophrenia [7, 13]. It is now evident that all SGAs, apart from clozapine, have propensity to cause certain degree of EPS. The results of recent clinical trials and meta-analyses have shown that there is no advantage of SGAs regarding tolerability and effectiveness compared with FGAs [13, 14]. Also, postmarketing followup of SGAs surfaced other adverse effects such as weight gain and metabolic side effects. However, notable metabolic side effects are also caused by FGAs and the higher cardiometabolic risk of SGAs versus FGAs has not been confirmed [15]. Therefore, the oversimplified distinction of antipsychotic drugs classes, in which FGAs are responsible for EPS and SGAs for metabolic side effects, though ingrained in clinical practice, is actually not supported by recent findings [1, 16].

Table 1: First- and second-generation antipsychotics and D2 antagonism.

Antagonistic D2 effect	First-generation antipsychotics	Second-generation antipsychotics
Low	Chlorpromazine Levomepromazine Thioridazine	Clozapine Quetiapine
Intermediate	Trifluoperazine Perphenazine	Olanzapine
High	Haloperidol Fluphenazine Flupentixol	Risperidone Ziprasidone Aripiprazole (possible D2 agonism)

This review summarizes the recent reported results regarding the risk of EPS development in patients treated with different classes of antipsychotic drugs.

2. Extrapyramidal Symptoms

EPS include acute dystonias, akathisia, Parkinsonism, and tardive dyskinesia (TD). EPS are serious, sometimes debilitating and stigmatizing adverse effects, and require additional pharmacotherapy. EPS develop into two phases. Early, acute EPS most often develop upon the beginning of treatment with antipsychotics or when the dose is increased. The later-onset EPS usually occur after prolonged treatment and present as tardive dyskinesia (TD). The motor manifestations include akathisia (restlessness and pacing), acute dystonia (sustained abnormal postures and muscle spasms, especially of the head or neck), and Parkinsonism (tremor, skeletal muscle rigidity, and/or bradykinesia) [13, 17]. TD is characterized by involuntary, repetitive facial movements such as grimacing, tongue protruding, oculogyric crisis, and lips puckering, as well as torso and limb movements. Acute EPS are one of the main causes of poor adherence to antipsychotic treatment due to the reversibility of symptoms, while late-onset TD has the most serious impact on patients and caregivers with respect to quality of life [18, 19]. TD may persist after the discontinuation of treatment or even be irreversible. It is estimated that approximately 50% of patients treated with high-potency FGAs (such as haloperidol) develop acute EPS within the first several days of treatment. The prevalence of TD is somewhat less known due to differences in design and methodologies among studies that have investigated this problem [13, 20, 21]. Prevalence of TD has been reported to be 0.5% to 70% of patients receiving FGAs, with the average rate being between 24% and 30% [22, 23].

Acute EPS usually respond to dose reduction of the antipsychotic agent or require additional pharmacological treatment.

Acute dystonia occurs within first few days after the initiation of the antipsychotic treatment and can be effectively prevented or reversed with anticholinergic drugs such as biperiden [24–26]. Risk factors for acute dystonia are young

age and male gender, history of substance abuse, and family history of dystonia [27, 28]. Acute dystonia is common with FGAs such as haloperidol [29] and less common with SGAs. It is reported that approximately 7.2% treated with long-acting parenteral risperidone develop acute dystonic reactions [30]. Also, case reports regarding acute dystonia after initiation of antipsychotic treatment with aripiprazole and ziprasidone have been published [31, 32].

Akathisia is very common (about one half of all cases of EPS), poorly understood, and difficult to treat. It occurs mostly within the first three months of treatment. Akathisia does not respond to anticholinergic medication, but antipsychotic dose reduction, liposoluble beta adrenergic blockers, and benzodiazepines have proved effective [24, 25]. The rough estimation is that about 25% of patients treated with FGAs develop akathisia, but it is also common with SGAs. Some researchers suggest that akathisia rates do not differ between FGAs and SGAs [24]. It was previously suggested that SGAs clozapine and quetiapine carry the lowest risk for akathisia, yet it was not confirmed in some blinded reviews [33]. Also, the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) study as a randomized, partially open-label study in which efficacy and side effects of multiple SGAs with an FGA perphenazine showed that akathisia remains a problem with SGAs, though at lower rates compared to FGAs [24, 34]. Based on CATIE study, it appeared that risperidone and perphenazine, for example, both cause akathisia in 7% of patients. Further analysis of the CATIE study data revealed no difference between any of the antipsychotics tested in this study regarding incidence of akathisia and other EPS in patients with chronic schizophrenia during maintenance of antipsychotic treatment for up to 18 months [35]. However, the well-known limitations of the CATIE (the choice of an intermediate-potency FGA perphenazine, the nonrandomized allocation of patients with the tardive dyskinesia to a SGA treatment) should be considered when interpreting these results.

Parkinsonism induced by antipsychotics occurs between few days and up to several months after the initiation of the treatment. Risk factors for this type of Parkinsonism are age (elderly), gender (females), cognitive deficit, and early onset EPS [36]. Antipsychotic-induced Parkinsonism is considered a reversible condition although its duration is variable. The treatment of choice is not established, but dose reduction and anticholinergic drugs may be useful. However, anticholinergics should be avoided in the elderly patients due to their side effects such as cognitive deterioration, urinary retention, dry mouth, and risk of glaucoma exacerbation. Although switching to SGAs is often recommended in cases of Parkinsonism, the rates of Parkinsonism induced by SGAs (e.g., 26% with olanzapine) are lower than those with the FGAs (55% with haloperidol), but not negligible [37]. Other evidence shows virtually no advantages of SGAs compared to FGAs in relation to Parkinsonism as an adverse effect, especially when the potency and dose are considered. It was shown that high doses of SGAs (such as olanzapine, risperidone, or quetiapine) caused Parkinsonism in high doses at a similar rate as low-potency FGA (chlorpromazine), but the risk was 50% higher in high-potency FGA group [38]. In CATIE study, the results regarding Parkinsonism were also conflicting. CATIE study includes patients with previous tardive dyskinesia, who at baseline were excluded from perphenazine branch. This could lead to potential bias, meaning that patients with previous vulnerability to EPS were allocated exclusively to SGA branch. In order to avoid this potential bias, only patients without previous TD were included in comparisons for Parkinsonism. The proportion of patients showing no evidence of Parkinsonism at baseline who met at least one of the three criteria for Parkinsonism during the subsequent follow-up period revealed no substantial differences between treatment groups. At the 12-month followup, covariate-adjusted rates of Parkinsonism were 37%–44% for SGAs and 37% for perphenazine [35]. However, the choice of an intermediate-potency FGA (perphenazine) as a comparator in modest doses in CATIE could probably be responsible for the lack of significant difference between FGAs and SGAs regarding incidence of Parkinsonism. The Cost Utility of the Latest Antipsychotics in Schizophrenia Study Band 1 (CUtLASS-1) as a randomized controlled trial (RCT) that tested the hypothesis that the clinical and cost-effectiveness of SGAs is superior in individuals whose antipsychotic treatment is changed due to insufficient efficacy or side-effects of previous treatment. This study also did not show statistically significant difference between the treatment groups in terms of Parkinsonism between SGA and FGA patients [39] between SGA and FGA patients. The results were similar regarding akathisia. As in CATIE study, the main limitation of this study is the choice of FGA comparator. Haloperidol as the high-potency FGA was a rare choice at baseline, while sulpiride was the most common. Sulpiride is considered as an FGA with atypical properties and its low propensity for EPS is well established [40].

Tardive dyskinesia occurs after months or years of antipsychotic therapy. The risk of TD development is highest in the first five years of treatment with FGAs [24]. Leading risk factors for TD are increased age, non-Caucasian race, female gender, a history of diabetes, organic brain damage, and the presence of negative symptoms of schizophrenia [41]. TD can also occur spontaneously in patients diagnosed with schizophrenia at the rate of 0.5% per year [42]. Management of TD is different than the management of acute EPS. Anticholinergic drugs are not recommended (actually, these drugs have been shown to exacerbate TD). The primary step is, according to guidelines, switching from the causative agent to an SGA followed by, if necessary, additional pharmacological treatment. An empirical treatment algorithm from Margolese et al. suggests tapering of anticholinergic drugs, switching to an SGA and, if necessary addition of tetrabenazine. Finally adding experimental therapy including donepezil/melatonin/vitamin E/vitamin B6/branched-chain amino acids (BCAAs) should be considered if previous steps do not provide relief [43]. Clozapine is considered the safest, even beneficial, SGA regarding TD due its ability to improve involuntary symptoms [41]. A recent prospective cohort study on TD incidence amongst outpatients on antipsychotic maintenance therapy showed some disappointing results regarding SGAs and TD incidence. While most of the previously conducted studies showed that the risk of TD with

SGAs is one-quarter that of FGAs, the results of this study suggest that the risk with SGAs is more than half that of FGAs (excluding clozapine patients) or more than two-thirds of the risk (including clozapine patients) [44]. The finding of surprisingly high rate of TD among clozapine patients in this study was attributed to certain confounding factors, such as confounding by indication (prescribing of clozapine to patients with TD or at-risk for TD), and should be interpreted with caution. In CATIE study, patients with TD were excluded from being randomized to perphenazine treatment. There were no statistically significant differences in the rate of new onset TD across the group of antipsychotic drugs. The rates ranged from 13% (quetiapine) to 17% (perphenazine) [13]. Since patients in the FGA (perphenazine) group were free from previous TD, CATIE study does not enable true comparison between FGAs and SGAs regarding TD, but it offers some valuable insight into predisposing factors for TD registered as baseline. These factors are older age, previous exposure to FGA and anticholinergic medication, previous longer antipsychotic treatment, and acute EPS [13, 24]. The CUtLASS-1 study showed unexpectedly the increase of TD incidence in the SGA group of patients during the 12th week of treatment, but this was probably due to switch of treatment (withdrawal of D2 blocking drug and the initiation of an SGA with more anticholinergic effects). This difference in the TD incidence was diminished by 52nd week of the followup [39].

Recent studies on the propensity of FGAs and SGAs to cause EPS yielded conflicting results [35, 37, 39, 45]. When interpreting these studies, it is of utmost importance to consider methodological issues and limitations, some of which are doses of antipsychotics, choice of an FGA comparator, duration of the study, inclusion and exclusion criteria, baseline patients' characteristics, and sensitivity of the criteria for EPS.

EPS remain the most serious problem among patients affected with schizophrenia, even in the era of new antipsychotics with less affinity towards D2 receptors. Upon the introduction of second-generation antipsychotics, these agents were defined as atypical based on their mechanism of action. Atypical antipsychotics expressed less affinity for striatal D2 receptors than typical, FGAs, and different levels of 5-HT2A antagonism, alpha-1 antagonism, or cholinergic antagonism. However, all SGAs still affect D2 receptors to some degree, with clozapine having the least affinity [7, 46] and therefore have some nonnegligible EPS liabilities.

3. Conclusion

SGAs have not completely fulfilled the expectation of being EPS-free antipsychotic drugs. Though recommended by current guidelines as the first-line therapy in the treatment of schizophrenia [47], the superiority of these drugs in terms of better efficacy and tolerability is not clear. Recent studies showed that SGAs do not significantly differ from FGAs in terms of efficacy (with the exception of clozapine for treatment-resistant patients) and have in general lower liability to cause EPS than FGAs, but with great variations within the class [48].

The likelihood of causing EPS with an SGA exists and depends on many factors. The patient's characteristics (age, gender, and concomitant conditions), history of the disease, previous treatment, the choice of a particular antipsychotic, its dose, and duration of treatment and adjuvant therapy should be taken into consideration in the order to minimize the risk of EPS and provide the best quality of care. At this moment, the trial-and-error approach is recommended, since the therapeutic outcome and adverse effects are not easily predictable. Hopefully, the recent, promising advances in pharmacogenomics and neurobiology could provide predictive markers of antipsychotic response and adverse effects and lead towards personalized therapy [48].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors have read and approved the final paper.

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Research Article

Long-Term Effects of Maternal Deprivation on the Neuronal Soma Area in the Rat Neocortex

Milan Aksić,[1] Nevena V. Radonjić,[2] Dubravka Aleksić,[1] Gordana Jevtić,[2] Branka Marković,[3] Nataša Petronijević,[2] Vidosava Radonjić,[1] and Branislav Filipović[1]

[1] *Institute of Anatomy "Niko Miljanic", Faculty of Medicine, University of Belgrade, dr Subotića 4, Belgrade 11000, Serbia*
[2] *Institute of Clinical and Medical Biochemistry, Faculty of Medicine, University of Belgrade, Pasterova 2, Belgrade 11000, Serbia*
[3] *Faculty of Sport and Physical Education, University of Belgrade, Blagoja Parovića 156, Belgrade 11000, Serbia*

Correspondence should be addressed to Milan Aksić; milanaksic@yahoo.com

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Early separation of rat pups from their mothers (*separatio a matrem*) is considered and accepted as an animal model of perinatal stress. Adult rats, separated early postnatally from their mothers, are developing long-lasting changes in the brain and neuroendocrine system, corresponding to the findings observed in schizophrenia and affective disorders. With the aim to investigate the morphological changes in this animal model we exposed 9-day-old (P9) Wistar rats to a 24 h maternal deprivation (MD). At young adult age rats were sacrificed for morphometric analysis and their brains were compared with the control group bred under the same conditions, but without MD. Rats exposed to MD had a 28% smaller cell soma area in the prefrontal cortex (PFCX), 30% in retrosplenial cortex (RSCX), and 15% in motor cortex (MCX) compared to the controls. No difference was observed in the expression of glial fibrillary acidic protein in the neocortex of MD rats compared to the control group. The results of this study demonstrate that stress in early life has a long-term effect on neuronal soma size in cingulate and retrosplenial cortex and is potentially interesting as these structures play an important role in cognition.

1. Introduction

Schizophrenia is a severe psychiatric disorder affecting 0.5–1% of general population. It is clinically characterised by disturbed thought processes, delusions, hallucinations, and reduced social skills [1]. The neuropathological and neuroanatomical findings in patients with schizophrenia have been proposed to arise from dysfunction of structural reorganization during early brain development [2, 3] or postnatally from altered maturation of synaptic elimination [4]. In the patients with first episode of schizophrenia several morphological observations have been described: reduced cortical thickness of anterior cingulate [5] and prefrontal cortex [6], enlarged ventricles, decrease in size of ventromedial temporal lobe structures, parahippocampal cortical thickness, and increase in the gyrification index [7–15]. Morphometric microscopy studies revealed alterations in neuronal density, size and shape in limbic, and temporal and frontal cortical regions [7, 16–25]. Moreover, there is growing evidence that patients with chronic schizophrenia have reduced cortical thickness predominantly in frontotemporal regions [26, 27].

The plethora of clinical evidence implies that schizophrenia has a neurodevelopmental component [28, 29] and has led to the development of novel animal models, focusing specifically on the long-term consequences of early environmental manipulations. Single 24-hour period of maternal deprivation (MD) of rat pups at postnatal day 9 (P9) leads to disturbances in prepulse inhibition and latent inhibition [30, 31] and induces neurochemical changes in brain structures implicated in the neuropathology of schizophrenia [32, 33].

The aim of this study was to elucidate long-term effects of maternal deprivation on the rat brain morphology. We have studied the areas important for information processing such as motor, prefrontal, and retrosplenial cortex. We observed reduced cell soma area of neurons in the cortex of MD animals compared to the controls. However, no difference was observed in expression of GFAP astrocyte marker. These

results suggest that early life stress can alter brain morphology and consequently impact its function.

2. Materials and Methods

2.1. Animals and Procedures. Male and four nulliparous female Wistar rats 3-month-old were put together in standard plexiglass cages with sawdust (26 × 42 × 15 cm), in a temperature controlled room (23 ± 1°C). The rats were on a standard 12 h light/dark cycle with lights on from 7:00 to 19:00 h, with water and food available *ad libitum*. Two weeks later, males were removed and the dams were checked twice daily for delivery. The day of delivery was denoted as a P0. On P9, two litters were subjected to the maternal deprivation procedure according to the previously published protocol [31, 34]. Briefly, dams were removed from the litter at 10:00 am, after which the pups were weighed and returned to their home cage. They remained in their home cage at room temperature for 24 h. On P10, the pups were weighed again and dams were returned to their cages. The dams of the control litters were briefly (3 min) removed from their home cages and the pups were weighed on both P9 and P10. All litters were later left undisturbed except for the routine cleaning of the cages until P21 when litters were weaned and classified according to sex. For morphological and biochemical studies only male rats were used in order to avoid sexual dimorphism [35] and many of the previous studies were performed on males [36, 37]. Animals were sacrificed at the period of young adulthood (P60). All efforts were made to minimize animal suffering and reduce the number of animals used in the study. All experiments were carried out according to the NIH Guide for Care and Use of Laboratory Animals and were approved by the Local Bioethics Committee.

2.2. Tissue Processing and Immunohistochemistry Staining. For morphological analysis, five male animals from the control and experimental group were anaesthetized with chloral hydrate (3 mg/kg, i.p.) and transcardially perfused with the fixative (4% formaldehyde in 0.1 M phosphate buffer). The brains were postfixed for 24 h at +4°C and cryoprotected by infiltration with sucrose for 2 days at 4°C (20% sucrose in 0.1 M phosphate buffer). Brains were frozen by immersion in 2-methyl-butane (Sigma-Aldrich, St. Louis, MO) precooled to −80°C and stored at −80°C until cutting. Serial transverse sections (25 μm thick) were cut on a cryostat (Leica Instruments, Nußloch, Germany). Sections were collected on SuperFrost Plus glass slides (Menzel, Braunschweig, Germany) in a standard sequence so that four sections 250 μm apart were present on each slide. Immunohistochemical staining was performed after water-bath antigen retrieval in 0.01 M sodium citrate solution, pH 9.0, for 30 min at 80°C. Nonspecific binding was blocked using 5% normal goat serum, dissolved in phosphate buffered saline (PBS), pH 7.3, and supplemented with 0.2% Triton X-100, 0.02% sodium azide for 1 h at RT. Incubation with the primary NeuN antibody (mouse monoclonal NeuN antibody, 1 : 1000; Millipore, Schwalbach, Germany), diluted in PBS containing 0.5% lambda-carrageenan (Sigma-Aldrich) and 0.02% sodium azide, was carried out for 2 days at 4°C. After washing in PBS (3×15 min at RT), the endogenous peroxidase activity was blocked by submerging sections in 3% H_2O_2 solution for 10 min. The sections were then incubated for 30 min at RT with EnVision + Dual Link System-HRP (Dako, Carpinteria, CA). After a subsequent wash in PBS, the sections were incubated with diaminobenzidine with chromogen (Dako, Carpinteria, CA) for approximately 20 min, until the immune reaction was visible. Finally, the sections were counterstained in Mayer's hematoxylin (Fisher Scientific, Leicestershire, UK) for 30 sec, rinsed with PBS, dehydrated, and mounted with DPX (Sigma Aldrich). Specificity of staining was controlled by replacing the primary antibody with the normal serum from the animal in which the antibody was produced, which resulted in the absence of signal.

2.3. Estimations of Cells Soma Area of NeuN Immunolabeled Neurons. Estimations of NeuN-positive (NeuN+) cells soma area were performed at the level of the largest cell body cross-sectional area. Coronal brain sections stained for NeuN were selected for analyses. Four sections 250 μm apart were analyzed per animal. NeuN immunolabeled neurons were identified by their position in the prefrontal, retrosplenial, and motor cortex. The sample size was between 20 and 30 neurons per animal. Areas were measured using the ImageTool 2.0 (University of Texas, San Antonio, TX).

2.4. Image Acquisition and Quantitative Analysis of Immunolabeled Neurons. Pictures were taken on optical microscope (DM4000 Leica) with a 40x objective and analyzed in Photoshop 7.0 software (Adobe, San Jose, CA), using a 1 cm grid. NeuN-immunoreactive cells were counted in stereological sections of the rat brains on the same distance from bregma (2.52 mm for prefrontal cortex and −2.76 mm for retrosplenial and motor cortex). The counted number of NeuN-immunoreactive cells was expressed per unit area (μm^2), and we will further refer to it as a profile density. At least 200 random microscope fields (area 20 μm^2) were counted in the retrosplenial, motor cortex and prefrontal cortex of each section.

2.5. Quantitative Western Blot Analysis. For Western blot analysis, five male animals from the control and experimental groups (P60) were killed by cervical dislocation and decapitation without anesthesia. After decapitation, the brains were quickly removed and transferred to liquid nitrogen. The dorsolateral frontal cortex (4.2 mm up to –1.32 mm from bregma; [38]) was homogenized in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail) on ice for 30 min, centrifuged at 18.000 g for 15 min at 4°C, and the supernatant was collected. An equal amount of protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK). Rabbit polyclonal anti-GFAP primary antibody (Dako, Denmark) was used. All membranes were stripped and reprobed with anti-actin antibody (Sigma-Aldrich) to ensure

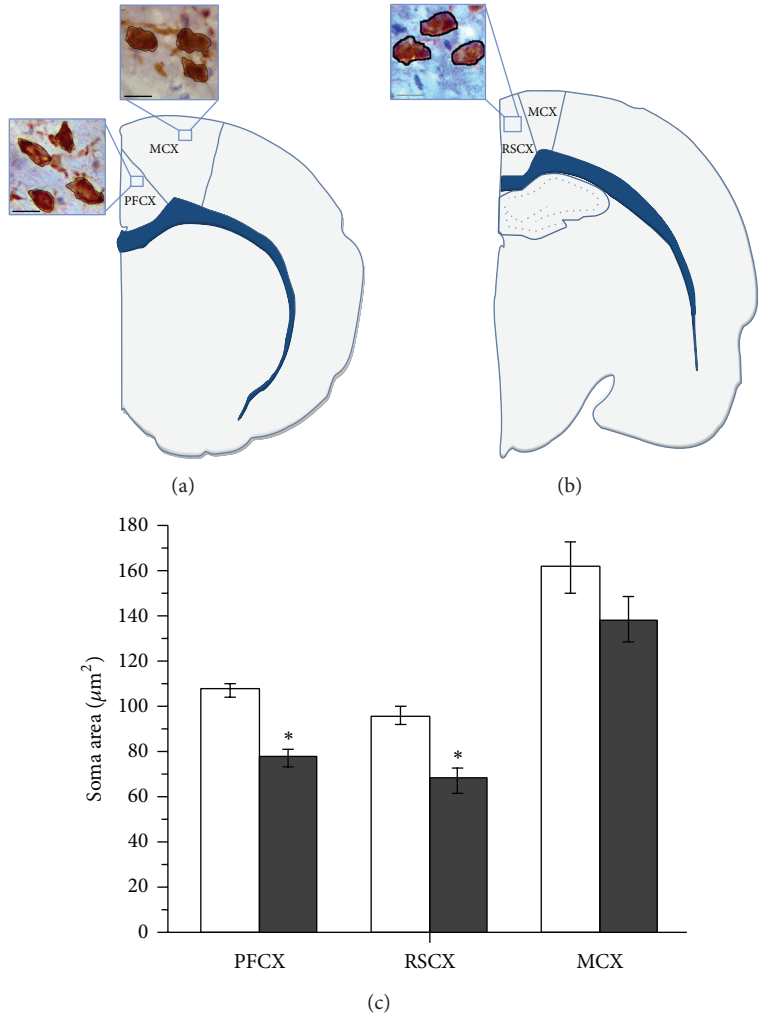

FIGURE 1: Cell soma area of NeuN-positive cells. Schematic representation of prefrontal (cingulate) cortex (PFCX) (a), motor cortex (MCX) (a, b), and retrosplenial cortex (RSCX) (b) on coronal sections of adult rat brain (according to Paxinos and Watson, 2005). Insets: NeuN cells on high magnification; scale bar 5 μm. (c) Profile densities of NeuN-immunolabeled cells in the PFCX, RSCX, and MCX of MD and control rats. Results are presented as the mean values ± SEM.

equal loading. Western blots were scanned and densitometric analysis was performed using ImageQuant 5.2 (GE Healthcare, Buckinghamshire, UK).

2.6. Statistical Analysis. All numerical data are presented as group mean values with standard errors of the mean (SEM). Morphological analysis was performed bilaterally, and if no difference was observed data were pooled together for presentation of results. All comparisons were performed by the Student's t-test, and the threshold value for acceptance of the difference was 5%.

3. Results

3.1. Cell Soma Areas of NeuN Immunolabeled Neurons in Prefrontal, Retrosplenial, and Motor Cortex. Previously, we have demonstrated that MD long-term results in decreased cortical thickness [39]. Here, we explored if this reduction is due to a decrease in size of neuronal soma area. We measured the cell soma area of the NeuN-positive (NeuN+) cells in retrosplenial, prefrontal, and motor cortex. The cell soma area of the NeuN+ cells in the control group of rats was 107.9 ± 2.8 μm^2 in prefrontal cortex, 97 ± 4.2 μm^2 in retrosplenial cortex, and 163.7 ± 10.4 μm^2 in motor cortex (Figure 1). In the MD group, the cell soma area of NeuN+ cells was 78.1 ± 5.1 μm^2 in prefrontal, 68.5 ± 6.1 μm^2 in retrosplenial, and 139.4 ± 9.8 μm^2 in motor cortex (Figure 1). Analysis of the obtained results by t-test showed that this difference is statistically significant in prefrontal and retrosplenial cortex demonstrating stressful effect of maternal separation (PFCX ($P = 0.002$), RSCX ($P = 0.005$), MCX ($P = 0.1$)) (Figure 1).

3.2. Expression of GFAP Protein in Neocortex. Next, we examined the expression of astrocyte marker, GFAP, in neocortex of MD animals and control group (Figure 2). Quantitative analysis of the immunoblot data did not show difference in GFAP expression in the neocortex ($P > 0.05$) (Figure 2). We

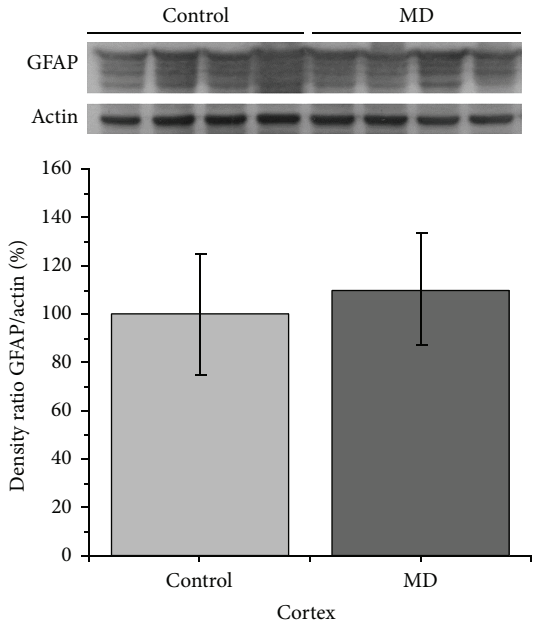

Figure 2: Expression of GFAP in neocortex homogenates. Figure is accompanied by representative immunoblots from the same gel. Results are presented as the mean values ± SEM.

can conclude that in neocortex substantial loss of neurons occurs in animals stressed by maternal deprivation and the levels of GFAP expression in astrocytes are not affected.

4. Discussion

In this study we demonstrate that MD used as a perinatal stressor has long-term effects on neuronal cell soma area and does not affect expression of astrocyte marker in the rat neocortex. Stress is an unavoidable part of human existence. Extreme forms of acute and chronic stress may cause an abnormal mental state and affect behaviour and represent risk factors for psychiatric disorders such as schizophrenia and mood disorders [40, 41]. Previously, we have demonstrated in maternally deprived animals reduced thickness of retrosplenial, prefrontal, and motor cortex and decreased density of neurons in retrosplenial and prefrontal cortex [39]. Also, we observed reduced expression of neuronal marker NeuN in the neocortex of MD rats [39]. Here, we further analyze this phenomenon and observe reduction in the neuronal cell soma area in the prefrontal and retrosplenial cortex. No difference in the neuronal soma size was observed in motor cortex.

Cerebral cortex and hippocampus play a central role in cognition and memory. Hyde and Crook [42] proposed that focal pathological changes in either the prefrontal cortex or mesial temporal lobe could lead to neurochemical changes in multiple neurotransmitter systems such as dopaminergic, glutamatergic, and cholinergic. Recently, our group demonstrated region-specific changes in the activity of acetylcholine esterase (AChE) and density of cholinergic fibers as a result of perinatal maternal deprivation [43].

The prefrontal cortex has been described as a region susceptible to detrimental effects of the exposure to chronic stress [44] and the most promising brain region in the terms of prediction of later psychosis [45]. The most consistent cognitive findings are those testing prefrontal cortical function, such as spatial working memory, antisaccade eye movements, olfactory identification, and tasks requiring rapid processing of information such as story recall [45]. Functional imaging studies showed structural and functional impairments in PFCX of patients with psychiatric disorders, especially in patients exposed to childhood maltreatment [46] or harsh corporal punishment [47]. Postmortem brain studies of schizophrenia patients demonstrated smaller neuron size and neuronal density in the cingulate cortex [22, 23]. Previous studies of PFCX in maternally deprived rats observed numerous impairments in neuronal activity [48, 49], dendritic morphology [50–52], and protein expression [53, 54].

Growing evidence indicates that entorhinal cortex, which has an important role in declarative memory, might be affected in patients with schizophrenia. Postmortem brain studies of schizophrenia patients revealed differences in neuron density, size, and arrangement, abnormalities in synapse-related proteins, alterations in monoaminergic and glutamatergic innervation, and receptor distribution and abnormalities in the expression of cytoskeletal proteins [55].

Lately, epigenetic factors are in the focus of etiological studies of psychiatric disorders. Cues from the social and physical environments in early life are considered to induce variations in epigenetic programing that functions as an adaptive response of the genome to the anticipated environment [56]. In mammalian development, the perinatal period represents a critical period when epigenetic programs are laid down resulting in changes in gene expression and to long-term influences on brain development and behavior. Recently, studies of non-human primate, *Rhesus Macaque*, revealed association of early maternal deprivation with DNA hydroxymethylation changes of promoters of genes in the adult monkey cortex related to neurological functions and psychological disorders [57]. In this study, we have characterised morphological changes induced by maternal deprivation in adult rat cortex. Further studies are necessary to clarify if MD used in this animal model results in epigenetic modulations.

5. Conclusion

In conclusion, this is the first study to provide evidence that early stress caused by MD in rats leads to alteration in size of neuronal cell soma area in the retrosplenial and prefrontal cortex and does not affect expression of astrocyte marker GFAP in the neocortex. These results further contribute to characterisation of MD model of animal perinatal stress and are potentially interesting as these structures play an important role in cognition.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Umbilical Cord Blood-Derived Stem Cells Improve Heat Tolerance and Hypothalamic Damage in Heat Stressed Mice

Ling-Shu Tseng,[1] Sheng-Hsien Chen,[2,3,4] Mao-Tsun Lin,[2,4] and Ying-Chu Lin[1]

[1] *School of Dentistry, Kaohsiung Medical University, Kaohsiung City 807, Taiwan*
[2] *Department of Medical Research, Chi Mei Medical Center, Tainan City 710, Taiwan*
[3] *Da-An Women and Children Hospital, Tainan City 710, Taiwan*
[4] *Department of Biotechnology, Southern Taiwan University of Science and Technology, Tainan City 710, Taiwan*

Correspondence should be addressed to Mao-Tsun Lin; 891201@mail.chimei.org.tw and Ying-Chu Lin; chulin@cc.kmu.edu.tw

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Heatstroke is characterized by excessive hyperthermia associated with systemic inflammatory responses, which leads to multiple organ failure, in which brain disorders predominate. This definition can be almost fulfilled by a mouse model of heatstroke used in the present study. Unanesthetized mice were exposed to whole body heating (41.2°C for 1 hour) and then returned to room temperature (26°C) for recovery. Immediately after termination of whole body heating, heated mice displayed excessive hyperthermia (body core temperature ~42.5°C). Four hours after termination of heat stress, heated mice displayed (i) systemic inflammation; (ii) ischemic, hypoxic, and oxidative damage to the hypothalamus; (iii) hypothalamo-pituitary-adrenocortical axis impairment (reflected by plasma levels of both adrenocorticotrophic-hormone and corticosterone); (iv) decreased fractional survival; and (v) thermoregulatory deficits (e.g., they became hypothermia when they were exposed to room temperature). These heatstroke reactions can be significantly attenuated by human umbilical cord blood-derived $CD34^+$ cells therapy. Our data suggest that human umbilical cord blood-derived stem cells therapy may improve outcomes of heatstroke in mice by reducing systemic inflammation as well as hypothalamo-pituitary-adrenocortical axis impairment.

1. Introduction

Heat exposure causes an increase in c-fos mRNA and protein in different brain regions including the hypothalamus [1]. Large releases have been reported in brain norepinephrine [2], dopamine, serotonin [3], and glutamate [4]. In addition, heat stress causes the increase in hypothalamic numbers of c-fos-positive cells [5, 6] as well as the increase in blood concentrations of both adrenocorticotrophic-hormone (ACTH) and corticosterone [7, 8], suggesting mobilization of the hypothalamic-pituitary-adrenocortical (HPA) axis. According to the findings of Michel et al. [9], intolerance to heat exposure is associated with HPA axis impairment.

Human umbilical cord blood cells (HUCBC) have emerged as an alternative to bone marrow since they have greater availability, lower risk of mediating viral transmission, and weaker immunogenicity [10]. It has also been documented that transplantation of HUCBC is a promising therapeutic strategy against stroke, traumatic brain injury, spinal cord injury, and heatstroke [11–14]. Although we have demonstrated that HUCBC therapy resuscitates heatstroke rats (under anesthesia) by reducing hypothalamic apoptosis [15], evidence is not available about the protective effect of HUCBC-derived $CD34^+$ cells against the heat intolerance, systemic inflammation, HPA axis impairment, and ischemic and oxidation damage to the hypothalamus in unanesthetized mice under heat stress.

To deal with the hypothesis, in the present study, heat tolerance was evaluated by assessing occurrence of thermoregulatory deficit as well as lethality after heat exposure [16, 17]. HPA axis impairment was reflected by the plasma levels of both ACTH and corticosterone in response to heat stress [9]. In addition, hypothalamic levels of cellular ischemia markers (e.g., cerebral blood flow [CBF], glutamate, and lactate/pyruvate ratio), oxidative damage indicators (e.g., malondialdehyde [MDA], oxidative- and reduced- form glutathione [GSH and GSSG], glutathione peroxidase [GP_x], glutathione reductase [GR], nitric oxide [NO_x^-], and 2,3-

dihydroxybenzic acid [2,3-DHBA]), and plasma levels of inflammatory indicators (e.g., tumor necrosis factor-α [TNF-α], interleukin-10 [IL-10], and ICAM-1) in heat stressed mice treated with $CD34^+$ cells or vehicle were assessed [18].

2. Material and Methods

2.1. Human $CD34^+$ Cell Preparation. Human $CD34^+$ cells were isolated from the cord blood of 15 females after informed consent from the mother and IRB approval. Single-cell suspensions of 1×10^5/0.3 mL of HUCBC were administered via the tail vein immediately after the termination of whole body heating (WBH). All protocols were approved by the Animal Ethics Committee of the Chi Mei Medical Center (Tainan, Taiwan) in accordance with the Guide for the Care and Use of Laboratory Animals of the National Science Council and the Guidelines of the Animal Welfare Act.

Human $CD34^+$ cells were isolated from cord blood using a Direct $CD34^+$ Progenitor Cell Isolation kit (Miltenyi Biotec, Bergisch Gladack, Germany) and $CD34^+$ Multisort kit (Miltenyi Biotec) according to the manufacturer's protocol. In brief, human cord blood lymphocytes and monocytes were suspended in 300 μL of phosphate buffered saline (PBS) and 5 mM EDTA. These cells were labeled with a hapten-conjugated monoclonal antibody against CD34 (PharMingen, San Diego, CA), followed by an antihapten antibody coupled with microbeads, and were incubated with beads at ratios of 100 μL of beads per 10^8 cells for 15 min at 4°C. FACS analysis using anti-CD34 antibodies (Phar Mingen) labeled with phycoerythrin (Becton Dickinson, Mountain View, CA) of MACS-sorted cells showed that 96 ± 3% of the selected cells were positive for CD34.

2.2. Murine Model of Heatstroke. ICR (Institute of Cancer Research) of the National Institutes of Health in the USA mice were purchased from the National Animal Center (Taipei, Taiwan) and kept under a 12 h light-dark cycle at controlled temperature (22 ± 2°C) with free access to food and tap water: ICR male mice 8- to 10- week-old were exposed to WBH (41.2°C, relative humidity 50%–55%, and for 1 hour) in an environmental temperature-controlled chamber [16, 17, 19]. The heated mice were returned to the normal room temperature (26°C) after the end of WBH. Mice that survived to day 4 of WBH were considered survivals, and the data were used for analysis of the results. In separate experiments, 4 h following WBH, all of the animals were killed and their organs were removed for histological and biochemical evaluation. The contents of NO_x^-, 2,3-DHBA, glutamate, lactate-to-pyruvate ratio, glycerol, MDA, GSSG, GSH, GP_x and GR, and number of neuronal damage scores in the hypothalamus were determined. In addition, systemic inflammatory responses molecules in the peripheral blood stream were assessed. For rectal temperature measurements, unrestrained, unanesthetized mice were used and measurements were collapsed into 10 min averages, taking one mouse each form each group and changing the sequence thereafter. Rectal temperatures were measured by a thermocouple probe (Bailey Instruments, Saddlebrook, NJ, USA).

2.3. Assessment of Thermoregulatory Function. Immediately after the termination of WBH, the animals were returned to a room temperature of 26°C for recovery. According to the findings of Chatterjee et al. [16, 17], WBH-treated mice became hypothermia, when they were exposed to room temperature (24°C).

2.4. Neuronal Damage Score. At the end of the experiments, animals were killed by an overdose of sodium pentobarbital, and the brains were fixed in situ and left in skull in 100% neutral-buffered formalin for at least 24 hours before removal from the skull. The brain was removed and embedded in paraffin blocks. Serial sections (10 μm thick) through the hypothalamus were stained with hematoxylin and eosin for microscopic examination. The extent of neuronal damage was scored on a scale of 1 to 3, modified from the grading system of Pulsinelli et al. [20], in which 0 is normal, 1 indicates approximately 30% of the neurons are damaged, 2 indicates that approximately 60% of the neurons are damaged, and 3 indicates that 100% of the neurons are damaged. Each hemisphere was evaluated independently by an examiner blinded to the experimental conditions.

2.5. Assessment of CBF and Cerebral PO_2. A 100 μm diameter thermocouple and two 230 μm fibers were attached to the oxygen probe. This combined probe measured oxygen, temperature, and microvascular blood flow. These measurements required OxyLite and Oxyflo instruments. OxyLite 2000 (Oxford Optronix Ltd., Oxford, UK) is a 2-channel device (measuring PO_2 and temperature at two sites simultaneously), whereas OxyFlo 2000 is a 2-channel laser doppler perfusion monitoring instrument. Under anesthesia, the mouse was placed in a stereotaxic apparatus and the combined probe was implanted in to the brain (or the hypothalamus) using the atlas and coordinates of Paxinos and Watson [21].

2.6. Extracellular Levels of Glutamate, Lactate-to-Pyruvate Ratio, Glycerol, NO_x^-, and 2,3-DHBA in the Hypothalamus. Hypothalamic samples were homogenized in 0.05 M phosphate buffer, pH7.0 and then centrifuged at 4000 ×g for 20 min at 4°C. The supernatants were used for determination of cellular levels of glutamate, lactate-to-pyruvate ratio, glycerol, NO_x^-, and 2,3-DHBA. The dialysis probe (4 mm in length CMA/12; Carnegie Medicine, Stockholm, Sweden) was put into the supernatants to obtain the dialysates.

The nitric oxide (NO_x^-) concentration in the dialysates of hypothalamus was measured with the Eicom ENO-20 NO_x^- analysis system [22]. In the Eicom ENO-20 NO_x^- analysis system, after the NO_2^- and NO_3^- in the sample have been separated by the column, the NO_2^- reacts in the acidic solution with the primary aromatic amine to produce an azo compound. Following this, the addition of aromatic amines to the azo compound results in a coupling that produces a diazo compound and the absorbance rate of the red color in this compound is then measured. For measurement of glutamate, lactate-to-pyruvate ratio, and glycerol, the dialysates were injected into a CMA600 microdialysis analyzer (Carnegie Medicine, Stockholm, Sweden). The concentrations of hydroxyl radicals were measured by a

modified procedure based on the hydroxylation of sodium salicylates by hydroxyl radicals, leading to the production of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid [12].

2.7. Determination of Lipid Peroxidation. Lipid peroxidation was assessed by measuring the levels of MDA with 2-thiobarbituric acid (TBA) to form a chromophore absorbing at 532 nm [23]. About 0.1 g of tissue was homogenized with 1.5 mL of 0.1 M phosphate buffer at pH3.5. The reaction mixture (0.2 mL of sample, 1.5 mL of 20% acetic acid, 0.2 mL of 8.1% sodium dodecyl sulfate, and 1.5 mL of aqueous solution of 0.8% TBA, up to 4 mL with distilled water) was heated to 95°C for 1 h, and then 5 mL of N-butanol and pyridine (15 : 1 vol/vol) was added. The mixture was vortexed vigorously, centrifuged at 1500 g for 10 min, and the absorbance of the organic phase was measured at 532 nm. The values were expressed as nanomoles of TBA-reactive substances (MDA equivalent) per milligram of protein.

2.8. Quantification of Total and Oxidized Glutathione. Tissues were homogenized in 5% 5-eslfoslicylic acid (1 : 10 wt/vol) at 0°C, and the supernatants were used for analysis of total and oxidized glutathione. Total glutathione [reduced-form glutathione (GSH) + oxidized-form glutathione (GSSG)] was analyzed according to the Tieze method [24], and GSSG was determined as described by Griffith [25]. The recycling assay for total glutathione is oxidized by 5,5-Dithiosis [2 acid] (DTNB) to give GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid. GSSG is reduced to GSH by the action of the highly specific glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (reduced form; NADPH). The rate of 5-thio-2-nitrobenzoic acid formation is followed at 412 nm and is proportional to the sum of GSH and GSSG present.

2.9. Determination of Glutathione Peroxidase (GP_x) and Glutathione Reductase (GR) Activity. Tissues were homogenized in 0.05 M phosphate buffer, pH7.0 and then centrifuged at 4000 ×g for 20 min at 4°C. The supernatants were used for GP_x and GR activity assay. The GP_x and GR activities were assayed with a commercial GP_x assay kit (Sigma, USA) and a GP assay kit (Sigma, USA), respectively. One unit of GP_x and GR activity was defined as the amount of sample required to oxidize 1 mmol of NADPH per minute based on the molecular absorbance of 6.22×10^6 for NADPH.

2.10. Plasma Concentrations of Inflammatory and Intracellular Adhesion Molecules and Cytokines. Blood samples were taken at 4 hours after the start of heat exposure for determination of TNF-α, IL-10, and intercellular adhesion molecule^{-1} (ICAM-1) levels. The amounts of the cytokines in serum were determined by double antibody sandwich enzyme-linked immunosorbent assay (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Optical densities were read on a plate reader set at 450 nm for TNF-α, IL-10, and ICAM-1. The concentration of TNF-α, IL-10, and ICAM-1 in the serum samples was calculated from the standard curve multiplied by the dilution factor and was expressed as picograms per milliliter [15].

2.11. Plasma Assessment of ACTH and Corticosterone. Plasma ACTH and corticosterone were assayed using ACTH (Rat, Mouse)-RIA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA) and Corticosterone Double Antibody RIA kit (MP Biomedicals, Solon, OH, USA), respectively. All analyses were performed according to manufacturers' instructions.

2.12. Statistical Analysis. All values in the figures and text are expressed as mean ± S.E.M. of n observations, where n represents the number of animals studied. Statistical evaluation was performed by using analysis of variance (ANOVA) followed by a multiple-comparison test (Scheffe's test). The Kaplan Meier analysis was used for determining the significant differences in the survival rate between control and drug-treated groups. The Wilcoxon tests were used for evaluation of neuronal damage scores. The Wilcoxon test converts the scores or values of a variable to ranks, requires calculation of a sum of the ranks, and provides critical values for the sum necessary to test the null hypothesis at a given significant levels. These data were presented as "median", followed by first (Q1) and third (Q3) quartile. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Thermoregulatory Outcome and Lethality Induced by WBH. Functional tests showed balanced thermoregulatory deficits between vehicle-treated WBH group and $CD34^+$ cells-treated WBH mice. $CD34^+$ cells-treated WBH mice showed significant ($P < 0.05$, $n = 12$/group) improvement of functional recovery on thermoregulatory test at 4–16 h compared with vehicle-treated WBH mice (Figure 1). The survival of $CD34^+$-treated WBH mice was 12 of 12 mice and one of 12 for vehicle-treated WBH mice (Figure 1).

3.2. Hypothalamic Cells Damage Induced by WBH. Histological verification showed that hypothalamic values of cell damage score (Table 1, Figure 2) for vehicle-treated WBH mice were significantly higher 4 h after WBH than they were for the non-WBH control mice. Vehicle-treated WBH mice displayed cell body shrinkage, pyknosis of the nucleus, loss of Nissl substance, and disappearance of the nucleolus (Figure 2). As compared to non-WBH control mice, vehicle-treated WBH mice also had significantly higher levels of a cellular damage marker (e.g., glycerol) (Table 2) in the hypothalamus [26, 27]. $CD34^+$ cells-treated WBH mice showed significant improvement of hypothalamic cell damage (Tables 1 and 2).

3.3. Hypothalamic Ischemia and Hypoxia Induced by WBH. Intracerebral assessment revealed that hypothalamic levels of both CBF and PO2 for vehicle-treated heated mice were significantly lower at 4 h after WBH than they were for

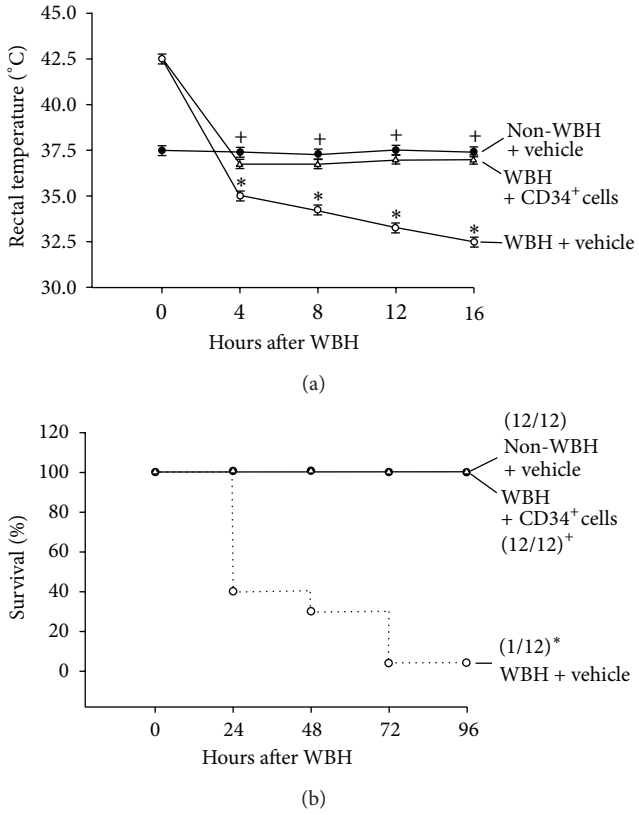

Figure 1: Thermoregulatory deficits and lethality by WBH. (a) Thermoregulatory dysfunction (or animals become hypothermia when exposing them to room temperature, 26°C) caused by whole body heating (WBH; 41.2°C for 1 h). Data are expressed as means ± S.E.M. of 12 mice per group. *At time "0" h after WBH (or immediately after the termination of WBH), heated mice treated with vehicle (WBH + vehicle; "○") or heated mice treated with $CD34^+$ cells (WBH + $CD34^+$ cells; "△") had significantly higher rectal temperature (~42.5°C) than those of non-WBH mice treated with vehicle (non-WBH + vehicle; "●"). In contrast, at time "4" h, "8" h, "12" h, or "16" h after WBH, (WBH + vehicle) group had significantly lower rectal temperature (Tco; ~35°C–~32.5°C) than those of (non-WBH + vehicle) group. +At time "4" h, "8" h, "12" h, or "16" h after WBH, (WBH + $CD34^+$ cells) group had significantly higher rectal temperature than those of (WBH + vehicle) group ($P < 0.01$). (b) Lethality (or decreased percentage survival) caused by WBH. *$P < 0.01$, (non-WBH + vehicle) mice versus (WBH + vehicle) mice. +$P < 0.01$, (WBH + $CD34^+$ cells) group versus (WBH + vehicle) group.

the non-WBH mice (Table 2). Again, the hypothalamic levels of cellular ischemia markers (e.g., glutamate and lactate/pyruvate ratio) [26, 27] for vehicle-treated WBH mice were significantly higher at 4 h after WBH than they were for the non-WBH control mice (Table 2). WBH mice treated with $CD34^+$ cells showed significant improvement of cerebral ischemia and hypoxia (Table 2).

3.4. Oxidative Stress Induced by WBH. Biochemical determination showed that hypothalamic levels of MDA, GSSG/GSH, NO_x^-, and 2,3-DHBA for vehicle-treated WBH mice were all significantly higher at 4 h after WBH than they were for the non-WBH control mice (Table 3). On the other hand, hypothalamic levels of GP_x and GR for vehicle-treated WBH mice were significantly lower than they were for the non-WBH control mice (Table 3). WBH mice treated with $CD34^+$ cells showed significant improvement of oxidative stress caused by WBH (Table 3).

3.5. Increased Plasma Levels of both ACTH and Corticosterone Induced by WBH. Biochemical verification showed that plasma levels of both ACTH and corticosterone for vehicle-treated WBH mice were significantly higher 4 h after WBH than they were for the non-WBH control mice (Table 4). WBH mice treated with $CD34^+$ cells showed significant enhancement of both ACTH and corticosterone in plasma by WBH (Table 4).

3.6. Increased Serum Levels of Systemic Inflammatory Response Indicator Induced by WBH. Biochemical determination showed that serum levels of several systemic inflammatory response indicators such as TNF-α and ICAM-1 for vehicle-treated heated mice were significantly higher 4 h after WBH than they were for the non-WBH mice (Table 4). WBH mice treated with $CD34^+$ cells showed significant reduction of the increased serum levels of these 2 inflammatory response indicators by WBH (Table 4). Table 4 also demonstrated that the serum levels of an anti-inflammatory cytokine, IL-10, were further significantly increased following $CD34^+$ cells therapy.

4. Discussion

Heat tolerance varies considerably among individuals [28]. When exposed to a certain extent of heat exposure, some subjects display a slightly elevated body core temperature (Tco < 40°C) while others become severely ill with a Tco above 40°C. When rats are exposed to heat, they also show a wide interindividual variability [9]. Heat tolerant rats showing the lowest Tco had a highest plasma ACTH and corticosterone levels. Conversely, heat intolerant rats exhibiting the highest Tco had the lowest plasma ACTH and corticosterone. These investigators also provide data to promote that decreased heat tolerance is associated with HPA axis impairment in rats. Consisting with the above hypothesis, we showed that vehicle-treated WBH mice exhibiting lowest survival showed the lowest plasma ACTH and corticosterone levels. In contrast, $CD34^+$ cells-treated WBH mice presented a greater percentage survival as well as a greater plasma level of both ACTH and corticosterone. The mobilization of HPA axis activity is associated with the increase in blood ACTH and corticosterone concentrations [7, 8]. In addition, our previous results have shown that corticosterone supplementation has beneficial effects in treating heatstroke in rats [28]. It is likely that CD34+ cells therapy may improve heat tolerance by attenuating HPA axis impairment in mice during heat exposure.

Accumulating evidence has demonstrated that $CD34^+$ cells transplantation is a promising therapeutic method against cerebral ischemic diseases, such as stroke, traumatic brain injury, and spinal cord injury [11, 13, 14]. Our previous

Table 1: Effects of heat exposure on neuronal damage score of the brain (or the hypothalamus) in different groups of mice.

Treatment groups	Neuronal damage score (0–3)
(1) Non-WBH mice	0 (0, 0)
(2) Non-WBH mice treated with $CD34^+$ cells (1×10^5 cells/0.3 mL, i.v.)	0 (0, 0)
(3) Heated mice treated with vehicle saline (0.3 mL, i.v.)	2 (2, 2)[a]
(4) Heated mice treated with $CD34^+$ cells (1×10^5 cells/0.3 mL, i.v.)	0.75 (0, 0.75)[b]

Samples were measured 4 hours after whole body heating (WBH; 41.2°C for 1 houer) or the equivalent time period for non-heated group. [a]compared with non-WBH group ($P < 0.01$); [b]compared with group 2 ($P < 0.05$). Data are means ± S.E.M. of 12 mice per group.

Table 2: Effects of heat exposure on hypothalamic levels of glutamate, lactate/pyruvate, glycerol, cerebral blood flow (CBF), and PO_2 in different groups of mice.

Treatment groups	Glutamate (percentage of baseline)	Lactate/pyruvate ratio	Glycerol (percentage of baseline)	CBF (BPU)	PO_2 (mmHg)
(1) Non-WBH mice	98 ± 5	10 ± 4	99 ± 6	328 ± 23	21 ± 2
(2) Non-WBH mice treated with $CD34^+$ cells (1×10^5 cells/0.3 mL, i.v.)	100 ± 6	12 ± 5	98 ± 7	307 ± 24	21 ± 3
(3) Heated mice treated with vehicle saline (0.3 mL, i.v.)	196 ± 22[a]	231 ± 32[a]	198 ± 16[a]	162 ± 11[a]	10 ± 1[a]
(4) Heated mice treated with $CD34^+$ cells (1×10^5 cells/0.3 mL, i.v.)	142 ± 10[b]	77 ± 11[b]	66 ± 12[b]	245 ± 16[b]	16 ± 2[b]

Samples were measured 4 hours after whole body heating (WBH) or the equivalent time period for non-heated group. [a]compared with non-WBH group ($P < 0.01$); [b]compared with group 2 ($P < 0.05$). Data are means ± S.E.M. of 12 mice per group.

Table 3: Effect of heat exposure on hypothalamic levels of malondialdehyde (MDA), oxidative-form glutathione (GSSG)/reduced-form glutathione (GSH), glutathione peroxidase (GP_x), glutathione reductase (GR), nitric oxide metabolites (NO_x^-), and 2,3-dihydroxy benzoic acid (2,3-DHBA) in different groups of mice.

Treatment groups	MAD (nmol/mg protein)	GSSG/GSH	GP (mU/mg protein)	GR (mu/mg protein)	NO_x^- (μM)	2,3-DHBA (percentage of baseline)
(1) Non-WBH mice	5 ± 2	0.45 ± 0.14	314 ± 36	175 ± 16	19 ± 2	100 ± 6
(2) Non-WBH mice treated with $CD34^+$ cells	4 ± 2	0.42 ± 0.16	302 ± 33	169 ± 17	17 ± 3	99 ± 5
(3) WBH mice treated with vehicle saline	12 ± 2[a]	2.43 ± 0.38[a]	83 ± 17[a]	81 ± 13[a]	115 ± 12[a]	188 ± 10[a]
(4) WBH mice treated with $CD34^+$ cells	4 ± 2[b]	0.42 ± 0.15[b]	257 ± 28[b]	166 ± 15[b]	21 ± 4	103 ± 5[b]

Samples were measured 4 hours after whole body heating (WBH; 41.2°C for 1 hour) or the equivalent period for non-WBH. [a]compared with non-WBH group ($P < 0.01$); [b]compared with group 2 ($P < 0.05$). Data are means ± S.E.M. of 12 mice per group.

Table 4: Effect of heat exposure on plasma levels of adrenocorticotrophic hormone (ACTH), corticosterone, tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), and ICAM-1 for various groups of mice.

Treatment groups	ACTH ($pg \cdot mL^{-1}$)	Corticosterone ($ng \cdot mL^{-1}$)	TNF (pg/mL)	IL-10 (pg/mL)	ICAM (pg/mL)
(1) Non-WBH mice	372 ± 79	32 ± 19	10 ± 6	5 ± 3	9 ± 4
(2) Non-WBH mice treated with $CD34^+$ cells	361 ± 72	29 ± 17	8 ± 5	6 ± 2	11 ± 5
(3) WBH mice treated with saline	601 ± 98[a]	256 ± 25[a]	415 ± 82[a]	11 ± 4[a]	496 ± 22[a]
(4) WBH mice treated with $CD34^+$ cells	1764 ± 116[b]	643 ± 30[b]	37 ± 6[b]	83 ± 11[b]	67 ± 18[b]

Samples were measured 4 hours after whole body temperature (WBH; 41.2°C for 1 hour) or the equivalent time period for non-WBH group. [a]compared with non-WBH group ($P < 0.01$); [b]compared with group 2 ($P < 0.01$). Data are means ± S.E.M. of 12 mice per group.

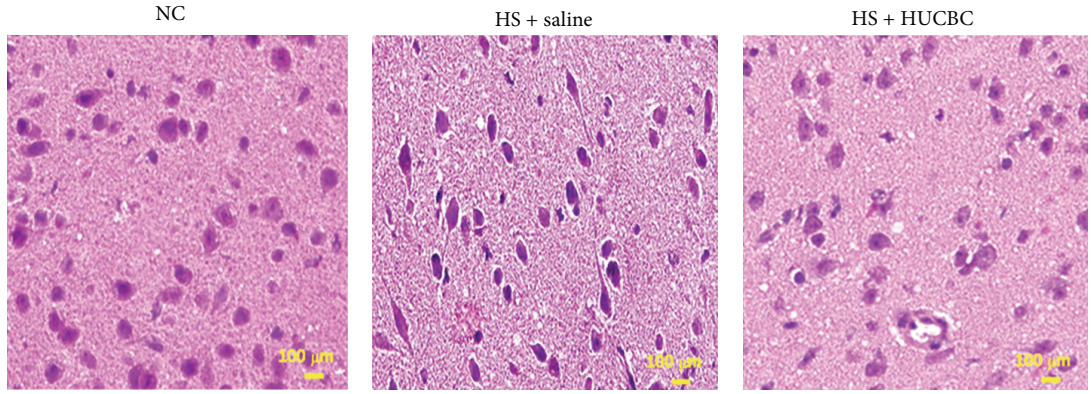

Figure 2: Hypothalamic hematoxylin-eosin (HE) staining 4 hours after WBH. Photographs showing hypothalamic HE staining for a (non-WBH + vehicle), a (WBH + vehicle) mouse, and a (WBH + $CD34^+$ cells) mouse. Samples were obtained 4 hours after WBH for the WBH groups or the equivalent time period for the non-WBH group. (WBH + vehicle) mice showed cell body shrinkage, pyknosis of the nucleus, loss of Nissl substance, and disappearance of the nucleolus which could be attenuated by $CD34^+$ cells treatment.

[12] and present results have also shown that HUCBC-derived $CD34^+$ cells therapy has beneficial effects in heatstroke. Severe heat stress decreases mean arterial pressure (MAP), increases intracranial pressure (ICP), and results in decreased cerebral perfusion pressure (CPP = MAP − ICP), which leads to cerebral ischemia and hypoxia [18]. In addition, hypothalamic and plasma values of cellular ischemia and damage markers, prooxidant enzymes, proinflammatory cytokines, inducible nitric oxide synthase-dependent nitric oxide, and myeloperoxidase activity were all significantly elevated after heatstroke occurrence [18].

In particular, heatstroke causes overproduction of proinflammatory cytokines in both the brain and the peripheral blood stream; this is associated with decreased MAP. In fact, activated inflammation is involved in the severity of acute heart failure [29], septic shock [30], and circulatory shock [31]. Systemic administration of interleukin-1 receptor [32] or glucocorticoids [28] immediately after the onset of heatstroke is able to attenuate arterial hypotension and cerebral ischemia and injury and to improve survival. In an anesthetized rat model of heatstroke, $CD34^+$ cell therapy significantly attenuates arterial hypotension, intracranial hypertension, cerebral ischemia, hypoxia, and injury, and TNF-α overproduction during heatstroke [33]. In order to avoid the influence of anesthetic state, our data further demonstrate that in an unanesthetized mouse model of heatstroke, $CD34^+$ cells therapy promotes survival by attenuating overproduction of systemic inflammatory response molecules (e.g., TNF-α and ICAM-1) and ischemic, hypoxic, and oxidative damage to the hypothalamus. In fact, both anti-inflammatory and proinflammatory cytokines normally have a role to fight infection and prevent immune pathology, respectively [34]. Interleukin-10 has important anti-inflammatory and immunosuppressive properties through attenuation of TNF-α and other proinflammatory cytokines [31]. Thus, it appears that $CD34^+$ therapy may improve brain inflammation during heatstroke by stimulating production of IL-10.

An occurrence of local inflammation process may be considered since TNF-α mRNA decreased in the tolerant rats' hypothalamus and pituitary as compared with control rats [9]. On the contrary, the occurrence of higher stimulation by free radicals led to an increase in the TNF-α mRNA level in the heat exhausted rats. [9, 35]. Heat tolerant rats exhibit low IL-1β and TNF-α mRNAs as well as high corticosterone levels, whereas heat exhausted rats present high IL-1β and TNF-α mRNA, but low corticosterone level [9]. As shown in Figure 1, animals displayed hyperthermia immediately after termination of WBH. Four hours after WBH, vehicle-treated WBH mice showed activated inflammation, hypothalamic ischemia, and HPA axis impairment, which could be significantly attenuated by $CD34^+$ cells therapy.

As compared to heated mice treated with vehicle solution, heated mice treated with $CD34^+$ cells displayed lower hypothalamic values of cellular ischemia (e.g., glutamate and lactate-to-pyruvate ratio), damage (e.g., glycerol) markers, and prooxidant enzymes (e.g., lipid peroxidation and glutathione oxidation). In contrast, CD34+ cells-treated heated mice had higher hypothalamic values of antioxidant defences (e.g., glutathione peroxidase and glutathione reductase). These observations suggest that heat-induced oxidative damage to hypothalamus in mice can be attenuate by $CD34^+$ cells therapy.

5. Conclusion

Heatstroke is characterized by excessive hyperthermia associated with systemic inflammatory responses, which leads to multiple organ failure, in which brain disorders predominates. This definition can be almost fulfilled by our present animal model [26, 36]. Heatstroke mice displayed (i) systemic inflammation; (ii) ischemic, hypoxic, and oxidative damage to the hypothalamus; (iii) hypothalamic-pituitary-adrenocortical axis impairment; (iv) decreased survival; and (v) thermoregulatory deficit. These heatstroke reactions can be significantly attenuated by HUCBC-derived $CD34^+$ cells therapy. Our data suggest that $CD34^+$ cells therapy may improve heat tolerance by reducing systemic inflammation and HPA axis impairment in heatstroke mice.

Abbreviations

WBH: Whole body heating
HUCBC: Hypothalamic-pituitary-adrenal
ACTH: Adrenocorticotropic hormone
NO_x^-: Nitric oxide metabolites
2,3-DHBA: 2,3-dihydroxybenzoic acid
GSSG: Oxidative-form glutathione
GSH: Reduced-form glutathione
GP_x: Glutathione peroxidase
GR: Glutathione reductase
CBF: Cerebral blood flow
ICAM-1: Intercellular adhesion molecule-1
MDA: Malondialdehyde
TBA: 2-Thiobarbituric acid
TNF-α: Tumor necrosis factor-alpha
IL-10: Interleukin-10
IL-1β: Interleukin-1β.

Ethical Approval

The animals and research protocols used in this study followed the guidelines of the Ethical Committee for Use of Animals of Chi Mei Medical Center (Tainan, Taiwan) and national law and policies of the National Science Council of China (Taipei, Taiwan). All efforts were made to minimize the number of animals used and their suffering.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Volumetric Analysis of Amygdala, Hippocampus, and Prefrontal Cortex in Therapy-Naive PTSD Participants

Ana Starcevic,[1] Srdjan Postic,[2] Zoran Radojicic,[3] Branislav Starcevic,[4] Srdjan Milovanovic,[5] Andrej Ilankovic,[5] Ivan Dimitrijevic,[5] Aleksandar Damjanovic,[5] Milan Aksić,[1] and Vidosava Radonjic[1]

[1] *Institute of Anatomy "NikoMiljanić", Medical Faculty, Dr Subotića 4, University of Belgrade, Belgrade, Serbia*
[2] *Clinic of Dental Prosthetic, The Faculty of Stomatology, University of Belgrade, Serbia*
[3] *Institute for Statistics, Faculty of Organizational Sciences, University of Belgrade, Serbia*
[4] *Institute for Traumatology, Emergency Center, Clinical Center of Serbia, Medical Faculty, University of Belgrade, Serbia*
[5] *Institute for Psychiatry, Clinical Center of Serbia, Medical Faculty, University of Belgrade, Serbia*

Correspondence should be addressed to Ana Starcevic; ana.starcevic22@gmail.com

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Objective. In our study we have hypothesized that volume changes of amygdala, hippocampus, and prefrontal cortex are more pronounced in male posttraumatic stress disorder participants. *Material and Methods*. We have conducted a study of 79 male participants who underwent MRI brain scanning. PTSD diagnosis was confirmed in 49 participants. After MRI was taken all scans were software based volume computed and statistically processed. *Results*. We found that left amygdala is the most significant parameter for distinction between PTSD participants and participants without PTSD. There were no significant differences in volumes of hippocampi and prefrontal cortices. Roc curve method outlined left amygdala AUC = 0.898 (95% CI = 0.830–0.967) and right amygdala AUC = 0.882 (95% CI = 0.810–0.954) in the group of PTSD participants which makes both variables highly statistically significant. *Conclusion*. The present investigation revealed significant volume decrease of left amygdala in PTSD patients. Concerning important functions of the amygdala and her neuroanatomical connections with other brain structures, we need to increase number of participants to clarify the correlation between impared amygdala and possible other different brain structures in participants with PTSD.

1. Introduction

Posttraumatic stress disorder or PTSD is an anxiety disorder that can occur following the experience or witnessing of traumatic event [1, 2]. Participants with migraine or tension headaches report high rates of exposure to traumatic events. In addition, about 17% have symptoms consistent with a PTSD diagnosis [3–7]. MRI guided studies revealed reduction of the limbic structures of the brain: hippocampus is believed to be the most frequently reduced structure [8–10], but also anterior cingulate cortex and amygdala are reported as the structures that undergo the volume changes in PTSD [11–17]. Despite some inconsistencies in findings, there is currently no clear evidence for abnormal amygdala volumes in PTSD. One study reported smaller amygdala volumes in a cohort of breast cancer survivors with intrusive recollections compared to survivors without intrusive recollections, but none of the participants met diagnostic criteria for PTSD [18]. Volumes of caudate nucleus, putamen, and lateral ventricles were found to decrease bilaterally in PTSD participants with headaches [19].

In the present study, we have hypothesized that volume changes in amygdala, hippocampus, and prefrontal cortex are more pronounced in male therapy-naive PTSD participants with headaches.

2. Materials and Methods

We have included 79 male participants from Psychiatric Hospital Clinical Centre, Serbia, and Psychiatric Clinic "Katarza." Recruiting criteria for this study were

(1) absence of comorbidity of any kind of psychiatric disorders or diseases;

(2) absence of comorbidity of any kind of neurological disorders or diseases;

(3) absence of previous psychotropic medication;

(4) absence of alcohol or substance abuse;

(5) no data of psychiatric illnesses, homicides, or suicides in participants family anamnesis;

(6) absence of memory impairment;

(7) absence of any kind of head trauma.

Excluding criteria were

(1) the traces of the psychoactive substances in the urine and blood;

(2) major brain abnormalities detected on MRI scans;

(3) evidence about the personality disorder.

Structured psychiatric interview was taken from all participants and they went for psychological testing and magnetic resonance imaging. All participants were followed up for a one-month period and were advised to undergo psychotherapy without taking any medication. They all underwent biochemical testing for illicit drugs. Diagnosis of disorder was assessed according to the guidelines in the 10th revision of the International Classification of Diseases (ICD-10). They were also told to record how often they had headaches and to mark on the pain rating scale when the strongest pain occurred during the day. The scale pain has ten segments, ranging from 0 (no pain) to 10 (the worst possible pain). Hamilton depression rating scale [20] was used for rating the depression level. After we collected all the data from the examination, the initial group of 79 subjects was divided into two groups. First group consisted of 49 participants with PTSD diagnosis and the second consisted of 30 participants without PTSD diagnosis. From the total amount of 79 participants, headache as a symptom appeared in 39 participants. Twenty-five of them (31.6%) had headaches at least twice a week. Fourteen participants (17.7%) had less than twice-a-week headaches and ten participants reported no headaches at all. We found that 49 participants (62%) were with PTSD diagnosis and 30 participants (38%) without PTSD diagnosis. In the range of Hamilton's scale of depression, from the total amount of 79 participants we found 33 participants (41.8%) with minor depression.

We included only males because of potential neuroendocrine involvement on headaches in females. The study was conducted in the period from June to February 2012. All participants signed a written consent.

3. Magnetic Resonance Imaging

This MRI study was performed using a 3.0 T whole body MRI scanner (Philips Medical Systems, Best, The Netherlands). After the scanning, all participants were coded in order to blind the volumetric evaluation team and sent for the subsequent volumetric analysis.

Volume measurements of the prefrontal cortex and hippocampus were performed on 3D T 1-weighted MR images (acquisition parameters were as follows: TR = 9.8 ms; TE = 4.6 ms; flip angle = 8; section thickness = 1.2 mm; number of sections = 120; no section gap; whole brain coverage; FOV = 224 mm; matrix = 192; reconstruction matrix = 256). Routine T 2-weighted MRI and FLAIR were performed to rule out a mass lesion as a contributory factor to memory loss or cognitive decline [21–23].

During registration, the input data (3D T1 images) were transformed to 152 standard spaces, by means of transformations based on 12 degrees of freedom (i.e., three translations, three rotations, three scalings, and three skews). After subcortical and cortical registration (MNI 152 space), a mask was applied to locate the cortical and subcortical structures of interest, followed by segmentation based on shape models and voxel intensities [24]. The absolute volumes of structures obtained were calculated, taking into account the transformations made in the first stage [21, 25]. Finally, a boundary correction was used to determine whether boundary voxels belonged to the structure examined or not. In this study, a Z-value of 3 was used, corresponding to a 99.998% certainty that the voxels belonged to the particular structure. After registration and segmentation of all 139 MR scans, all segmented the regions of interest were visually checked for errors in registration and segmentation. Brain tissue volume was estimated with MIPAV software package, Medical Image Processing, Analysis, and Visualization (National Institute of Health, Bethesda, USA).

4. Results

There was no correlation between PTSD participants and associated parameters except in case of left and right amygdala. The same is with participants without PTSD where we did not find any correlation between hippocampi and prefrontal cortices. We found no difference in the parameters and observed groups and the same parameters.

Using the t-test for the independent samples, we found that both left ($t = 8.453$, df = 72.700; $P < 0.001$) and right ($t = 8.228$; df = 68.675; $P < 0.001$) amygdala volumes have statistically significant difference between PTSD participants and participants without PTSD (Table 1).

We found that left amygdala is the most significant parameter (Wald = 16.476; df = 1; $P < 0.001$) for distinction between PTSD and participants without PTSD, using multivariate logistic regression forward method (Table 2).

The receiver operating characteristic (ROC) curve is the plot that displays the full picture of trade-off between the sensitivity and across a series of cut-off points. Area under the Roc curve is considered as an effective measure of inherent validity of a diagnostic test. This curve is useful in evaluating the discriminatory ability of a test to correctly pick up diseased and nondiseased subjects or find optimal cut-off point to the least misclassify diseased and nondiseased subjects.

Roc curve method outlined left amygdala AUC = 0.898 (95% CI = 0.830–0.967) and right amygdala AUC = 0.882

Table 1: t-test (other parameters showed no statistically significant differences).

Parameters		N	Mean	Std. Deviation	t	df	P
Age	PTSD+	49	46.4694	8.16523	−0.208	77	0.836
	PTPD−	30	46.8667	8.39020			
Left hippocampus	PTSD+	49	3.5616	0.71178	−0.965	55.577	0.339
	PTPD−	30	3.6637	0.15949			
Right hippocampus	PTSD+	49	3.5704	0.70175	−1.200	55.774	0.235
	PTPD−	30	3.6957	0.15939			
Left amygdala	PTSD+	49	1.6645	0.20737	−8.453	72.700	<0.001
	PTPD−	30	1.9553	0.09587			
Right amygdala	PTSD+	49	1.6760	0.22086	−8.228	68.675	<0.001
	PTPD−	30	1.9677	0.08854			
Left prefrontal cortex	PTSD+	49	164.7553	21.71777	0.445	77	0.657
	PTPD−	30	162.4967	22.14106			
Right prefrontal cortex	PTSD+	49	162.2963	21.39362	0.445	77	0.657
	PTPD−	30	160.0714	21.81059			

Table 2: Left amygdala is shown to be important parameter.

Variables in the equation		Wald	df	Sig.
Step 1	Amygdala left	16.476	1	<0.001
	Constant	16.419	1	<0.001

(95% CI = 0.810–0.954) in the group of PTSD participants which makes both variables very statistically significant (Figure 1). Correlation between left and right amygdala in the group of PTSD participants was $r = 0.878$; $P < 0.001$. The same correlation is with participants without PTSD $r = 0.830$; $P < 0.001$. There was no difference between these two groups $P > 0.05$.

The comparison to the cut-off point shows 81.6% sensitivity and 80% specificity, which means that left amygdala can clearly indicate who is PTSD participant and who is not, $P < 0.001$. The classification of up to and above the limit of 1.87 indicates that it can be used as the test considering that OR = 17.778 with 95% CI of 5.627–56.162; that is, those with lower left amygdala score are in the relative risk of 4.082 with 95% CI of 1.971–8.452.

Additionally, the limit for the right amygdala is 1.88, because different scores were obtained only for amygdala. We noticed the association between the group of participants without PTSD and PTSD participants and the right amygdala scores of up to and above 1.88. In this case, the sensitivity of 77.6% and specificity of 73.3% are observed, which is less compared to the left amygdala but still statistically significant $P < 0.001$.

Results for right amygdala that showed statistical significance are OR = 9.500 with 95% CI from 3.320–27.181 and the relative risk of those with scores lower than 1.88 shows that the RR = 2.908 (1.577–5.346).

We used positive/negative ratio for finding cut-off point of the following variables: for left amygdala it is 1.87 and for right amygdala it is 1.88. Those numbers divide initial group of 79 participants in two groups on those with PTSD diagnosis (PTSD participants) and participants without PTSD (Table 3).

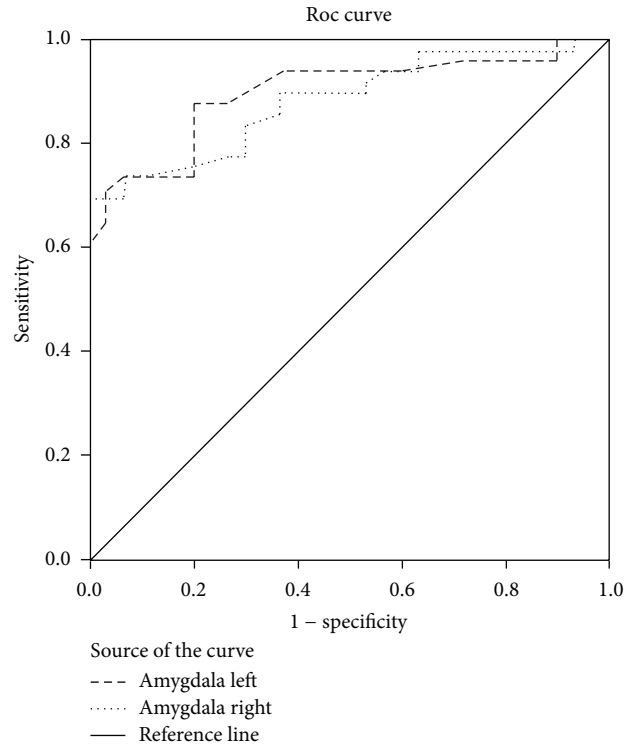

Figure 1: Roc curve method outlined left amygdala AUC = 0.898 (95% CI = 0.830–0.967) and right amygdala AUC = 0.882 (95% CI = 0.810–0.954). Both variables are statistically significant.

5. Discussion

In our study we compared volumes of amygdala, hippocampus, and prefrontal cortex in male PTSD participants who were therapy-naive and participants without PTSD. Headaches occurred in 39 participants (49.3%). Several recent publications have emphasized the relationship between life stressors and recurrent headaches. According to de Leeuw et al. [5], almost 64% of the headache participants reported one or more major traumatic stressors. It is not

Table 3: In the prediction to the specified limits of up to 1.88 and 1.87 can be significant, primarily those for amygdala left ($P = 0.007$) while those for amygdala right show not statistical significance ($P = 0.822$) with the Nagelkerke R Square of 0.438, which is significant.

	Wald	df	Sig.	HR	95% C.I. for HR	
					Lower	Upper
Amygdala left on cut-off point 1.87	7.177	1	0.007	22.200	2.298	214.443
Amygdala right on cut-off point 1.88	0.051	1	0.822	0.771	0.081	7.384
Constant	18.098	1	<0.001	0.152		

entirely clear why people with PTSD may be more likely to experience problems with headaches. However, stress has been linked to the occurrence of headaches, and the symptoms of PTSD can definitely contribute to very high levels of stress and emotional strain. In addition, headache patients tend to have more stressful events in their daily lives. PTSD can greatly interfere with many aspects of a person's life, such as at work and in relationships. This is likely going to cause more stress, increasing the likelihood of headaches. In some cases, the type of traumatic event a person with PTSD has experienced may increase the likelihood of headaches. There are several potential mechanisms for the association between PTSD and migraine. Potential mechanisms include dysfunction of the central monoaminergic system and the hypothalamic-pituitary-adrenal (HPA) axis. It is unknown why the PTSD-migraine association is stronger in men than women. Genetic sex differences and sex differences in the stress response of the HPA axis may play a role [26]. Trauma survivors who have PTSD may have trouble with their close family relationships or friendships. Their symptoms can cause problems with trust, closeness, communication, and problem solving, which may affect the way the survivor acts with others. In turn, the way a loved one responds to him or her affects the trauma survivor. A circular pattern may develop that could harm relationships. That can lead to anxiety, general anxiety disorder and depression. In our investigation, from the total amount of 79 participants, in the range of Hamilton's scale of depression, we found 33 participants (41.8%) with minor depression.

Our analysis showed hippocampal decrease consistent with the finding of Kitayama et al., [9]. Karl et al. [8] showed significantly smaller hippocampal volumes in persons with PTSD compared to controls with and without trauma exposure. Smith [10] conducted a study in which on average PTSD participants had a 6.9% smaller left hippocampal volume and a 6.6% smaller right hippocampal volume compared with control subjects. Ahmed et al., [27] noted decreased volumes of the prefrontal cortex in their MRI study. We did not find any correlation between hippocampi and prefrontal cortices.

In our investigation of volumes of amygdala, hippocampi, and prefrontal cortices, the interesting finding is that the left amygdala was significantly smaller than right. Gurvits et al., [28] found greater right amygdala volume in their meta-analysis, and Wignall et al., [29] showed postmortem asymmetry in amygdala in healthy adult human brains. Woon and Hedges [30] found no significant volume differences in the left and right amygdala in comparing subjects with PTSD both with healthy comparison subjects not exposed to trauma and with healthy, trauma-exposed subjects without PTSD.

Although most previous studies reported nonsignificant findings, some studies showed a trend towards significance for smaller amygdala volumes in PTSD [31–34]. The investigations of PTSD and amygdala volume with the largest samples included PTSD groups with $n = 4443$ and $n = 2844$ but were conducted in children and therefore do not generalize well to adults due to developmental changes in brain structure and connectivity. All studies of adults had a sample size of fewer than 20 in the PTSD group, with the majority of studies having 15 or fewer participants [22, 23, 25, 30, 35]. Consistent with our findings and relevant to the core symptom cluster of reexperiencing symptoms in PTSD, smaller amygdala volume was associated with the presence of cancer-related intrusive recollections in a sample of 76 breast cancer survivors [3]. It is worthwhile to consider the factors that may have produced a significant association of decreased amygdala volume given the preponderance of negative findings in prior studies. The small effect sizes observed from the meta-analyses suggest that they were underpowered to detect significant differences. Normal amygdala volumes do not necessarily preclude functional abnormalities in the amygdala in participants with PTSD. As a case in point, the results of a functional neuroimaging meta-analysis in participants with PTSD found evidence of amygdala abnormalities, particularly in the left amygdala, where two distinct clusters of abnormal function were identified: a ventral anterior hyperactivation cluster and a dorsal posterior hypoactivation cluster [34]. The distinct functional expression within the subregions of the amygdala highlights the need for more focused studies of PTSD with high-resolution structural MRI technology.

6. Conclusion

Our results provide evidence of an association between a smaller amygdala, hippocampus, and prefrontal cortices volumes and PTSD therapy-naive participants suffering from headaches. The headaches that occur at least twice a week might be possible sign of the decrease in volumes of amygdala. Results of our MRI research study provide us significant findings which can contribute to better understanding of the neuroanatomical substrate of PTSD, but the increase of the number of participants to clarify further significance of changed volumes of different brain structures in participants with PTSD, as well as the establishment of a reliable animal model, is mandatory.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Long-Term Effects of Maternal Deprivation on Cholinergic System in Rat Brain

Branka Marković,[1] Nevena V. Radonjić,[2] Milan Aksić,[3] Branislav Filipović,[3] and Nataša Petronijević[2]

[1] *Faculty of Sport and Physical Education, University of Belgrade, Blagoja Parovića 156, 11000 Belgrade, Serbia*
[2] *Institute of Clinical and Medical Biochemistry, Faculty of Medicine, University of Belgrade, Pasterova 2, 11000 Belgrade, Serbia*
[3] *Institute of Anatomy "Niko Miljanić", Faculty of Medicine, University of Belgrade, Dr. Subotića 4, 11000 Belgrade, Serbia*

Correspondence should be addressed to Nataša Petronijević; natasapetronijevic@yahoo.com

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Numerous clinical studies have demonstrated an association between early stressful life events and adult life psychiatric disorders including schizophrenia. In rodents, early life exposure to stressors such as maternal deprivation (MD) produces numerous hormonal, neurochemical, and behavioral changes and is accepted as one of the animal models of schizophrenia. The stress induces acetylcholine (Ach) release in the forebrain and the alterations in cholinergic neurotransmitter system are reported in schizophrenia. The aim of this study was to examine long-term effects of maternal separation on acetylcholinesterase (AChE) activity in different brain structures and the density of cholinergic fibers in hippocampus and retrosplenial (RS) cortex. Wistar rats were separated from their mothers on the postnatal day (P) 9 for 24 h and sacrificed on P60. Control group of rats was bred under the same conditions, but without MD. Brain regions were collected for AChE activity measurements and morphometric analysis. Obtained results showed significant decrease of the AChE activity in cortex and increase in the hippocampus of MD rats. Density of cholinergic fibers was significantly increased in CA1 region of hippocampus and decreased in RS cortex. Our results indicate that MD causes long-term structure specific changes in the cholinergic system.

1. Introduction

Animal model of maternal deprivation (MD) is based on exposure to stress in early postnatal life. It has repeatedly been shown that early perinatal stress can cause various short- and long-term disturbances in cognitive, emotional, and other behavioral performances [1, 2]. Nonetheless, there is evidence that early stressful life events can increase the risk of developing schizophrenia [3–5]. Schizophrenia is a chronic, severe, and disabling brain disorder. Typical symptoms of schizophrenia can be divided into positive, negative, and cognitive ones. Typical antipsychotic drugs are effective in reducing the positive symptoms, but there is no efficacy against the negative symptoms and cognitive disorder [6–8]. Cholinergic system is a target for drug development aimed at improving treatments [9, 10]. Cholinergic disturbance in basal forebrain structures and their projections in schizophrenia could be notable for cognitive dysfunction given their known functional roles in conscious awareness and components of information processing, including attention, working memory, encoding memory consolidation, and retrieval [11, 12]. Recent studies show that selective muscarinic receptor agonist (xanomeline) can improve cognitive dysfunction in patients affected with schizophrenia [13].

The stress response includes acetylcholine (Ach) release in the forebrain, which plays an important role in many cognitive functions like learning [14, 15], attention [16], memory [17], and cortical modulation of sensory information [18]. This release of Ach is responsible for physiological and emotional responses, in particular through its action on the hypothalamic-pituitary system [19], one of the main physiological systems mediating the neuroendocrine response to stress [20]. Alterations in acetylcholine neurotransmission have been commonly reported in schizophrenia [21, 22].

The aim of this study was to examine long-term effects of maternal separation on cholinergic system by measuring AChE activity in different brain structures and density of cholinergic fibers in the hippocampus and retrosplenial (RS) cortex of rats.

2. Methods

2.1. Animals and Procedures. Male and nulliparous female Wistar rats at the age of 3 months were put together in standard Plexiglas cages with sawdust (26 × 42 × 15 cm), in a temperature controlled room (23 ± 1°C). The rats were on a standard 12 h light/dark cycle with lights on from 7:00 to 19:00 h, with water and food available *ad libitum*. Two weeks later, the males were removed and the dams were checked twice daily for delivery. The day of delivery was denoted as postnatal day (P) 0. On P 9, pups from four litters were subjected to the maternal deprivation procedure according to the previously published protocol [23]. Briefly, the mothers were removed from the cage around 10:00 a.m. and placed in a single cage housed in the same room as the pups. The pups were weighed and left in the home cage at room temperature. In the control four litters, mothers were removed at P 9, pups were weighed, and mothers were returned immediately (within 3 minutes). At P 10 (24 hours later), the deprived pups were weighed again and the mothers were placed back in the home cage. In the control litters, the mothers were again removed, the pups were weighed, and the mothers were placed back in the cage. All litters were later left undisturbed except for the routine cleaning of the cages, until P 21. On P 21, the litters were weaned. Rats were sacrificed at 2 months of age (P60). All experiments were performed on male animals.

All efforts were made to minimize animal suffering and reduce the number of animals used in the study. All experiments were carried out according to the NIH Guide for Care and Use of Laboratory Animals and were approved by the Local Bioethics Committee.

2.2. Brain Preparation for Measurements of Acetylcholinesterase Activity. Eight animals from control and eight from experimental groups were used for biochemical analysis of acetylcholinesterase activity. Four brain regions, dorsolateral frontal cortex, hippocampus, thalamus, and caudate nuclei, were dissected and the crude synaptosomal fraction was prepared according to the method of Whittaker and Barker [24–26]. Briefly, isolation of specific brain structures from individual animals was performed quickly on ice. Isolated tissue was homogenized in ice-cold buffer, pH 7.0, containing 0.25 M sucrose, 0.1 mM EDTA, and 50 mM K–Na phosphate buffer. Homogenates were centrifuged twice at 1000 ×g for 15 min at 4°C. The supernatant was further centrifuged at 20,000 ×g for 20 min. Supernatant obtained by this procedure represents crude synaptosomal fraction containing membrane vesicles (microsomes) from smooth and rough endoplasmic reticulum, Golgi and plasma membrane, and all of the soluble components of the cytoplasm.

2.3. Immunohistochemistry. For morphological analysis, five male animals from the control and five from experimental groups (P 60) were anaesthetized with chloral hydrate (3 mg/kg, i. p.) and transcardially perfused with fixative (4% formaldehyde in 0.1 M phosphate buffer solution). The brains were postfixed for 24 h at +4°C and cryoprotected by infiltration with sucrose for 2 days at 4°C (20% sucrose in 0.1 M phosphate buffer). Brains were frozen by immersion in 2-methyl-butane (Fluka) precooled to −80°C and stored at −80°C until cutting. Serial transverse sections (25-μm-thick) were cut on a cryostat (Leica Instruments, Nußloch, Germany). Sections were collected on Super Frost Plus glass slides (Menzel, Braunschweig, Germany) in a standard sequence, so that four sections 250 μm apart were present on each slide. Immunohistochemistry was performed according to a published protocol [27]. The commercially available goat anti-choline acetyltransferase antibodies (ChAT, 1 : 100; Chemicon, Hofheim, Germany) were used at optimal dilutions. Water-bath antigen unmasking was performed in 0.01 M sodium citrate solution, pH 9.0, for 30 min at 80°C [28]. Nonspecific binding was blocked using 5% normal serum from the species in which the secondary antibody was produced, diluted in phosphate-buffered saline, pH 7.3 (PBS), and supplemented with 0.2% Triton X-100 and 0.02% sodium azide for 1 h at RT. Incubation with the primary antibody, diluted in PBS containing 0.5% lambda-carrageenan (Sigma) and 0.02% sodium azide, was performed for three days at 4°C. After washing in PBS (three times for 15 min at RT), the appropriate Cy3 conjugated secondary antibody, diluted 1 : 200 in PBS-carrageenan solution, was applied for 2 h at RT. Following a subsequent wash in PBS, cell nuclei were stained for 10 min at RT with bisbenzimide solution (Hoechst dye 33258, 5 μg/mL in PBS; Sigma). Finally, the sections were washed again, mounted in antifading medium (Fluoromount G; Southern Biotechnology Associates via Biozol, Eching, Germany), and stored in the dark at 4°C.

To estimate the densities of projecting immunolabeled fibers, pictures were taken on a fluorescent microscope (DM4000 Leica) with a 40x objective. Images from the RS and following subdivisions and layers of the hippocampus were taken: stratum moleculare, stratum granulosum, and stratum polymorphe of the dentate gyrus; stratum oriens, stratum pyramidale, and stratum lucidum of the CA3 region; and stratum oriens, stratum pyramidale, and stratum radiatum of the CA1 region. For each animal and layer, four pictures were taken. To estimate fiber density, the images were overlaid with a 1 cm stereological test grid (grid C4) using Photoshop 7.0 software (Adobe, San Jose, CA), and the number of intersections of fibers with the grid was counted.

3. Results

3.1. The Acetylcholinesterase Activity Is Differentially Changed in the Cortex and Hippocampus of Maternally Deprived Rats. The acetylcholinesterase activity in the synaptosomal fraction of cortex (Figure 1(a)) of MD rats was significantly decreased ($P < 0.05$) while in the hippocampus (Figure 1(b))

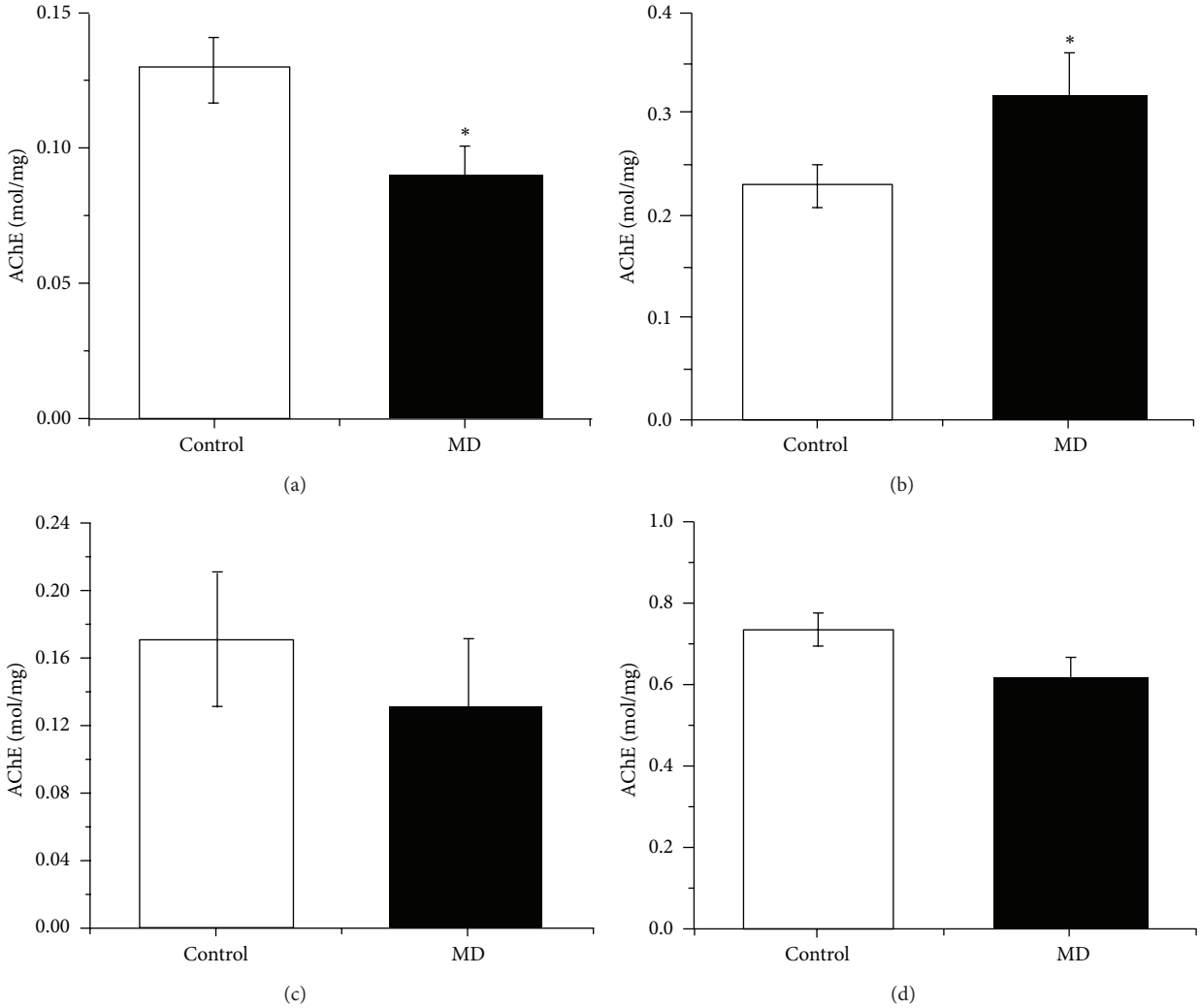

Figure 1: The activity of AChE in MD and control groups of animals (P 60) in synaptosomal fraction in cortex (a), hippocampus (b), thalamus (c), and caudate nuclei (d). Results are presented as mean ± SE. $^{*}P < 0.05$.

it significantly increased ($P < 0.05$) comparing to the values measured in the control group. In thalamus and caudate nuclei, no change in the acetylcholinesterase activity was observed (Figures 1(c) and 1(d)).

3.2. Immunohistochemistry Revealed Increase of the ChAT Positive Fibers Density in the Hippocampal CA1 Sector and Decrease in RS Cortex of MD Rats. Representative immunohistochemical staining of the ChAT positive fibers in the hippocampus is presented in Figure 2(a). Measurements of ChAT positive fibers density have shown significant increase in CA1 region while no change in CA3 and DG was noticed (Figure 2(b)). The density of ChAT positive fibers in RS cortex was significantly decreased (Figure 2(c)) in MD animals.

4. Discussion

The results of our study have revealed region-specific changes in the AChE activity in cortex and hippocampus in the maternally separated rats. Also, the density of cholinergic fibers was decreased in the retrosplenial cortex but increased in CA1 region of hippocampus. These results are important since the hippocampus and cerebral cortex are critical areas to the processes of human's memory and cognition [29]. The cholinergic system has been proposed to contribute to the pathophysiology of schizophrenia probably as a result of an imbalance between central cholinergic and dopaminergic systems [30]. Growing body of evidence showed that patients affected with schizophrenia demonstrate cognitive deficit [31, 32] and cholinergic abnormalities, such as reduced acetylcholine in different brain structures [33] as well as widespread decrease in the level of muscarinic receptors in the postmortem brain [34–36]. Cholinergic involvement in schizophrenia is further supported by the fact that muscarinic antagonists can evoke a psychotic state ("antimuscarinic psychosis/syndrome"), which includes a range of cognitive and psychotic symptoms resembling schizophrenia [37]. Muscarinic cholinergic receptors (mAChRs) are G-protein-coupled receptors for the acetylcholine and consist of five

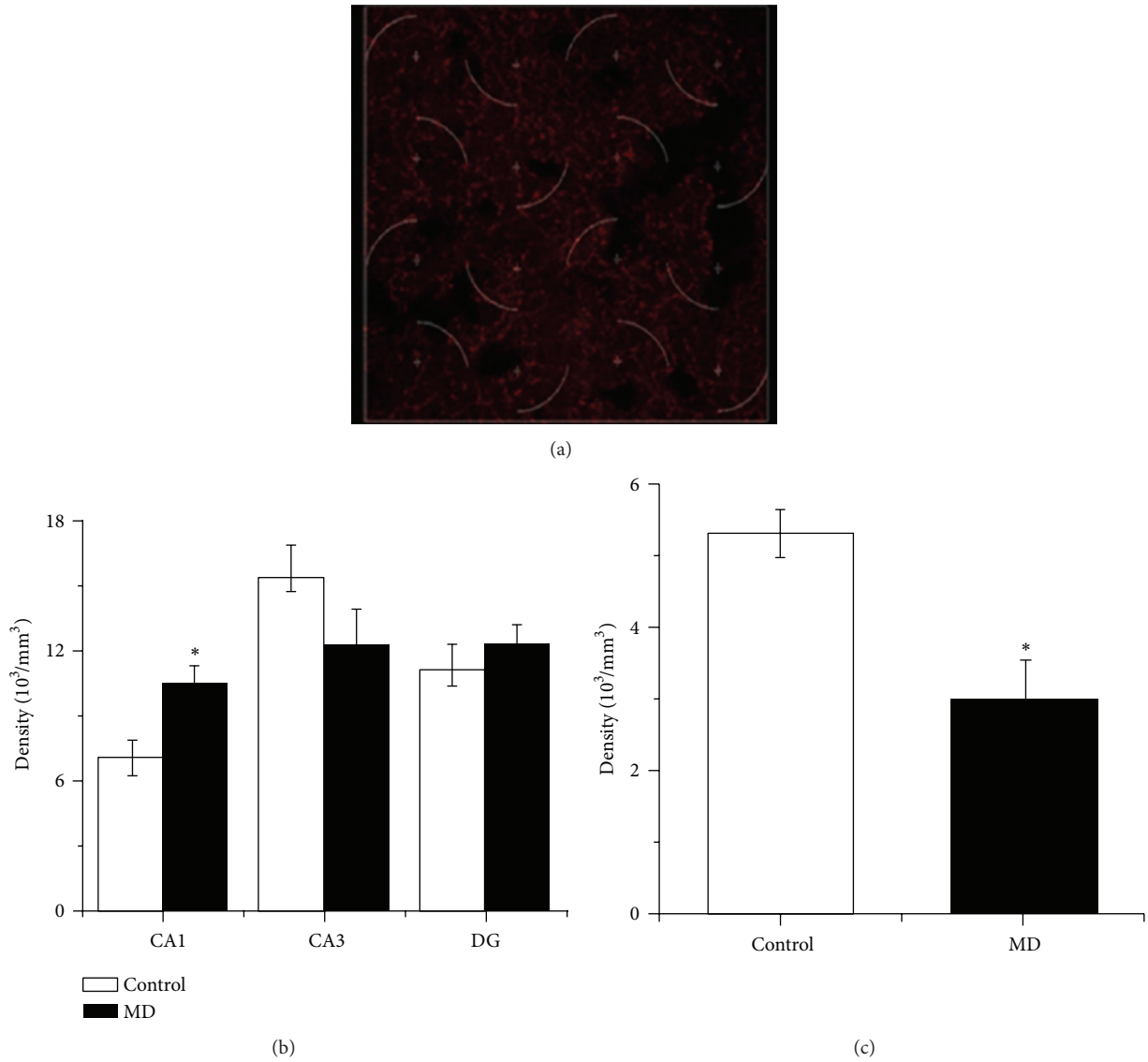

Figure 2: Representative immunohistochemical staining of the ChAT positive fibers in the hippocampus (a). Density of the ChAT positive fibers in the hippocampus (b) and RS cortex (c).

different subtypes, termed M_1–M_5. Numerous preclinical and clinical studies with nonselective mAChR agonists suggest that activation of mAChRs improves cognitive function in patients suffering from various central nervous system disorders, and these studies, along with genetic studies, indicate that M_1 is the mAChR subtype mediating the procognitive effects. Receptor protein and mRNA levels of M_1 have been shown to be decreased in frontal cortex of schizophrenic patients. In addition, circulating antibodies against M_1 have been found in the serum of schizophrenics, suggesting a link between the immune system and M_1 in schizophrenics [38]. Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels, existing as combinations from a family of similar but distinct subunits α1–α10, β1–4, γ, δ, and ε. The most predominant receptor in the mammalian brain is the $\alpha4\beta2$ nAChR, while there is also high expression of the $\alpha7$ nAChR. Both receptors are widely expressed in areas of the brain important to cognition, such as the hippocampus, thalamus, frontal, cingulate, and occipital cortices. It has been proposed that the significantly higher levels of smoking seen in patients with schizophrenia might be due to an implicit desire to activate the $\alpha7$ nAChR. Several lines of evidence suggest that the $\alpha7$ nicotinic acetylcholine receptor (nAChR) could be an important pharmacological target for the treatment of cognitive deficits in schizophrenia. Polymorphisms in the promoter region of the $\alpha7$ nAChR gene have been linked to sensory gating deficits in schizophrenia and some studies have found a reduced expression of $\alpha7$ nAChRs in the frontal cortex of patients with schizophrenia [39].

On the other hand, disturbances of cholinergic system have been shown in animal models of schizophrenia. Treatment of adult rats with phencyclidine (PCP), antagonist

of NMDA glutamate receptors, used to mimic some signs and symptoms of schizophrenia, was followed by increased efflux of acetylcholine [40] and alterations in the behaviors modulated by muscarinic receptors [41]. Furthermore, Du Bois et al. [42] have found changes in the expression of M1/4 receptors in the prefrontal cortex and hippocampus at different developmental time points following perinatal PCP treatment. Recently, Zugno et al. [43] have demonstrated that three hours of maternal deprivation daily, during first ten postnatal days (P 1 to P 10), caused an increase of AChE activity in prefrontal cortex, hippocampus, and striatum, as well as behavior alterations in adult animals. The authors have also investigated the effects of three different doses of ketamine in adult rats that were perinatally exposed to maternal deprivation and found that perinatal maternal deprivation made the animals susceptible to ketamine effects. In our study, the maternal deprivation on P 9, which lasted 24 hours, has produced different effects. We have noticed decrease of AChE activity in cortex and increase in hippocampus. Furthermore, our study has demonstrated significant increase of the ChAT positive fibers density in RS cortex and selectively in the hippocampal CA1 sector. The different pattern of AChE activity changes, seen in our study and the study of Zugno et al. [43], indicates that different protocols of maternal separations are reflected on the biochemical changes in the brain of adult animals. We can suppose that the different timing of early stress could be followed by different biochemical changes and interfere with developmental process. The alterations of AChE activity are particularly important since, independently of its catalytic function, AChE exhibits multiple biological actions on neuronal cell differentiation, adhesion, and neuritogenesis [44].

On the other hand, overexpression of AChE disrupts the glutamatergic system and results in damage of synaptic structures and excitatory function [45]. All of these findings indicate that excess of AChE that emerges under conditions of stress [46] could contribute to the pathophysiological processes developed after procedure of maternal separation.

Our study is in agreement with the findings of cholinergic abnormalities in schizophrenia and further supports the need for investigation of therapeutic strategies involving this neurotransmitter system. Whereas drugs, which have specific dopaminergic system as targets, are effective in reducing the positive symptoms in schizophrenia, they are not sufficient against the negative symptoms and cognitive disorder [47]. The neurotransmitter acetylcholine which plays an important role in the cognitive processes [11, 48] and learning and memory functions [49, 50] can play a key role in the development of new drugs. Use of acetylcholinesterase (AChE) inhibitors represents a promising possibility for treatment of cognitive disorder in schizophrenia and other psychiatric disorders [51].

However further experiments that will include the measurement of the density of cholinergic fibers in other cortical regions especially frontal cortex, the density of neuronal cell bodies in the nucleus basalis Meynert, and the density of mAChR and nAChR are needed for better understanding of the long-lasting cholinergic system changes initiated with early temporarily maternal separation.

5. Conclusion

Maternal deprivation causes region-specific changes of the AChE activity and cholinergic fiber density. In the cortex, the activity of AChE and the density of ChAT positive fibers were decreased while in the hippocampus AChE activity in whole structure and the density of ChAT positive fibers in CA1 region were increased. These results are important since the hippocampus and cerebral cortex are critical areas to the processes of human's memory and cognition and further support the need for investigations of therapeutic strategies involving cholinergic system in the treatment of schizophrenia.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors have read and approved the final paper.

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Research Article

Simple Method for Evaluation of Planum Temporale Pyramidal Neurons Shrinkage in Postmortem Tissue of Alzheimer Disease Patients

Martina Kutová,[1] Jana Mrzílková,[1] Denisa Kirdajová,[1] Daniela Řípová,[2] and Petr Zach[1]

[1] *Institute of Anatomy, Third Medical Faculty, Charles University at Prague, Ruská 87, Prague 10, 100 00 Praha, Czech Republic*
[2] *Prague Psychiatric Center, Ústavní 91, Prague 8, 181 03 Praha, Czech Republic*

Correspondence should be addressed to Martina Kutová; martinakutova@volny.cz

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We measured the length of the pyramidal neurons in the cortical layer III in four subregions of the planum temporale (transitions into superior temporal gyrus, Heschl's gyrus, insular cortex, and Sylvian fissure) in control group and Alzheimer disease patients. Our hypothesis was that overall length of the pyramidal neurons would be smaller in the Alzheimer disease group compared to controls and also there would be right-left asymmetry in both the control and Alzheimer disease groups. We found pyramidal neuron length asymmetry only in controls—in the transition into the Sylvian fissure—and the rest of the subregions in the control group and Alzheimer disease patients did not show size difference. However, control-Alzheimer disease group pyramidal neuron length comparison revealed (a) no length difference in superior temporal gyrus transition area, (b) reversal of asymmetry in the insular transition area with left insular transition significantly shorter in the Alzheimer disease group compared to the control group, (c) both right and left Heschl's gyrus transitions significantly shorter in the Alzheimer disease group compared to the control group, and (d) right Sylvian fissure transition significantly shorter in the Alzheimer disease group compared to the control group. This neuronal length measurement method could supplement already existing neuropathological criteria for postmortem Alzheimer disease diagnostics.

1. Introduction

Neurodegeneration in Alzheimer disease (AD) affects structures of the temporal lobe (MTA) and in particular the supratemporal plane of the temporal lobe—planum temporale (PT) (for review see [1]). PT is mostly a heteromodal auditory association region whose asymmetry is established by 31 weeks of gestation. Functionally, it is involved in auditory and spatial objects processing, auditory-motor integration, music pitch and tune recognition, and sound localization functions [2], and its structural and functional organization was recently revised. Neuroanatomical and neurophysiological evidence suggest subdivision of the PT into anterior and posterior parts. The anterior part belongs to the auditory cortex proper supporting spatially related but not spatially specific functions like stream segregation. The posterior part is not part of the auditory cortex and it supports sensory-motor integration of the vocal tract actions [3]. At the gross anatomy level, the PT exhibits leftward asymmetry (approx. in 60% of the population) of its length and/or area. In clinical populations was observed reversal of this asymmetry in dyslexia and a loss of asymmetry combined with an increase in right PT size in schizophrenia [4]. Anatomical changes of the PT in dementia were not extensively studied. Microanatomical changes were found in the cortex of the PT in patients with AD in the form of minicolumn thinning, although this finding was not well correlated with decline in cognitive functions [5]. Age associated minicolumn thinning in normally aged people was found in the medial temporal gyrus and temporal lobe association cortex but not in Heschl's gyrus [6]. Our previous anatomical study of PT in AD revealed overall volume and cortical width decrease in AD compared to controls together with rightward asymmetry reversal in AD [7]. We were interested in whether this gross anatomical finding could be correlated with the histological level. If so, PT could be another brain region (besides

the hippocampus, prefrontal cortex, gyrus collateralis, locus coeruleus of the brain stem, and cerebellum) that is included in neuropathological examinations in postmortem AD diagnostics.

In our present study, we measured shrinkage of layer III pyramidal neurons in four different parts of the PT (transition to superior temporal gyrus, transition to Heschl's gyri, and transition into insula and posterior part of Sylvian fissure) on the left and right side. Cytoarchitectonically, the first three parts are classified as auditory parakoniocortices (internal: PaAi, external: PaAe, and caudodorsal: PaA) with prominent granularity in layer IV and sparse layer V. The fourth part is nonauditory temporal-parietal area—Tpt—occupying the posterior side of the PT with a weak layer IV and prominent layer V. The reasons for selecting layer III pyramidal neurons for shrinkage measurement were anatomical abnormalities of the PT were observed particularly in the upper cortical layers I–III of the Tpt part of PT in the left hemisphere in schizophrenics [8], axons of layer III pyramidal neurons do not project outside the cortex and their apical dendrites are most prominent, and finally relative homogeneity in the presence of layer III pyramidal neurons across the PT compared to other layers.

We expected more prominent pyramidal neuron size loss in AD compared to controls and also a change towards rightward asymmetry in AD compared to controls.

2. Materials and Methods

This study uses the same brain PT tissue samples that were conserved from our past anatomical study on a new volumetric method of AD postmortem diagnosis. Clinical diagnosis of AD as well as postmortem verification on autoptic tissue, demographic data, method of extraction of PT samples from postmortem brains, brain tissue fixation, control patient characterization, and other data were fully described in our previous study [7]. Out of 84 formerly studied postmortem brains, we selected 10 AD PT and 7 control PT (age and sex matched, similar severity of neuropathological findings in the case of AD).

Out of each PT embedded in paraplast four samples were cut at the following anatomical localizations: (1) at the lateral border as the superolateral margin of the superior temporal gyrus (STG), (2) at the medial border of the PT at the point of transition into the insular cortex (I), (3) at the ventral border at the point of transition into one of the Heschl's gyri (HG), and (4) at the dorsal border at the point where the Sylvian fissure terminates or bifurcates into posterior descending ramus (SR) (Figure 1) (for more detailed regional description see [1]). All samples were cut on a standard rotary microtome Leica (30 μm slice thickness) with the knife positioned vertically to the cortical surface. Slices were mounted on poly-lysine coated glass slides and stained according to standard Nissl cresylviolet staining. Pyramidal neurons from layer III of the cortex (where most disorganization was previously reported [6]) were studied on a Leica DMLB microscope with 10x magnification and images were digitally captured. Digital images were transferred onto a standard PC and opened in freeware image analysis program

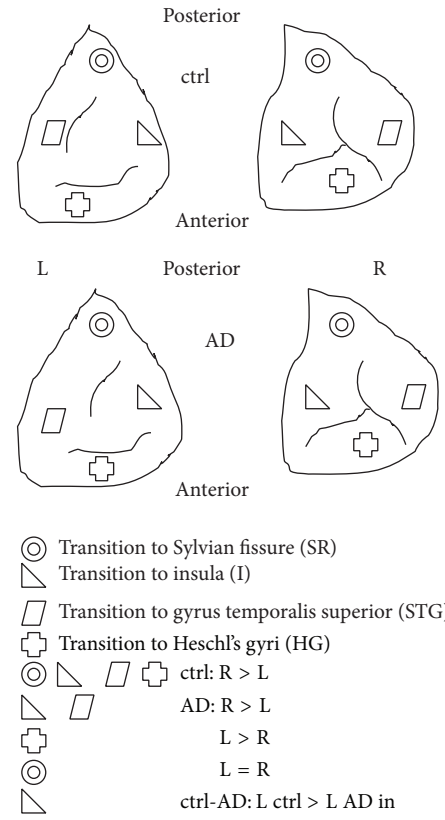
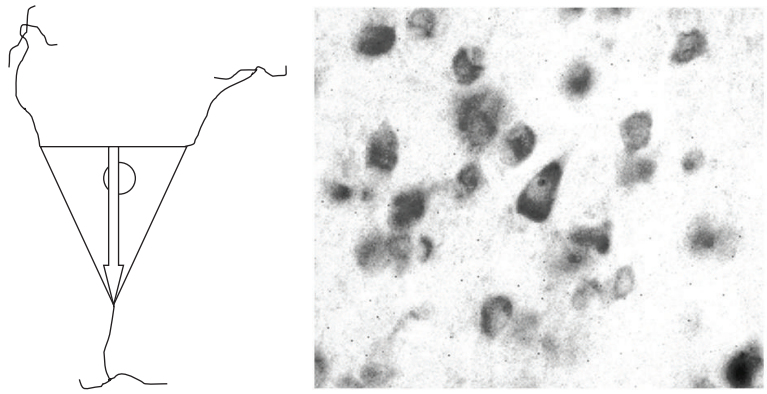


Figure 1: Example of the PT sample from ctrl and AD group (above view, left and right control and AD). Four symbols indicate four positions within the PT where brain tissue was collected and where pyramidal neurons in the cortical layer 3 were measured (STG, HG, I, and SR). Ctrl = control group, AD = Alzheimer disease group, L = left, and R = right. Equations in the lower half of the picture show asymmetries within ctrl, AD, and between ctrl and AD.

Image J 1.47 (http://rsb.info.nih.gov/ij/download.html) with implemented stereological tools. Image J was first calibrated using a calibration slide micrometer (OptixCam, USA). Length of the pyramidal neurons was measured manually by PC mouse pointer after software magnification of the images. Briefly, a thin line was drawn by mouse from the apical dendrite towards the midst of the base of the pyramidal neuron where basilary dendrites were emerging. Measured data in micrometers were stored in the Excel database. All length measurements were done blind to the status of the AD or controls using double blind labeling system of the glass slides.

Measured neurons were selected according to their pyramidal shape and horizontal position. Their length was estimated by our modification of the nucleator method [9]. Instead of measuring the length of 3 isotropic random lines from a nucleolus to the cell periphery, we measured a straight line connecting the apical dendrite with the point on the opposing cell membrane in between two emerging basilary dendrites. Criteria for inclusion of pyramidal neuron into measurement were position in the cortical layer III, triangular shape of the cell body and clearly visible whole cytoplasmic



(a) (b)

Figure 2: Shape of selected pyramidal neurons of the PT cortical layer III from STG, HG, I, and SR subregions. (a) Base of the arrow represents the starting and tip of the arrow represents the terminating point of the length of the cell body measurement. Tip of the arrow is pointing to the apical dendrite. Only neurons having at the same time visible apical dendrite, both basilary dendrites emerging from the cell body and full contour of the cytoplasm membrane with nucleus inside, were selected into measurement. The ratio of the size of cell, basilary dendrites, and apical dendrite is only for illustration in this picture. (b) Typical pyramidal neuron selected for measurement (in the middle), Nissl stained section, 30 μm thickness, magnification 400x.

membrane and clearly visible apical dendrite leaving the cell body and at the opposite side of two or more emerging basilary dendrites. This way we believe we avoided anisotropy in the shape and orientation of the sampled neurons as mentioned in [9]. Such criteria should guarantee horizontal position of the pyramidal neurons relatively to the camera view (see Figure 2). Always five randomly selected neurons were measured in all of the samples (STG, I, HG, SR) on the left (L) and right (R) sides so we measured 680 neurons in total.

The data were exported from Excel into Statistica 10 software. ANOVA with repeated measures was selected for control-AD group differences and Newman-Keuls post hoc analysis for statistical significance [10]. t-test for dependent samples was used to evaluate R/L asymmetries within control and AD group. Statistical significance was accepted at $P = 0.05$.

3. Results

In all subregions (STG, I, HG, SR), length of pyramidal neurons was higher in the control group compared to the AD group. Dependent t-test showed that within the control group, there was R > L length for all subregions except for I (L > R) (Figure 4). Within the AD group, there was R > L for STG, reversal of asymmetry (L > R) for HG, decrease of L > R asymmetry (but still L > R) for I, and R = L for SR. Comparison of control-AD group by ANOVA with repeated measures yielded mixed results: no significance in STG, L AD < L control in I, both L and R AD < control in HG and R AD < R control in SR (Figure 3). In terms of within groups asymmetry, we found significance only in the SR of the control group (R > L).

3.1. STG. R > L in both control (w/o significance) and AD groups (w/o significance). No significant difference exists between control and AD groups on both sides.

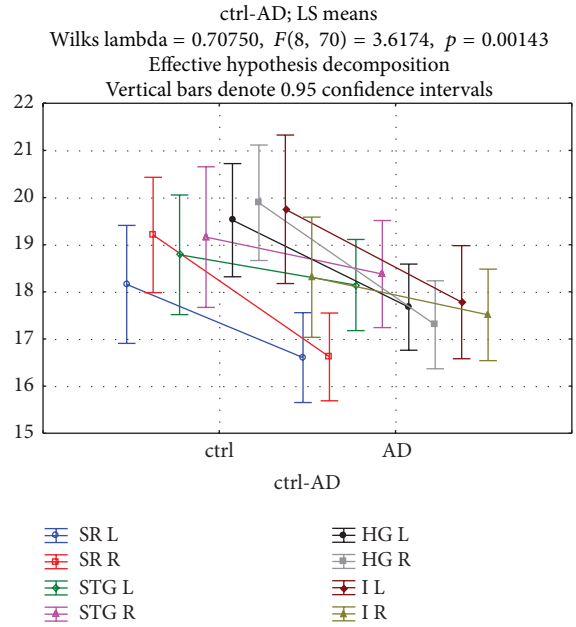


Figure 3: Lenght of pyramidal neurons in cortical layer 3 of the PT subregions in control and AD groups for R and L sides. Length of the cell bodies is in μm. Abbreviations: R: right, L: left, STG: PT transition to superior temporal gyrus, HG: PT transition to Heschl's gyri, I: PT transition to insula and SR: PT transition to Sylvian fissure, ctrl: control group, and AD: Alzheimer disease group.

3.2. I. L > R in control (w/o significance) and AD groups (almost equal, $P = 0.78$). L is significantly shorter in AD compared to control group ($P = 0.04$).

3.3. HG. R > L in the control group (w/o significance) and L > R in the AD group (w/o significance). Both R and L are significantly shorter in the AD group compared to control group (R; $P = 0.001$, L; $P = 0.01$).

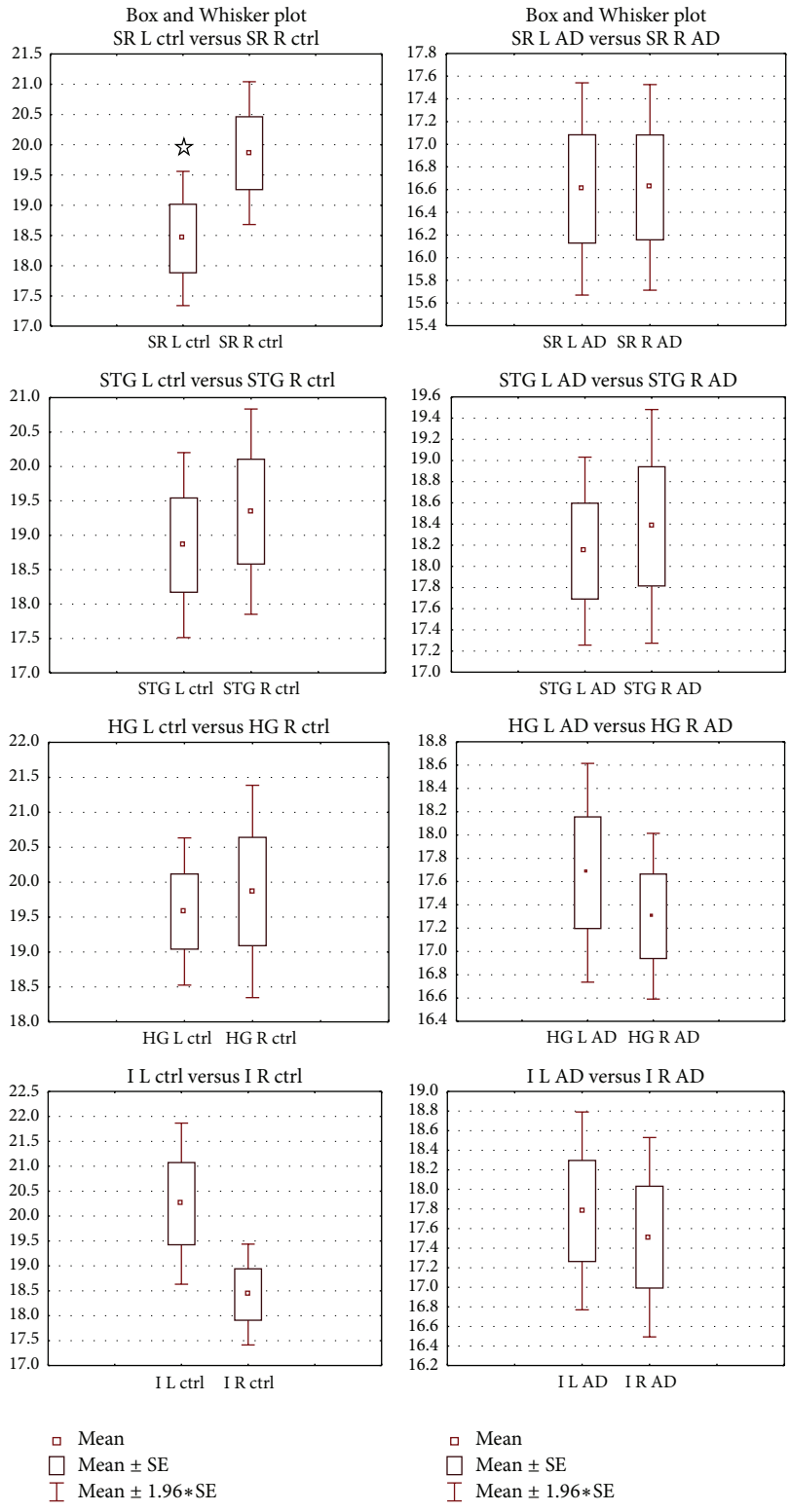


Figure 4: Left-right asymmetries in the length of the layer III pyramidal neurons in the PT of the control (left) and AD (right) groups. Y-axis data are in μm. Data are presented as means ± SE. Abbreviations: SR: transition to Sylvian fissure, STG: transition to gyrus temporalis superior, HG: transition to Heschl's gyri, I: transition to insula, L: left side, and R: right side. Significance between left and right side estimated by dependent samples t-test at $P < 0.05$ is marked by ☆.

3.4. SR. R > L in the control group ($P = 0.04$) and R = L in the AD group ($P = 0.98$). R is significantly shorter in AD compared to control group ($P = 0.003$).

4. Discussion

We measured length of PT cortical layer III pyramidal neurons in 7 controls and 10 AD patients on postmortem brain tissue. As mentioned in our previous study [7], PT tissue samples of AD patients and controls were embedded in paraplast in 2009, so we expected unavoidable tissue shrinkage. We presume similar tissue shrinkage due to the same paraplast embedding protocol for all samples. Also, all paraplast embedded PT tissue samples were stored in the same dry boxes at room temperature until sectioning. Interestingly, although we did not sort out pyramidal neurons according to their cell body size, our results ranged typically from 15 to 22 μm regardless of AD or control groups. If there would be a significant difference in shrinkage between samples, then the neurons would exhibit greater size variability. This is the reason we did not calculate tissue shrinkage corrections [11].

Our findings show that only one subregion of PT, SR, had larger cell bodies (R > L) and only in the control group. This part of the PT is a nonauditory cortex involved in sensorymotor integration of vocal tract actions [3]. In the AD group in all subregions, we did not find significant R/L asymmetry. One region (I) showed that L > R, and one region (SR) is almost equal to R = L. This is in contrast with a study reporting that L > R in controls (cells in the auditory or speech areas—but only magnopyramidal cells accounting for 10% of the cells sorted for binomial analysis for hemisphere lateralization out of great size variability of total pyramidal neurons were measured [12]). Our observation of the lack of R/L asymmetry in the AD group could be seen as a decrease of rightward dominance that is normally present in controls. Similarly, well-known rightward decrease in hippocampal volume in AD leads to call-down of normally present asymmetry in controls [13]. Therefore, the posterior part of the PT can be another area specifically lesioned by AD, in addition to the gyrus collateralis, entorhinal cortex, hippocampus, and brain stem. Due to participation of Tpt in the vocal processing streams, its decreased size layer III pyramidal neurons could contribute to vocalization problems in patients with AD in combination with declarative memory impairment. This would however be valid only for population with rightward lateralized auditory and speech centers since we did not find a decrease in neuronal size on the left side. Reduced neuronal size in layer III of the PT was also observed in bipolar disorder [14] but not in schizophrenia and major depressive disorder, and significance was lost when adjusted for six layerwise comparisons. Also reduction of somal volume of the layer III pyramidal neurons of the temporal association cortex was described in schizophrenia [15], so our finding may not be limited to the extent of the PT but rather with overlaps to neighbouring regions. Our observed reversal of asymmetry in the HG region (R > L to L > R) is opposite to reversals of PT asymmetry observed in schizophrenia (R > L) [1].

Layer III: external pyramidal layer of the cortex predominantly contains small- and medium-sized pyramidal neurons and also nonpyramidal cells. Pyramidal cells are principal targets of interhemispheric corticocortical afferents and also a primary source of corticocortical efferents. Rightward lateralized decrease of their size in AD may not be restricted only to the layer III, and that could be a topic of further studies.

5. Conclusion

We present neurohistological methodology of measuring length changes in layer III pyramidal neurons in the PT (especially in its subparts of I, SR, and HG) in patients with progressed Alzheimer disease. The data from our previous study [7] showed right/left laterality in the cortical thickness, area and volume of the PT in controls, and change into left/right laterality in the AD group. In the present study, we found similar right/left laterality in the control group and a decrease, disappearance, or reversal of this asymmetry in the AD group. Therefore, gross changes in the PT in AD may be at least partially attributed to neurohistological changes in the layer III pyramidal neurons. We suggest that this method of pyramidal neuron length measurement could be used in addition to existing clinical neuropathological evaluations of AD in hospitals.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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