Nicotinamide Reverses Deficits in Puberty-born Neurons and Cognitive Function after Maternal Separation

Gaohua Wang (✉ wanggaohua6402@163.com)

Renmin Hospital of Wuhan University

Keke Hao

Renmin Hospital of Wuhan University

Huiling Wang

Renmin Hospital of Wuhan University

Yuejin Zhang

Department of Physiology, School of Basic Medicine, Tongji Medical College

Xinhui Xie

Renmin Hospital of Wuhan University

Huan Huang

Renmin Hospital of Wuhan University

Cheng Chen

Renmin Hospital of Wuhan University

Shilin Xu

Renmin Hospital of Wuhan University

Rui Xu

Renmin Hospital of Wuhan University

Chang Shu

Renmin Hospital of Wuhan University

Zhongchun Liu

Renmin Hospital of Wuhan University

Yuan Zhou

Renmin Hospital of Wuhan University

Gavin P Reynolds Reynolds

Biomolecular Sciences Research Centre, Sheffield Hallam University

Research Article

Keywords: Schizophrenia, Early life stress, Maternal separation, Cognitive impairment, Neuroinflammation, Neuronal loss, Nicotinamide, Sirt3

Posted Date: January 31st, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1288332/v1>

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Abstract

Background: Neuronal loss is one of the important pathological features of schizophrenia. Early life stress (ELS) is associated with the development of schizophrenia. The hippocampus undergoes significant development during childhood and is extremely reactive to stress. In rodent models, ELS can induce neuroinflammation and hippocampal neuronal loss, and a schizophrenia-like behavioral phenotype. Nicotinamide (NAM) administration is found to inhibit microglial inflammation. Whether treatment with NAM during adolescence can reduce hippocampal neuronal loss and abnormal behaviors induced by ELS is unknown.

Methods: The present study used 24h maternal separation (MS) as ELS to Wistar rat pups on postnatal day (PND) 9. On PND 35 animals were given a single intraperitoneal injection of BrdU to label dividing neurons, and gaved with NAM from PND35 to PND65. Schizophrenia-like behavior tests were performed. Western blot and immunofluorescence staining detected the NAD⁺/Sirt3/SOD2 pathway-related proteins.

Results: Compared with the control group, only at the adult stage (PND56-65) but not the adolescent stage (PND31-40), animals of the MS group exhibited pre-pulse inhibition (PPI) deficits and cognitive impairment that mimic symptoms of schizophrenia. The survival and activity of puberty-born neurons, the expression of nicotinamide adenine dinucleotide (NAD⁺) and Sirtuin3 (Sirt3) in the hippocampus were decreased in the MS group rats at adulthood stage, followed by acetylated superoxide dismutase 2 (SOD2) increase, and microglial activation, and a significant increase of IL-1 β , TNF- α , IL-6 expression. All the effects of MS at PND9 could be reversed by NAM administration at the adolescent stage (PND 35-65).

Conclusions: The results demonstrated that MS may lead to schizophrenia-like phenotypes and lasting effects on the hippocampus, and highlight the utility of NAM during adolescence for restoring normal hippocampal function, ameliorating schizophrenia-like behavior.

1. Introduction

Schizophrenia is a devastating mental disease caused by complex genetic and environmental factors. Epidemiological studies have repeatedly shown that stressful early life experiences such as abuse and neglect are associated with higher rates of schizophrenia[1]. Mother-infant interaction may be a key factor in brain maturation and stress associated with maternal separation (MS) may induce the development of psychoses or susceptibility for psychotic diseases including schizophrenia. The abnormal behaviors and molecular changes taking place after a single 24 h period of MS on postnatal day PND9 in rats successfully recapitulate several features of schizophrenia [2-4]. Therefore, the MS rat model has become a powerful tool for exploring the neurobiological bases of schizophrenia.

Neuronal loss is an important pathological feature of schizophrenia, and early life stress (ELS) may induce neuronal loss. The hippocampus is a key brain area in stress response and is enriched with corticosteroid receptors, particularly in dentate granule (DG) cells [5]. As there is also significant maturation of the hippocampus during childhood and adolescence, this region has been predicted to be

especially vulnerable to the effects of ELS[6]. Previous neuropathological studies showed that hippocampal neurogenesis and dentate gyrus volume are decreased in schizophrenia patients[7, 8]. The hippocampus is involved in cognitive function, and its dysfunction is thought to be related to the cognitive impairment seen in schizophrenia[9-12]. Meta-analyses have reported significant associations between childhood adversity and both reduced hippocampal volume and impaired hippocampal function[13, 14]. Rodent MS models demonstrated that ELS induces abnormal behaviors reminiscent of schizophrenia symptoms in the adult by affecting the generation of neurons in the hippocampus[15-18]. However, the underlying specific molecular and pathological mechanisms have not been fully elucidated.

Many studies have revealed that a neuroinflammatory state may contribute to the pathogenesis of many mental disorders including schizophrenia. Microglia cells are the main immune effector cells of the brain [19]. Microglial function is also essential for successful development of the central nervous system (CNS), such as neurogenesis, synaptic maturation, axonal growth, and myelination[20]. Normal microglial cells can release neurotrophic factors to promote neuron growth and migration[21-23]. Over-activated microglia can induce further neuronal damage and loss, enhancing the release of pro-inflammatory signals, activating microglia to an even greater extent, and inducing a self-propagating cycle of inflammatory neuronal damage [24, 25]. It is hypothesized that this hyperactivation of microglia, especially during critical growth periods of the brain, would lead to structural and behavioral alterations that persist into adulthood [26-28]. Accordingly, we have assumed in the present study that activation of microglia following ELS plays a critical role in development of behaviours reminiscent of schizophrenia.

Nicotinamide adenine dinucleotide (NAD⁺) is an essential cofactor for multiple cellular metabolic reactions, and a high NAD⁺ level facilitates activation of proteins involved in mitochondria quality control, such as Sirtuin3 (Sirt3). Sirt3 reinforces mitochondrial antioxidant defense by deacetylating and increasing the activity of superoxide dismutase 2 (SOD2) [29, 30]. A recent study has suggested that mitochondrial NAD⁺ and Sirt3 activation prevented metabolic reprogramming of macrophages and restrain their pro-inflammatory phenotype [31]. NAD⁺ precursors have been suggested to be a possible augmentation treatment for schizophrenia [32]. One such precursor is nicotinamide (NAM), from which NAD⁺ is produced by the NAD⁺ salvage pathway. NAM administration is considered to be protective of neuron viability and brain function [33] and has been studied extensively in the adult rat model. However, there is little information about how NAM acts as a neuroprotective agent in the developing brain.

The primary aim of the present study was to investigate whether NAM administration during adolescence could rescue MS-induced neuronal loss and cognitive impairment in adult offspring. Secondly, we investigated the effects of NAM administration on microglial inflammation, expression of NAD⁺ and activity of Sirt3, as well as changes in other oxidative stress parameters in order to determine the possible underlying mechanism.

2. Materials And Methods

2.1 Animals and Maternal separation

Twenty nulliparous female and twenty male eight-week-old Wistar rats were obtained from Beijing Vital Rival Laboratory Animal Technology Co., Ltd. (Beijing, China). Rats of the same sex were caged together, with 3 or 4 per cage. The animals were mated at age of 3 months and the males removed one week later. The mated female rats were housed individually in ventilated plastic cages in a temperature- and humidity-controlled ($22 \pm 20^{\circ}\text{C}$, $50 \pm 10\%$) holding facility with a constant 12h day-night cycle (lights: 08:00 – 20:00). All animals had free access to food and tap water. The MS protocols were performed according to previous research [3]. Females were checked twice daily for delivery (08:00 and 17:00). The day of delivery was considered as PND 0. Each pregnant rat provided on average 10 ± 2 offspring. On PND9, about ten litters of rats were randomly subjected to MS for 24h. In brief, the mothers were removed at 10:00. The pups remained in their home cages at room temperature for 24h. On PND10, the mothers were placed back in their cages. The control groups grew naturally to adulthood. All the litters were otherwise left undisturbed except for the routine cleaning of the cages. On PND 21, the MS and the control group of pups were weaned, and then group-housed by sex (3-4 per cage). All the following determinations were carried out only on male offspring. All procedures involving animals were approved and carried out according to the guidelines of the Institutional Animals Care Committee of Renmin Hospital of Wuhan University.

2.2. Experimental design

2.2.1. Experiment 1: The effects of MS on behavior, neuronal loss, M1 microglial activation, pro-inflammatory cytokines, and NAD⁺, Sirt3, and SOD2 expression in rats at adolescent and adult stage

On PND9, the dams with their pups were randomly divided into control group and MS group (55pups in each group). The rats in the control group were untreated (the normal controls), whereas the rats in the MS group were exposed to MS as described above. During the adolescent stage (PND30-40) and the adult stage (PND56-65), 15 randomly selected pups from each group were subjected to behavioral tests and then euthanized (PND40 and PND65). The rest of the pups were used in Experiment 2.

2.2.2. Experiment 2: The effects of NAM administration during adolescence on behaviors, neuronal loss, microglial activation, pro-inflammatory cytokine and NAD⁺, Sirt3 and SOD2 expression in the hippocampus of adult MS rats

On PND10 after MS, each infant group was randomly divided into two subgroups of 10 each. The resulting 4 groups (PND35) were: control and saline group (Con + Saline), MS and saline group (MS +

Saline); control and NAM group (Con + NAM); MS and NAM group (MS + NAM); The NAM groups were gavaged with NAM (100mg/kg/d, diluted in saline) for 30 days from PND35 to PND65. The doses and treatment protocol of NAM used were based on previous studies[33, 34]. The saline groups received saline (1ml/kg/d) from PND35 to PND65 as vehicle control. All rats were sacrificed on PND65 after behavioral tests. The experimental flowchart was shown in Figure 1A.

2.3. Behavioral testing of offspring

The experimental rats were cognitively evaluated at adolescent (PND30–40) and adult (PND56–65) stages of development. The novel object recognition test was completed during the first three days. A standard behavioral protocol, the Barnes maze test, was completed between the 4th and 8th days to evaluate cognition, including working and spatial recognition memory. On the last day, a pre-pulse inhibition (PPI) test was completed, to measure sensorimotor gating. All behavioral tests were performed between 08:00 and 18:00. Each rat was assessed for each behavioral measure. After each trial, the apparatus was cleaned with 75% alcohol.

Novel object recognition test

The novel object recognition test was performed using the open-field apparatus and consisted of three components: habituation, training, and testing. Rats were placed into the center of the open field to adapt to the apparatus for 24 hours. During the training experiment, the rats were allowed to explore two identical objects for 10 minutes. The test experiment started 24 hours later. Rats were allowed to explore one familiar and one novel object, replacing a training object, for 10 minutes and locomotion was tracked by video and analysed using SpainPan lab Smart 3.0. The calculation formula of preference for exploring the novel object was: $[(\text{time spent in the novel subject}) / (\text{total time spent in two subjects}) \times 100\%]$. See figure 1D.

Barnes maze test

We examined spatial learning ability and memory with the Barnes maze, which is elevated 140 cm above the floor and consists of 20 holes located evenly on the surface periphery, each of which is 10 cm in diameter. The target box was one hole that connected to a dark chamber, allowing the animal to escape from bright light exposure. The day before the formal experiment, animals were adapted to the target box for 4 minutes. On the first day, each animal was placed in the center black cube of the maze for 5 seconds and permitted to explore the maze to find the target box, when the cube was removed. Once the animal entered the escape box, it was left there for 30 seconds, if it failed to find the target box within 3 minutes it was taken to the target box and allowed to remain in the target box for 1 minute. Each animal underwent two trials during the day with an interval of 4 hours. The time of latency to reach the target box

was recorded. The test was performed over 4 days. The whole process was monitored by a digital camera and a computer system. See Figure 1G.

Pre-pulse inhibition (PPI) test

The test was measured in a sound-attenuated chamber equipped with a small Plexiglas cage mounted on a motion-sensitive transducer platform. Following acclimatization for 5 minutes with a background noise of 70 dB, first a sequence of 5 startle stimuli (120 dB of white noise for 20 ms) was applied. During the testing phase, the initial delay is about 50 ms, followed by a 20 ms white noise impulse stimulus (75, 78, or 82 dB), then a 100 ms delay, followed by a 40 ms white noise startle stimulus of 120 dB, randomly applied over 40 trials by software control. The formula for calculating the percentage of PPI caused by the intensity of each pre-pulse was $[1 - (\text{startle amplitude on pre-pulse trial}) / (\text{startle amplitude on pulse alone})] \times 100\%$.

2.4. Sample collection

After completion of behavior testing on PND40 and PND65, the animals were anesthetized using sodium thiopental (50 mg/kg, i.p.). Rats were sacrificed. From about ten animals for each group, hippocampal tissue was taken and frozen immediately on dry ice and then transferred to -80°C storage until required for further dissection and analysis for gene and protein expression. Four animals for each group, previously given a single intraperitoneal injection of BrdU (200 mg/kg in 0.9% sterile saline solution) at PND 35, were perfused with 4% paraformaldehyde and brains removed for immunofluorescence analysis.

2.5. NAD⁺ quantification

NAD⁺ levels in the hippocampus were measured using NAD⁺ assay kits according to the manufacturer's instructions (Bio Chain, Hayward, CA, USA). The principle of the method is based on glucose dehydrogenase cycling reaction, in which tetrazolium dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) is reduced by NADH in the presence of phenazine methosulfate. The product absorbance, measured at 565 nm, is proportionate to the NAD⁺ concentration of the sample. NAD⁺ concentration was calculated based on a standard curve.

2.6. Protein extraction and western blot analysis

Total protein was extracted from hippocampal tissues with protein lysis buffer (Beyotime Biotech, China). The protein concentration was determined with a BCA kit (P0010S, Beyotime Biotech). The proteins in each sample were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto

polyvinylidene fluoride (PVDF) membranes. Western blot analysis was carried out using the following primary antibodies raised against target proteins: rabbit anti-acetyllysine (dilution 1:1000, ab190479, Abcam, UK), rabbit anti-Sirt3 (dilution 1:1000, ab189860, Abcam), rabbit anti-SOD2 (dilution 1:1000, ab68155, Abcam), rabbit anti-Acetyl-SOD2(dilution 1:1000, AF3751, Affinity Biosciences, China) and rabbit anti-β-actin (dilution 1:1000, ab181602, Abcam) antibodies overnight at 4 °C. The following secondary horseradish peroxidase (HRP)-conjugated antibodies were used at 1: 5000 dilution: goat anti-rabbit HRP (12–348) (Millipore, Watford, UK); and goat anti-mouse (sc-2005) (Santa Cruz, Insight Biotechnology, Wembley, UK). The blots were visualized with enhanced chemiluminescence detection reagents using a Chemidoc TM Touch Imaging System (Bio-Rad).

2.7. RNA preparation and gene expression analysis of pro-inflammatory cytokines by quantitative Real-Time polymerase chain reaction (qPCR)

For total RNA isolation and PCR, RNA was isolated with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer, followed by chloroform extraction and isopropanol precipitation. The RNA was resuspended in nuclease-free water. The RNA concentration and purity of RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Then, the total RNA (2μg) was reverse transcribed into cDNA using a Prime Script RT Kit (RR820A, Takara, Japan). The reaction mixture was added to the RNA solution, and the RNA/reagent mixture was incubated at 42 °C for 1 h, further heated at 70 °C for 5 min, and cooled to 48 °C. qRT-PCR was performed on a BioRad Connect Real-Time PCR platform (Bio-Rad, USA). PCRs were performed in a 20μl volume with SYBR Master Mix and 1μl pre-primer and 1μl post-primer PCR conditions were: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s, 95 °C for 10 s, and 60 °C for 5 s. GAPDH was used as a housekeeping gene. The qRT-PCR primer sequences are listed in Table 1. The Cq values were normalized to the Cq values of GAPDH to calculate the expression levels (using the $2^{-\Delta\Delta CT}$ method).

Table 1

Specific primers sequences of target genes

Gene	Sense primer	Anti-sense primer
TNF-α	CTGGCGTGTTCATCCGTTCT	AGCCCATTTGGAATCCTTGC
IL-1β	TGTGATGTTCCCATAGAC	AATACCACTTGTTGGCTTA
IL-6	CCACTGCCTTCCCTACTT	TTGGTCCTTAGCCACTCC
GAPDH	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT

2.8. Immunofluorescent staining assay

Rats were anesthetized, perfused through the heart initially with PBS, followed by 4% paraformaldehyde. The brain was then post-fixed in 4% paraformaldehyde overnight. Brain sections were embedded in paraffin and were cut at 4 µm thickness. The paraffin-embedded tissue cross-sections were dewaxed in xylol, rehydrated, placed in sodium citrate, and microwaved for antigen retrieval, and then washed with PBS, blocked with 1% BSA (Roche, Switzerland) in PBS for 2 h at room temperature. The sections were incubated at 4 °C overnight with primary antibodies against rabbit anti-Sirt3 (1:100, ab189860, Abcam, UK), goat anti-Iba1 (1:100, ab5076 Abcam), mouse anti-NeuN (1:100, ab104224 Abcam), goat anti-BrdU (1:100, ab1893, Abcam), rabbit anti-c-fos (1:100, ab190289, Abcam), followed by incubation with an Alexa Fluor CY3-conjugated goat anti-rabbit IgG secondary antibody (1:250, Bost Biotech, China) and an Alex Fluor FITC-conjugated donkey anti-goat IgG secondary antibody (1:200, Bost Biotech, China); another Alexa Fluor FITC-conjugated goat anti-mouse IgG secondary antibody (1:250, Bost Biotech, China), and Alexa Fluor CY5-conjugated goat anti-rabbit IgG secondary antibody (1:200, Bost Biotech, China) and an Alex Fluor CY3-conjugated monkey anti-goat IgG secondary antibody (1:200, Bost Biotech, China) for 60min. DAPI (Thermo Fisher) was used as a nuclear stain. The images were obtained under an inverted fluorescence microscope (IX53, Olympus, Japan). Image J (National Institutes of Health, Bethesda, MD) was used to analyze the integral optical density (IOD) of the target protein.

2.9. TUNEL assay

TUNEL staining was performed on paraffin-embedded sections using the In Situ Cell Death Detection Kit, Fluorescein (ZSGB-Bio, China). This kit labels DNA strand breaks that are generated during apoptosis, allowing for the differentiation of apoptosis from necrosis. The procedure was carried out according to the manufacturer's instructions. The slides were incubated for 30 minutes at 37°C in a 20µg/ml Proteinase K working solution. The slides were rinsed with PBS, and the area around the sample was dried. The slides were then incubated with 50µl of the TUNEL reaction mixture containing Terminal deoxynucleotidyl transferase (TdT) for 60 minutes in a dark, humidified atmosphere at 37°C. After rinsing with PBS 3 times, they were analyzed for the positive cells.

3. Data Analysis And Statistics

The data are presented as means ± standard error of the mean (SEM) and were analyzed with SPSS Statistics version 20.0 (SPSS Inc.). Data from Experiment 1 were analyzed using an independent sample t-test. In experiment 2, the data were analyzed by analysis of variance (ANOVA) to determine the group difference. A probability level less than 0.05 was accepted as significant.

4. Results

4.1 The effects of MS on behavior, neuronal loss, M1 microglial activation, pro-inflammatory cytokines, and NAD⁺, Sirt3, and SOD2 expression in rats at PND40 and PND65

4.1.1 MS rats exhibit abnormal PPI and cognitive dysfunction on PND65 but not PND40

To investigate the effect of MS on behaviors in rats, we performed several behavioral tests. No significant difference between the MS groups and the control group at PND40 were found during the PPI test (Figure 1B), the novel object recognition test (Figure 1E), and the Barnes maze test (Figure 1H). While at PND65, in the PPI test, MS rats exhibited impaired PPI at varying degrees of pre-pulse, indicating a reduced inhibition of the startle stimulus compared to the control rats (Figure 1C). We then assessed the cognitive function of MS rats using the novel object recognition test; the MS and the control group showed a similar time to explore the two identical objects in the adaption stage (Figure 1F, left panel). However, the MS group spent relatively less time exploring the novel object when one of the objects was replaced after 24 h (Figure 1G, right panel). As for the Barnes maze test, MS rats showed deficiencies in spatial learning and memory at PND65. Compared to the controls, the MS rats showed a greater latency to escape on day 1 to day 4 (Figure 1I).

4.1.2 Neuronal loss in the hippocampus in MS rats.

We gave two groups of rats a single intraperitoneal injection of BrdU during adolescence, to mark the number of newborn neurons that survived successfully from adolescence to adulthood period. The survival of these puberty-born neurons was reduced throughout the DG area in MS animals, as measured by BrdU/NeuN (Figure 2A, B). MS reduced the number of active puberty-born neurons in the entire DG as measured by BrdU/NeuN/c-fos staining (Figure 2A, C).

Neuronal apoptosis was assessed by TUNEL staining. As illustrated, there was no difference between the groups at PND40 (Figure 3A-B), while at PND65 the number of TUNEL-positive cells increased in the MS group in the DG area compared with the control group (Figure 3C-D).

4.1.3 Depletion of NAD⁺ and Sirt3 increased protein acetylation, microglial pro-inflammatory responses and cytokine expression in the hippocampus in MS rats.

We measured the concentration of NAD⁺, the expression of Sirt3 and SOD2 on PND40 and PND65. There was no difference at PND40. The levels of NAD⁺ and Sirt3 in the hippocampus of the MS group on PND65 decreased significantly than the control group. Although the total expression of SOD2 was not changed, on PND65 there was approximately 30% increase in SOD2 acetylation (Figure 4E-H). Figure 4M-O showed that the proportion of co-stained area decreased significantly in the MS rats through Sirt3 and Iba1 co-labeling. Figure 4M and Figure 4P showed the number of Iba1 immunostained cells was significantly increased in the DG area of the MS group. Furthermore there was a significant increase of IL-1 β , TNF- α , IL-6 expression in the hippocampus of the MS rats at PND65 (Figure 4Q-R).

4.2 The effects of NAM administration during adolescence on behaviors, neuronal loss, microglial activation, pro-inflammatory cytokine, and NAD⁺, Sirt3 and SOD2 expression in the hippocampus of adult MS rats

4.2.1 Changes in behavior and cognitive function

Figure 5A showed that the impaired PPI of the MS group was ameliorated to some extent after the rats were administrated with 100 mg/kg NAM from PND35 to PND65. As for the novel object recognition test, the MS+NAM group spent more time sniffing the novel object after 24 h than the MS + Saline group (Figure 5B, right panel). In addition, the MS+NAM group had decreased latency entering the target hole compared to the MS+ Saline group during the Barnes maze test (Figure 5C-D).

4.2.2 The expressions of NAD⁺, Sirt3, SOD2, Microglial pro-inflammatory responses and Pro-inflammatory cytokine expression in the hippocampus

Figure 6A showed the recovery of NAD⁺ in the hippocampus of MS rats after NAM administration. Compared with the MS group, the MS+NAM group had increased Sirt3 expression and decreased SOD2 acetylation in the hippocampus (Figure 6B-D). Figure 6E-F showed that the proportion of co-stained positive cells increase in MS+NAM rats through Sirt3 and Iba1 co-labeling. Figure 6E and Figure 6H showed the number of Iba1 immunostained cells decrease in the DG area of the MS+NAM group. And Figure 6I showed the decrease of IL-1 β , TNF- α , and IL-6 expression in the hippocampus in MS+NAM rats at PND65.

4.2.3 Neuronal loss in the hippocampus

NAM administration in the MS+NAM group decreased neuronal apoptosis, as assessed by TUNEL staining, compared with MS+Saline group (Figure 7A, B). MS reduced the survival of puberty-born neurons in the DG, as measured by BrdU/NeuN, which was abolished by NAM administration (Figure 8A, B). NAM administration significantly increased the proportion of active adult-born neurons in the MS group, measured by dividing the number of cells co-labeled with BrdU/NeuN by those triple labeled with BrdU/NeuN/c-fos (Figure 8A, C).

5. Discussion

A growing body of evidence has shed light on the neurodevelopmental nature of schizophrenia, while symptoms typically emerge during late adolescence or young adulthood[35]. Following MS on PND9, we compared behaviors at PD40 and PD65, corresponding to adolescence and adulthood and likely to reflect pre-symptomatic and symptomatic periods of development in schizophrenia. We found that PPI deficit and other cognitive impairment were present on PND65 rather than PND40, as is in accordance with other studies [2-4], and suggesting that the effect of MS in early life can last into adulthood. Furthermore, this age-related abnormal behavioral phenotype is also found in other animal models of schizophrenia that involve early-life trauma such as those derived from poly I:C-induced gestational inflammation [36].

One question is why early adverse life experiences in this model do not show adverse effects until adulthood. Adolescence is a sensitive period of brain development. Perinatal stress is known to modulate the progress of pubescent brain development. There is evidence implicating newborn neurons during the adolescent stage in emotional regulation and hippocampal-dependent behaviors, including trace and contextual fear conditioning, spatial navigation, pattern separation, and cognitive flexibility[37-39]. Given the important roles of these puberty-born neurons, we gave two groups of rats a single intraperitoneal injection of BrdU on PND35, to label neuronal neogenesis at this age and track neuronal survival through adolescence to adulthood. We found a reduced survival of these mature puberty-born neurons in the hippocampus in the MS rats on PND65. We further confirmed a significant increase in neuronal apoptosis in the DG area in these adult animals through TUNEL staining, while there was no difference in adolescence. Puberty-born cells differentiate into neurons and glia and are functionally incorporated into neural circuits that subserve behaviors [40]. The juvenile brain is more plastic than in adulthood and the puberty-born neurons are especially responsive to learning, which can have significant consequences for the functional integrity of the hippocampus[41]. Thus, mature puberty-born neurons would be functionally relevant to behavior. Ablation of this neuronal population might therefore result in abnormal behaviors.

In this study, we found an over-activation of microglia and the release of pro-inflammatory factors in MS rats on PND65, while there was no difference on PND40. ELS is known to cause the microglia to be in a pre-activated state for years, and primed microglia have an exaggerated response to future life challenges such as stress, infection, and changes in hormone levels [42, 43]. The levels of various hormones in puberty change significantly, and previous study has suggested that androgens promote activation of microglia and increase phagocytosis mediated by microglia [44]. This might partly explain our results. Microglia are the main effectors in the inflammatory process of the central nervous system (CNS), and

they can respond to even small changes in the CNS. The view that over-activated microglial-mediated neuroinflammation contributes to neurotoxicity in the developing brain is supported by both in vitro and in vivo studies [45-47]. Therefore, the reduced survival of newborn neurons in the MS group on PND65 may be caused by over-activated microglia induced by ELS. Avoiding or reducing neuronal loss induced by microglial inflammation is therefore a potential mechanism to prevent the development of disease during the asymptomatic period of schizophrenia.

Previous study has suggested that NAM contributes to the control of inflammatory diseases by increasing NAD⁺ [48]. The primary purpose of our study is to explore whether the use of NAM, as a precursor of NAD⁺, can prevent or reduce ELS-induced adverse changes in CNS. This study provides evidence showing beneficial effects of NAM supplementation during adolescence, effectively inhibiting microglial inflammation, and avoiding both ELS-induced reduction in puberty-born neurons and the consequent impairment of cognition. The results of the present study are somewhat consistent with studies using adult animals indicating that NAM treatment attenuates cortical loss following closed-cortical injury [49-52]. To our knowledge, this is the first study demonstrating that NAM as a neuroprotective agent can be used during puberty to prevent neuronal loss. NAM administration would be a potential approach for preventing neuronal loss and improving hippocampal function in individuals who had undergone ELS.

In addition, we investigated the specific molecular mechanism of NAM in reducing microglial inflammation in the MS model. We found that NAM can reverse the MS-induced deficit of NAD⁺. NAD⁺ is a fundamental mediator for antioxidant defenses, and mitochondrial NAD⁺ degradation would therefore reduce antioxidant capacity [53]. The brain is particularly vulnerable to oxidative stress due to the high content of highly oxidizable polyunsaturated fatty acids and the relatively low antioxidant load in comparison with other tissues [54, 55]. Evidence from proteomic and metabolomic studies in recent years suggested elevated oxidative stress in schizophrenia, with disturbed mitochondrial function and reduced antioxidant capacity [56]. A recent clinical study has shown redox dysregulation in first-episode schizophrenia patients demonstrated by a decreased ratio of NAD⁺/NADH in the prefrontal cortex measured by 31P MRS [57]. Reduced antioxidant capacity in the brain would cause neuroinflammation and affect neuronal function such as neurotransmitter synthesis and synaptic plasticity [58]. Sirt3 is a mitochondrial deacetylase, a class of proteins involved in aging and stress damage, and is NAD⁺ dependent [59]. Previous study has shown that Sirt3 has an important role in the inhibition of neuroinflammation and in protecting blood-brain barrier integrity in ischemia stroke [60]. During our research, we found a decrease in NAD⁺ and Sirt3 levels in brain tissue in the MS group at the adult stage (PND65), along with an increase in acetylated SOD2. SOD2 plays a key role in the mitochondrial antioxidant defense mechanism, and increased acetylation of SOD2 can decrease antioxidant function [61-63]. To confirm the importance of mitochondrial antioxidant dysregulation for the pro-inflammatory response in microglia, we examined the expression of Iba1 and Sirt3 by immune co-labeling and found that the expression of Sirt3 in microglia decreased significantly in the MS group on PND65 rather than on PND40. The number of microglia significantly increased, accompanied by pro-

inflammatory cytokines (TNF- α , IL-1 β , IL-6) upregulating in the MS group at the adult stage. NAM administration can reverse or attenuate all these consequences of MS.

The findings of the present study suggest that in the MS model, NAM administration may act through up-regulation of the expression of NAD⁺ and Sirt3 to increase the antioxidant activity of SOD2 and thereby reduce microglial inflammation and neuronal loss. However, we did not explore whether it can inhibit microglial inflammation and improve cognitive impairment in model animals by regulating the expression of Sirt3. Further investigations are necessary to fully elucidate the exact mechanisms behind this connection. Furthermore, we only explored the changes at PND40 and PND65, and so could not identify the time point when immune activation began. Additionally, we only administered NAM to model rats during adolescence, but not adulthood, and thus could not determine the effect of NAM administration on emerging or established abnormal behaviours reminiscent of schizophrenia.

Conclusions

In conclusion, this study shows that exposure to ELS may result in age-dependent cognitive impairment, concomitant with an ongoing decrease in survival and activity of puberty-born neurons in the hippocampus. NAM administration throughout adolescence rescued behavioral performance and restored survival and activity of puberty-born neurons to normal levels. Our study provides extra clues for the etiological research of schizophrenia. NAD⁺ supplementation may provide a novel preventative strategy for humans who have undergone childhood trauma and are at higher risk of developing psychiatric disorders such as schizophrenia.

Abbreviations

ELS: early life stress; NAM: Nicotinamide; MS: maternal separation; PND: postnatal day; PPI: pre-pulse inhibition; NAD⁺: Nicotinamide adenine dinucleotide; Sirt3: Sirtuin3; SOD2: Superoxide Dismutase 2; NAM: Nicotinamide; DG: dentate granule; CNS: central nervous system

Declarations

Acknowledgements

We wish to thank Minglang Gao for his excellent technical assistance.

Authors' Contributions

Authors G-HW, H-LW, Y-Z, GPR and K-KH designed the study and wrote the protocol. Authors K-KH and Y-JZ performed the experiments and analyzed the data. Authors K-KH, H-H, ZC-L, C-C, R-X, C-S and S-LX managed the literature searches and figure drawing. Author K-KH wrote the manuscript. H-LW, X-HX and GPR revised the manuscript. All authors contributed to and have approved the final manuscript.

Funding

This research was conducted with funding by grants from the National Natural Science Foundation of China (91132301), and the Natural Science Foundation of Hubei Province (2014CFB732).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Ethics approval and consent to participate

All procedures involving animals were approved and carried out according to the guidelines of the Institutional Animals Care Committee of Renmin Hospital of Wuhan University (IACUC no: 20201201).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The MS rats exhibit age-related impairment in sensorimotor gating and cognitive behaviors at PND 65.

(A) Experimental program. Litters were subjected to MS protocols during lactation on PND9 and then injected intraperitoneally with BruU on PND35. Behavior tests were performed during PND30-40 and PND56-65.

PND, postnatal day; MS, maternal separation; BrdU, 5-Bromo-2'-deoxyuridine.

(B) Percentage of pre-pulse inhibition of the auditory startle reflex across different pre-pulse intensities on PND40

(C) Percentage of pre-pulse inhibition of the auditory startle reflex across different pre-pulse intensities on PND65

(D) Schematic illustration of the novel object recognition experiment

(E, F) On day2, time was spent exploring the two identical sample objects within the the10-min period. On day3, time was spent exploring novel and old objects during the the10-min period on PND40 and PND65.

(G) The trajectory graphs provide examples of the route to explore the target location by all groups in the Barnes maze test. The red dot and blue dot represent the start and end positions respectively. The green line represents the trajectory. The target hole is labeled with a black circle.

(H) The latency to the target hole in the Barnes maze test at PND40

(I) The latency to the target hole in the Barnes maze test at PND65.

PND40 (CON, n = 9; MS, n = 10) and PND65 (CON, n = 13; MS, n = 15). n.s. - not significant; *p < 0.05, **p < 0.01, and ***p < 0.001 as compared to the controls. The data are represented as mean ± SEM.

Figure 2

The Survival and activity of puberty-born neurons.

(A) Immunofluorescence was used to characterize the survival of puberty-born neuron cells. BrdU/NeuN assay shows the survival of puberty-born neurons in the DG till PND 65; BrdU/NeuN/c-fos assay shows the active puberty-born neurons. Original magnification ×200, scale bar = 50 μm.

(B) The percentage of puberty-born neurons in neurons of DG.

(C) The percentage of active puberty-born neurons in BrdU/NeuN cells.

P values were calculated using the Student's t-test. The data are presented as mean ± SEM for each group (n = 4 per group). *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 3

The neuronal apoptosis in the hippocampus dentate gyrus by TUNEL.

(A, B) Neuronal apoptosis in the DG was assessed by TUNEL staining at PND 40.

(C, D) Neuronal apoptosis in the DG was assessed by TUNEL staining at PND 65.

Original magnification $\times 200$. The data are presented as mean $\pm$ SEM for each group (n = 4 per group). *P* values were calculated using the Student's t-test. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Figure 4

The expression of NAD⁺, Sirt3, acetylated SOD2 and inflammation of microglia.

(A) NAD⁺ levels in the hippocampus at PND40 (CON, n = 8; MS, n = 8);

(B-D) Immunoblots and quantification analysis of the level of Sirt3/acetylated SOD2 and total SOD2 in the hippocampus (HIPPO) at PND40 (CON, n = 4; MS, n = 4)

(E) NAD⁺ levels in the hippocampus at PND65 (CON, n = 10; MS, n = 16);

(F-H) Immunoblots and quantification analysis of the level of Sirt3/acetylated SOD2 and total SOD2 in the hippocampus at PND 65 (CON, n = 6; MS, n = 6)

(I) Representative immunofluorescence images show the expression of Iba1 (green pixels) and Sirt3 (red pixels) in the DG at PND40 (n = 4 per group).

(J) Quantitative analyses of the percentage of Sirt3 expression.

(K) Quantitative analyses of the percentage of Iba1 and Sirt3 co-labeling.

(L) Quantitative analyses of the Iba1+ immunostained cells.

(M) Representative immunofluorescence images show the expression of Iba1 (green pixels) and Sirt3 (red pixels) in the DG at PND65 (n = 4 per group).

(N) Quantitative analyses of the percentage of Sirt3 expression.

(O) Quantitative analyses of the percentage of Iba1 and Sirt3 co-labeling.

(P) Quantitative analyses of the Iba1+ immunostained cells.

(Q) Real-time qPCR analysis of TNF- α , IL-1 β , IL-6 in the hippocampus and PFC at PND40 (CON, n = 4; MS, n = 4)

(R) Real-time qPCR analysis of TNF- α , IL-1 β , IL-6 in the hippocampus and PFC at PND65 (CON, n = 6; MS, n = 8).

Original magnification $\times 200$. The data are presented as mean $\pm$ SEM for each group. *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 5

NAM administration attenuates pre-pulse inhibition and cognitive deficits induced by MS.

(A) Effects of NAM administration (100 mg/kg) on PPI at different pre-pulse intensities.

(B) Effects of NAM administration on the novel object recognition assay in different groups.

(C) Effects of NAM administration on the latency to the target hole in the Barnes maze test.

CON + Saline, n = 10; MS + Saline, n = 10; CON+NAM, n = 10; MS+NAM, n = 10. n.s. - not significant; *p < 0.05, **p < 0.01, and ***p < 0.001 as compared to the controls. The data are represented as mean $\pm$ SEM.

Figure 6

NAM administration reverses the effects of MS on NAD⁺, sirt3, protein acetylation, and inflammation of microglia.

(A) NAD⁺ levels in the hippocampus (CON + Saline, n = 10; MS + Saline, n = 10; CON+NAM, n = 8; MS+NAM, n = 8);

(B-D) Immunoblots and quantification analysis of the level of Sirt3/acetylated SOD2 and total SOD2 in the hippocampus (n = 6, per group)

(E) Representative immunofluorescence images show the expression of Iba1 (green pixels) and Sirt3 (red pixels) in the DG (n = 4 per group);

(F) Quantitative analyses of the percentage of Sirt3 expression.

(J) Quantitative analyses of the percentage of Iba1 and Sirt3 co-labeling.

(H) Quantitative analyses of the Iba1+ immunostained cells.

(I) Real-time qPCR analysis of TNF- α , IL-1 β , IL-6 in the hippocampus (n = 6 per group).

Original magnification $\times 200$. The data are presented as mean $\pm$ SEM for each group. *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 7

NAM administration reduces neuronal apoptosis in the DG.

(A, B) The neuronal apoptosis in the DG was assessed by TUNEL staining at PND 65.

Original magnification $\times 40$ (upper), $\times 400$ (lower). The data are presented as mean $\pm$ SEM for each group (n = 4 per group). *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 8

The NAM administration recovers the survival and function of puberty-born neurons.

(A) BrdU/NeuN assay shows the survival of puberty-born neurons in the DG till PND 65. BrdU/NeuN/c-fos assay shows the active puberty-born neurons. Original magnification $\times 200$.

(B) The percentage of puberty-born neurons in neurons of DG.

(C) The percentage of active puberty-born neurons in BrdU/NeuN cells.

The data are presented as mean $\pm$ SEM for each group (n = 4 per group). *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 9

A proposed schematic diagram illustrating how NAM administration might attenuate cognitive impairment in schizophrenia through the NAD⁺/Sirt3 pathway.

The deficit of NAD⁺ in brain tissue downregulates the expression of Sirt3 in the microglia, followed by increased protein acetylation and redox abnormalities. Microglia are activated and neuroinflammation enhanced, which would induce neuronal damage and result in cognitive deficits. NAM administration can reverse these changes.