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LONG-TERM EFFECTS OF MATERNAL DEPRIVATION ON THE VOLUME, NUMBER AND SIZE OF NEURONS IN THE AMYGDALA AND NUCLEUS ACCUMBENS OF RATS

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SUMMARY

Background: *Maternal deprivation (MD) in rodents is an important neurodevelopmental model for studying a variety of behavioral changes which closely resemble the symptoms of schizophrenia in humans.*

Subjects and methods: *To determine whether early-life stress leads to changes in the limbic system structures: the amygdala and the nucleus accumbens, 9-day-old Wistar rats were exposed to 24 hour MD. On P60 the rats were sacrificed for morphometric analysis and their brains were compared to the control group.*

Results: *Results show that MD affected important limbic system structures: the amygdala and the nucleus accumbens, whose volume was decreased (17 % of the control value for the amygdala and 9% of the control value for the nucleus accumbens), as well as the number of neurons (41 % of the control value for the amygdala and 43% of the control value for the nucleus accumbens) and the size of their cells soma (12% of the control value for the amygdala and 33% of the control value for the nucleus accumbens).*

Conclusion: *This study indicates that early stress in life leads to changes in the morphology of the limbic areas of the brain, most probably due to the loss of neurons during postnatal development, and it further contributes to our understanding of the effects of maternal deprivation on brain development.*

Key words: *amygdala - nucleus accumbens – neurons – volume - cell soma - maternal deprivation – schizophrenia - limbic system – rats*

Abbreviations:
Amyg= amygdala, HPA= hypothalamic-pituitary-adrenal, MD= maternal deprivation, P= postnatal, PPI= prepulse inhibition, Neu N= neuronal-specific nuclear protein, PFCX= prefrontal cortex, RSCX= retrosplenial cortex, MCX= motor cortex, fMRI= functional magnetic resonance imaging, NMDA= N-methyl-d-aspartate, Nuc. acc= nucleus accumbens

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INTRODUCTION

Adverse early life experiences can alter brain development (Teicher et al. 2003) and subsequently increase the risk of psychiatric disorders, such as schizophrenia (Chocyket et al. 2011). Stress in early life potentially impairs essential processes in the brain development, such as neurogenesis, migration, differentiation, synaptogenesis, myelination, neurite extension followed by pruning, gliogenesis and naturally occurring apoptosis (Bandeira et al. 2009).

Schizophrenia has been considered as a neurodevelopmental disease, manifested by cortical and subcortical volumetric and microstructural abnormalities (Spoletini et al. 2009). Morphometric studies of individuals with schizophrenia confirm gray matter volume loss across a range of brain structures, such as the basal ganglia, the amygdala, the temporal and the parahippocampal lobe cortex. The limbic system has been suggested to be a possible focus of pathological change in schizophrenia (Bogets 1997). The symptoms of schizophrenia such as inappropriate or flat affect might be related to the change in structure of amygdala and its connections (Benes & Berretta 2000, Ledo-Varela et al. 2007). Amygdaloid complex is thought to play an important role in assigning emotional and motivational valence to sensory inputs, in social communication, and in the processing of the representation, disposition and the intentionality of others (Holland 1999, Cardinal et al. 2002). Male - female differences in amygdaloid complex have also been discussed: extensive dendritic arbors with short branches but with unchanged spine density of the basolateral amygdala were obtained to be more pronounced in male than in female population. (Wang 2008, Ledo-Varela et al. 1998). The nucleus accumbens has an important role in the pathophysiology of schizophrenia as an integral part of limbic and prefrontal-cortico-striato-palilidal-thalamic circuits. Developmental disturbances within the entorhinal cortex and hippocampus induce a dysregulation of inputs to the nucleus accumbens resulting in behavioral abnormalities (Gray 1998, Grace 2000, Carlson 2013).

Animal models are an important development in the investigation of the mechanisms underlying mental human disorders (Lipska & Weingerger 2000). None of the models are perfect, as none of them reflect the full clinical picture observed in humans. As found by some authors (Grace 2000), the key factors determining the pathophysiological image of schizophrenia with typical symptoms of the disease are neuronal structure disorders in the following brain regions: the ventral tegmentum, the prefrontal cortex, the hippocampus, and the limbic system (Ratajczak et al. 2013). The maternal deprivation (MD) model consists of separating newborn infants from their mothers for a period of 24 hours, on day 9 after birth (during this period they are not fed by their mothers) (Ellenbroek & Cools 1998, 2000, Husum & Mathe 2002). Exposure of mammals to early-life stress, such as MD or social isolation, adversely affect brain development and adult behavior (Harlow et al. 1965, Heim et al. 2004, Rapoport et al. 2005). Maternal deprivation significantly reduces prepulse inhibition and sensitivity to dopaminergic drugs and stress, but also reduces latent inhibition (Ellenbroek & Cool 2002). The main tools used to verify the symptoms in this model are the PPI sensorimotor gating test, latent inhibition mechanism weakening, auditory sensory gating (N40), and startle habituation (Ellenbroek et al. 1998, 2004).

The authors hypothesized that maternal deprivation has long term effects on the rat brain morphology. Changes in the morphology of the important structures in the limbic system, the amygdala and the nucleus accumbens, were the focus of this study.

SUBJECTS AND METHODS

Animals and Procedures

Male and four nulliparous female 3-month-old Wistar rats 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 12h light/dark cycle with lights on from 07:00 am to 07:00 pm, and 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 zero (P0). On P9, two litters were subjected to the MD procedure according to the previously published protocol (Ellenbroek et al. 1998, Roceri et al. 2002). The 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 the dams were returned to their cages. The dams of the control litters (2 groups) were briefly (3 min) removed from their home cages and the pups were weighed on both P9 and P10. All the litters were later left undisturbed except for the routine cleaning of the cages, until P21 when the litters were weaned and classified according to the sex. In order to avoid sexual dimorphism, only male rats were used for morphological study (Woolley & McEwans 1992) as was the case with all previous studies (Owen & Patel 2013, Vivinetto et al. 2013). The animals were sacrificed in 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.

Tissue Processing

For the purpose of morphological analysis, 5 male animals were anaesthetized with chloral hydrate from both the examined and the control group (3 mg/kg, i. p.) and transcardially perfused with a fixative (4% formaldehyde in 0.1 M phosphate buffer). The brains were post-fixed 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). The brains were frozen by immersion in 2-methylbutane (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 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

Immunohistochemical staining was performed after water-bath antigen retrieval in a 0.01 M sodium citrate solution, pH 9.0, for 30 min at 80°C. Nonspecific binding w with PBS, dehydrated, and mounted with DPX (Sig as 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, 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 the sections in a 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 s, rinsed ma Aldrich).

Image Acquisition and Quantitative Analysis of Immunolabeled Neurons

Images were taken using a DM4000 Leica with 40× 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 at the same distance from the bregma (2.52 mm for the nucleus accumbens and −2.76 mm for the amygdala). The counted number of NeuN-immunoreactive cells was expressed per unit area (mm^2), which will further be referred to as profile density. At least 200 random microscope fields (area of 400 μm^2) were counted in the nucleus accumbens and the amygdala of each section.

Estimations of the Amygdala and Nucleus Accumbens Volume

The whole nucleus accumbens and the amygdala were delineated and the area was measured on the micrographs using Image Tool 3.0 software (Fig.1B, C). The volumes of the whole amygdala and nucleus accumbens were calculated according to Cavalieri's principle. All measurements were performed bilaterally.

Estimates of the Soma Area of Neuronal Cells

Estimates of neuron soma areas were performed at the level of the largest cell body cross-sectional area. Coronal brain sections stained for NeuN were selected for analysis. Four sections, 250 μm apart, were analyzed per animal. The sample size was between 20 and 30 neurons per animal. Areas were measured using Image Tool 2.0 (University of Texas, San Antonio, TX).

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 for two independent samples, with the threshold value for acceptance of the difference set at 5%.

RESULTS

The Effect of Maternal Deprivation on the Reduction in the Volume of the Nucleus Accumbens and Amygdala

The body weight of the control and MD group of rats was measured on P9 and showed that groups were matched (16.05±2 g and 15.37±1 g, respectively; p=0.8). We, also, measured body weights of maternally deprived pups before (15.37±1 g) and after (14.72±0.84 g) separation and compared them. The results shown no reduction in the body weight (p=0.6).

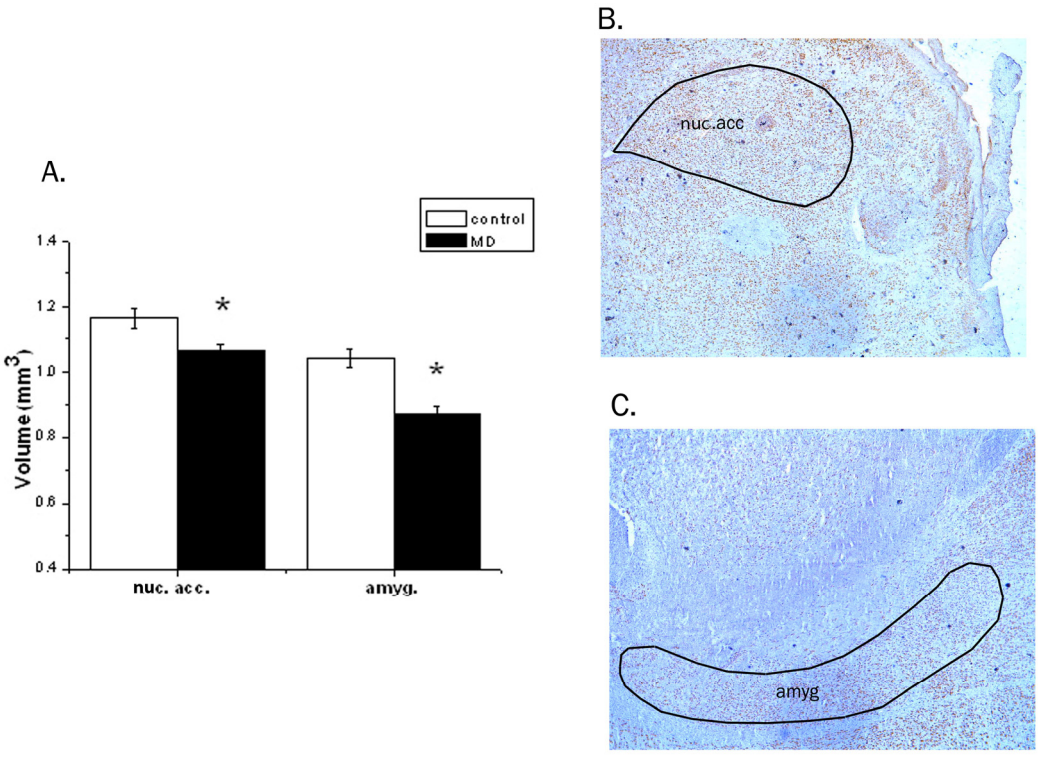

Figure 1. Volume of the nucleus accumbens and amygdala. Results presented as mean values + SEM. The asterisk indicates significant differences between group mean values (Student's t test for two independent groups, $p<0.05$) (A). Representative micrograph of the Nissl stained section of the nucleus accumbens and amygdala with traced areas for volume analysis (B, C)

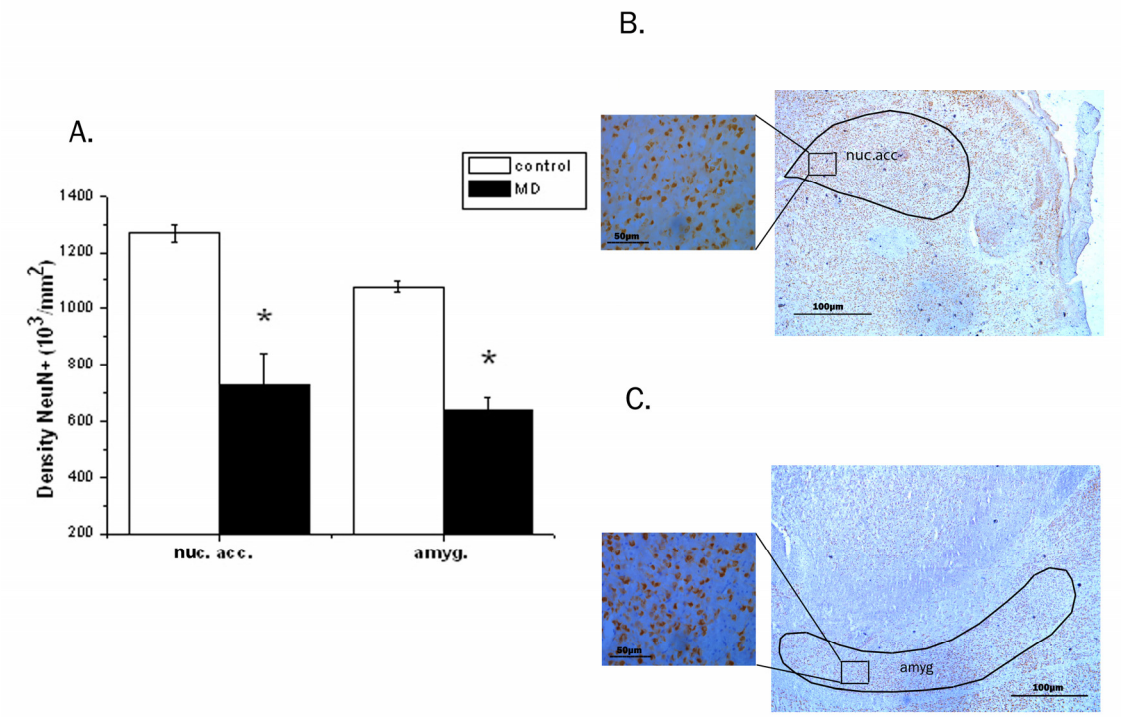

Figure 2. Profile densities of NeuN-positive neurons in the nucleus accumbens and amygdala. Results are presented as the mean values + SEM. The asterisk indicates significant differences between group mean values (Student's t test for two independent groups, $p<0.05$) (A). Representative micrographs, in high magnification, of the NeuN-positive section of the nucleus accumbens and amygdala (B, C)

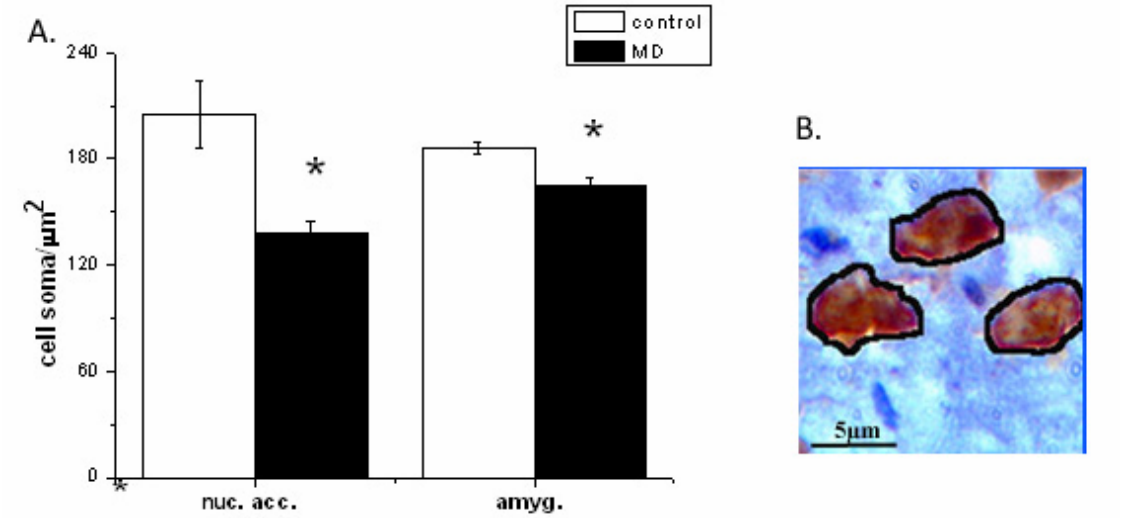

Figure 3. The cell soma area of the NeuN-immunolabeled neurons in the nucleus accumbens and amygdala. Results are presented as the mean values + SEM. The asterisk indicates significant differences between group mean values (Student's t test for two independent groups, $p<0.05$) (A). Representative micrographs, in high magnification, of the NeuN-immunolabeled neurons in the nucleus accumbens and amygdala (B)

Examination (P60) commenced with the computing of the volume of key limbic system brain structures, the nucleus accumbens and the amygdala (Figure 1). The volumes of the whole nucleus accumbens were 1.16±0.03 mm³ and 1.06±0.02 mm³ for the control and the MD group, respectively ($p<0.05$).The volume of the whole amygdala in the control group was 1.04±0.04 mm³ and in the MD group (Figure 1A) was 0.87±0.025 mm³, respectively ($p<0.01$).

Effects of Maternal Deprivation on NeuN-positive neurons in the Nucleus Accumbens and Amygdala

Profile densities of NeuN-positive neurons in stereological sections of the rat brains were counted at the same distance from the bregma (Figure 2). The profile density of NeuN-positive neurons in the nucleus accumbens of the control group was 1268.06±32.48 cell/mm², whereas

in the MD group the density was 729.17±111.83 cell/mm². This difference was statistically significant ($p<0.01$) (Figure 2A). The profile density of the NeuN-positive neurons in the amygdala in the control group was 1075±19.24 cell/mm², while in the MD group it was 636.11±46.73 cell/mm², with the difference being statistically significant ($p<0.01$) (Figure 2A).

The Effect of Maternal Deprivation on the Reduction of Cell Soma Areas of NeuN Positive Cells in the Nucleus Accumbens and Amygdala

It was also speculated in the study that the neurons present in these structures may be smaller in size after maternal deprivation which is why the cell soma area of the NeuN positive cells in the nucleus accumbens and amygdala (Figure 3) was measured. The cell soma area of the NeuN+ cells in the nucleus accumbens of the control group was 205.3±18.85 μm^2, while in the amygdala it was 186.05±3.34 μm^2. In the MD group, the cell soma area of the Neu N+ cells in the nucleus accumbens was 137.87±6.67 μm^2, and in the amygdala it was 164.94±4.67 μm^2 (Figure 3A). This difference was statistically significant for both structures ($p<0.01$).

DISCUSSION

In the present study, we have investigated the long term effects of the maternal deprivation on the nucleus accumbens and the amygdala. Maternal deprivation has been recognized as a relevant animal model for schizophrenia studies.

In human population based studies, earlier, using slices thicker than 1 mm, it was almost impossible to delineate amygdaloid complex from hippocampus. Newer technologies, however, introduced thinner slices and allowed to evaluate amygdala isolated from the hippocampal formation. Relative volume changes of the amygdaloid complex in early onset of schizophrenia, owing to the application of newer techniques, have been revealed in recent investigation (Rich et al. 2016). Amygdala has been found as relatively smaller in schizophrenia patients which was interpreted by a higher level of stress among these patients. Furthermore, smaller amygdaloid complex volume appeared to be in correlation with schizophrenia symptom severity (Anticevic et al. 2014). Same authors reported a decreased connectivity between amygdala and orbitofrotnal cortex on one, and increased amygdala connectivity with brain stem noradrenergic centers, on the other side. Liu and co-workers (2014) emphasized disrupted connectivity between amygdaloid complex and prefrontal cortex schizophrenia patients. Dividing amygdaloid complex to anatomical subunits, basolateral and corticomedial segments, a significant resting state functional connectivity decrease has been obtained between basolateral part of the amygdala and dorsolateral, prefrontal cortex, as well as with left middle cingulate cortex (Liu et al. 2014). A fMRI based meta analysis study comprising 450 patients with schizophrenia and 422 healthy controls revealed reduced activity of amygdala and prefrontal cortex in cognitive task performance (Taylor et al. 2012). Oppositely, Maat et al. (2015) claimed that only the decreased volume of prefrontal cortex but not amygdaloid complex, despite the fact that amygdaloid complex has been outlined as a structure with the decreased volume in schizophrenia patients, has impacted poorer cognitive tasks performance in fMRI investigation. Facial emotional recognition impairment, in schizophrenia suffering individuals, seems to be related with the volume decrease of the amygdaloid complex (Namiki et al. 2007). Moreover, the level of sadness recognition has been related to the left amygdala volume decrease, as claimed by the same team. Meta analysis published by Okada et al. (2016) involving 1680 healthy individuals and 884 schizophrenia patients, outlined both, amygdala and nucleus accumbens as structures with reduced volume in schizophrenia patients None the less, definitive significance of the volume reduction of the amygdaloid complex and nucleus accumbens and their influence to the schizophrenia symptoms, remains yet to be clear.

In maternally deprived rats, the volume of the nucleus accumbens and the amygdala was significantly smaller compared to the controls. Sensitivity to stress and consequent morphological changes of the nucleus accumbens and amygdala in the maternally deprivated group of rats, result from stress early in development (Coplan et al. 2001). Rodent models of early adverse experience, such as maternal deprivation, demonstrate long-term changes in neuroendocrine responses to stress, cognition, basal ganglia morphology, and emotional and behavioral regulation (Sanchez et al. 2001). This early stress in rodents can reprogram the brain to have increased stress-induced dopamine release in the nucleus accumbens (Cabib et al. 2002). In the nucleus accumbens shell glucocorticoid receptors are the only corticosteroid receptors. The glucocorticoids are currently known to be the only known endogenous compounds that can induce psychotic problems, such as delusions and hallucinations. Hormonal dysregulation in maternal separation leads to decrease of the volume of the limbic system structures and developing psychotic symptoms (Loi et al. 2015). The decrease in the number of NeuN-immunolabeled neurons in the nucleus accumbens and the amygdala was also present in this study, suggesting that the reduction in volume was due to neuronal loss during postnatal development. The glucocorticoids may play a contributing role toward neuron death, endanger the amygdala and nucleus accumbens neurons by enhancing glutamatergic signals (Lee et al. 1999). In stress glutamate accumulating in the synapse which, at sufficiently high concentrations, no longer functions as an excitatory neurotransmitter, but instead becomes an `excitotoxin' (Lee et al. 2002). Excess cytosolic calcium is mobilized, producing promiscuous over activity of

calcium-dependent enzymes. This produces cytoskeletal degradation, protein malfolding and oxygen radical generation which collectively lead to neuron death (Sapolsky 2000). Our results demonstrate that there is a reduction of the cell soma area in both structures, the nucleus accumbens and the amygdala in maternally deprived group of rats. The reduction of the cell soma area was,also, found in both, the shell of nucleus accumbens and the amygdala in rats that underwent adrenalectomy, after a mild stress (vehicle injection), subcutaneous administration of morphine (2mg/kg) or intraperitoneal injection of cocaine (15 mg/kg) (Barret et al. 2000). The shell of the nucleus accumbens may represent a transition zone between the striatum and the amygdala and is related to mesolimbic system (Salgado & Kapitt 2015). Postnatal stress in rats heightened the complexity of dendritic morphology of the accumbens, altering branching, length and spine density of neurons (Muhammad et al. 2012). Rats born by mothers stressed in mid-pregnancy by injections of saline or amphetamine in saline show reduction in the volume of the nucleus accumbens and a decreased total number of cells (Keshavan et al. 1998). The dysfunction in sensory gating and attentional processes observed in rats with thalamic reticular nucleus lesions may be related to neuronal atrophy in the limbic region such as the prefrontal cortex, the hippocampus and the nucleus accumbens (Torres-Garcia et al. 2012). Environmental factors induced damage of the thalamic reticular nuclei results as decrease of dendritic surface and of the medium spine neuron of the nucleus accumbens, (Torres-Garcia et al. 2012, Salgado & Kapitt 2015). The reduction of the accumbens volume may also be present as a result of the disturbances of the connection between the shell of accumbens and the prefrontal cortex as well as subcortical motor areas, including the extended amygdala and lateral hypothalamus (Zahm & Brog 1992). In our previous study, we demonstrated that in maternally deprived rats prefrontal cortex volume is also decreased. Therefore, the decrease of the nucleus accumbens and the amygdala volume, number and size of neurons could be a consequence of the altered connection between these structures (Aksic et al. 2013). Dopamine neurons coming in to the prefrontal cortex hold projection glutamatergic pyramidal neurons under tonic inhibition. If these inhibitory dopaminergic afferents are disabled, heightened glutamatergic activity renders the nucleus accumbens hyperresponsive to stressful experiences (Deutch et al. 1990, McClure et al. 2004). The nucleus accumbens is posited to be relevant to the attribution of incentive salience. Heinz (2002) has theorized that stress-induced or chaotic activation of dopamine release may attribute incentive salience to otherwise irrelevant stimuli and thus be involved in the pathogenesis of delusions and other positive symptoms. The disruption in the interaction between mesocortical dopaminergic neurons and dopaminergic neurons projecting to the nucleus accumbens shell is involved in those symptoms of schizophrenia that are influenced by stress (King et al. 1997). The lesions of the mesocortical dopamin innervation enhance stress-evoked dopamin efflux in the nucleus accumbens shell support the suggestion that the nucleus accumbens shell is functionally related to the limbic system. In so far as the mesocortical dopamin regulation of subcortical dopamin is related to the pathophysiology of schizophrenia, these data implicate the human homolog of the nucleus accumbens shell as being involved in the stress-induced symptomology of schizophrenia (King et al. 1997).

In our previous study we have exhibited that MD influence other structures important for schizophrenia like neocortex and hippocampus (Aksic et al. 2013). Reduction in the hippocampal volume was at least in part due to a reduction of the volume of pyramidal and granular cell layers as well as a decrease in pyramidal and granular cell soma size. Furthermore, MD leads to a reduction of the cortical thickness in the PFCX, RSCX and MCX. These results were further corroborated with reduced NeuN expression in hippocampus and neocortex by Western blot analysis. Decrease in the number of NeuN-immunolabeled neurons in the hippocampus and neocortex was also present, suggesting that the reduction in volume was due to neuronal loss during postnatal development. Decreased thickness of the motor cortex with no changes in density of NeuN-positive cells implies that there is a reduction in dendritic branching and synapse formation in the motor cortex upon MD. Also, in our previous study, we hypothesized that even though in motor cortex there was no change in neuron density, reduced NeuN expression in neocortex reflects overall result, presumably due to changes in prefrontal and retrosplenial cortex.

Postmortem studies of individuals with schizophrenia and in vivo magnetic resonance (MR) imaging studies, also, offer evidence of a progressive effect of this disease on the limbic brain structures like the nucleus accumbens and the amygdala (Benes 2000, Byne et al. 2000). Through their extensive cortical connections, the nucleus accumbens and the amygdala can influence both motor and cognitive functions (DeLong 2000) which is why they are involved in cognitive and behavioral syndromes (Levy et al. 1997).

Pakkenberg (1990) reported that the volume of the nucleus accumbens of schizophrenic patients was reduced by 50% and that the number of cells in this nucleus was reduced by a similar percentage. Changes in the nucleus accumbens volume in patients with schizophrenia, is possibly without significance or insufficiently pronounced to be detected by magnetic resonance imaging (Wang et al. 2012). The meta-analysis, by Wright et al. (2000) found that the amygdala was 94% its normal size in both hemispheres, relative to cerebral volume differences in schizophrenia. The reduction of the amygdala volume has also been reported in post-mortem material (Corson et al. 1999). Decrease in the volume of the amygdala, is more often found in

male schizophrenic patients, whereas female patients had an increased volume of the amygdala, probably as a result of the protective influence of estrogen (Gur et al. 2000). If settled, reduced volume of the amygdala in women usually occurs only unilaterally (Ledo-Varela et al. 2007). Chatterjee (2007) demonstrated that artificially reared rats (no maternal contact) had a decrease in the rate of the apoptosis in the cortex, the amygdala and the nucleus accumbens. The decrease of the total number of neurons in the basolateral amygdala, which leads to a reduction of total volume, was also detected in schizophrenic patients (Berretta et al. 2007). Magnetic resonance imaging studies of first-episode schizophrenia patients receiving minimal or no antipsychotic treatment found similar (Shihabuddin et al. 2001, Gunduz et al. 2002) or smaller (Cahn et al. 2002) basal ganglia in these patients as compared with healthy volunteers. The reduction in the volume of the amygdala was also present in patients who had discontinued treatment with antipsychotic drugs (Boonstra et al. 2011). Basal ganglia enlargement, when found, has usually been interpreted to be the result of exposure to antipsychotic drugs (Mamah et al. 2007). This finding suggests that morphometric alterations in the amygdaloid complex are more diffuse and more severe in schizophrenia suffering men (Ledo-Varela et al. 1998).

Overall, these data show that there is a relationship between early life stress, brain development and mental health, which supports the opinion that schizophrenia, as one of the very common psychiatric disorders, may be considered as a neurodevelopmental disease.

CONCLUSION

In conclusion, this study has shown that accumbens nucleus and amygdala are among the structures affected by the stress caused by maternal deprivation. This early stress in rats leads to changes in morphological parameters, including the size of the amygdala and the nucleus accumbens and the number of neurons and size of the cell soma area in these structures of the limbic system. The alterations are the possible consequence of disturbances in the HPA axis and mesolimbic pathway due to stress. Since the changes of the accumbens nucleus and amygdaloid complex are similar to those already outlined in schizophrenia patients, we have the stand point that the maternal deprivation represents a reliable animal model for the further studies of schizophrenia genesis regarding the affection of the basal brain structures.

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Conflict of interest: None to declare.

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The idea for the study: *Prof.dr Branislav Filipović;*
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Interpretation of the results and conclusion : *Prof.dr Vidosava Radonjić, Prof.dr Nataša Petronijević;*
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