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ORIGINAL PAPER

Optogenetic Stimulation of the Anterior Cingulate Cortex Ameliorates Autistic-Like Behaviors in Rats Induced by Neonatal Isolation, Caudate Putamen as a Site for Alteration

Elham Sadat Sayed Javad Javaheri[1] · Mohammad Reza Bigdeli[1,2] · Mohammad Ismail Zibaii[3] · Leila Dargahi[4] · Hamid Reza Pouretemad[2]

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Abstract

Epigenetic agents, such as neonatal isolation during neurodevelopmental period of life, can change various regions of the brain. It may further induce psychological disorders such as autistic-like phenomena. This study indicated the role of chronic increased anterior cingulate cortex (ACC) output on alteration of caudate putamen (CPu) as a main behavior regulator region of the brain in adult maternal deprived (MD) rats. For making an animal model, neonates were isolated from their mothers in postnatal days (PND 1–10, 3 h/day). Subsequently, they bilaterally received pLenti-CaMKIIa-hChR2 (H134R)-mCherry-WPRE virus in ACC area via stereotaxic surgery in PND50. After 22 days, these regions were exposed to blue laser (473 nm) for six consecutive days (15 min/day). Then, behavioral deficits were tested and were compared with control group in the following day. Animals were immediately killed and their brains were prepared for tissue processing. Results showed that neonatal isolation induces autistic-like behaviors and leads to overexpression of NMDAR1 and Nox2-gp91^{phox} proteins and elevation of catalase activity in the CPu regions of the adult offspring compared with control group. Chronic optogenetic stimulation of ACC neurons containing (ChR2+) led to significant reduction in the appearance of stereotypical behavior and alien-phobia in MD rats. The amount of NMDAR1 and Nox2-gp91^{phox} expression and the catalase activity in CPu were reduced after this treatment. Therefore, autistic-like behavior seems to be related with elevation of NMDAR1 and Nox2-gp91^{phox} protein levels that enhance the effect of glutamatergic projection on CPu regions. Optogenetic treatment also could ameliorate behavioral deficits by modulating these protein densities.

Keywords Neonatal isolation · Autistic-like behavior · Optogenetic treatment · Anterior cingulate cortex · Caudate putamen · NMDA receptor

Introduction

Autism is the spectrum of neurodevelopmental disorder that is characterized by (i) social connection deficit and (ii) appearance of stereotypical behavior (Association 2013; Luo et al. 2018). Multiple agents such as genetic, epigenetic, and pathologic events cause people vulnerable to autism spectrum disorder (ASD). Despite the broad role of genetic disruption in ASD, only 6–15% of cases of autism are directly related to genetic defects, and 85–95% of them are affected by epigenetic factor (Alexander and Poletaev 2016). Stress factors are found as important epigenetic agents that affect throughout the lifespan on the brain (Curley et al. 2011; Gudsnuk and Champagne 2012; Lee et al. 2003). Neonatal isolation (or maternal deprivation) is one of the most potent stressors in neurodevelopmental period after literation in the

✉ Mohammad Reza Bigdeli
bigdelimohammadreza@yahoo.com

[1] Department of Animal Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, IR, Iran

[2] Institute for Cognitive and Brain Science, Shahid Beheshti University, Tehran, Iran

[3] Laser and Plasma Research Institute, Shahid Beheshti University, Tehran, Iran

[4] NeuroBiology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

majority of mammalians. In this critical time, low levels of primary maternal care can lead to persistent alteration in expression and function of several proteins in various parts of the offspring brain. It might result in long-term behavioral abnormalities, similar to those seen in ASD (Lupien et al. 2009; Tan et al. 2018; Wu et al. 2014). For instance, exposure of rodents to neonatal isolation has been shown to significantly reduce social interaction contact (Tsuda and Ogawa 2012) but increase repetitive behaviors (Bahi 2017). In order to reveal the etiology of the effects of maternal deprived stress on behavioral malfunction such as autistic-like phenomena, it is necessary to show the role of neuronal circuits in the occurrence of natural behavior and identify deviation factors that disrupt these performances in disorder conditions. Today, the optogenetic approach provides a new method for investigating the role of specific neurons' function in brain networking involved in the occurrence of behavioral deficits (Deisseroth 2012; Yizhar 2012). For instance, Lee et al. (2018) concluded that optogenetic stimulation of the nigro-striatal dopamine circuit induced the social deficit and repetitive behavior in relation to the pathology of ASD in an animal model. These behaviors were inhibited using a D1 receptor antagonist (Lee et al. 2018). In another similar study, Kim et al. (2017) reported that the inhibition of pre-limbic cortical neuro-projection to dorsal striatum, via an optogenetic method, improved the social defect behavior in transgenic autistic-like mouse (AC5 KO), with knock-out adenylyl cyclase gene type 5 (Kim et al. 2017). Burguière et al. (2013) also indicated that increasing function of orbitofronto–striatal circuit, via optogenetic stimulation, reduced the stereotypical behavior in transgenic autistic-like mice (Burguiere et al. 2013). However, little is known about the molecular mechanisms underlying the therapeutic effects of optogenetic stimulation on ASD. Previous studies have shown that striatal circuits' disturbance has been indicated as a common node for behavioral deficit in ASD (Fuccillo 2016; Kim et al. 2016). This region receives glutamatergic input from cerebral cortex and dopaminergic projections from ventral tegmental area and substantia nigra (Fonnum et al. 1981; McGeer et al. 1977). The main population of neurons of the striatum are GABAergic medium spiny neurons (MSNs). They express high rate of *N*-methyl-D-aspartate receptors (NMDARs) on their surfaces (Albin et al. 1992; Fonnum et al. 1981; McGeer et al. 1977; Standaert et al. 1999). There are molecular interactions between NMDARs and dopamine receptors (D1 and D2) in MCNs. For example, NMDARs strap diffusible D1 receptors by direct physical interaction, and D2 receptors can also inhibit NMDA receptor-mediated currents in MSNs. It has been revealed that NMDARs are responsible for integration of these two neurotransmitter systems, and disruption of this integration can associate with various behavioral malfunctions, including ones in the ASD (Lee et al. 2015).

Medial prefrontal cortex (mPFC), especially the anterior cingulate cortex (ACC), with the intermediary of glutamate sends facilitator messages to the dorsomedial striatum, called the caudate putamen (CPu). This connection regulates CPu function to issue the inhibitory or excitatory emotional messages following the calculation of the profit and cost algorithm (Karreman and Moghaddam 1996; Vertes 2006). Mutually, striatum indirectly affects ACC function for effort-based decision making via dopaminergic projections (Hauber and Sommer 2009). The normal function of this circuit is very important for the occurrence of normal motor behaviors. It has been proven that increased communication between the prefrontal cortex and caudate nucleus plays an essential role in behavioral deficits especially in ASD (Fuccillo 2016).

Evidence has shown that exposing to the long-term stressful condition can change glutamate effect on post-synaptic neurons via intracellular mechanisms that is regulated by NMDARs (Calabrese et al. 2012; Lee et al. 2003; Martin and Wellman 2011). On the other hand, psychological stress elevates NADPH oxidase type2 (Nox2) enzyme activity and causes ROS accumulation in the brain (Markovic et al. 2017; Sorce and Krause 2009). There is a correlation between NMDAR and Nox2 activities. Generally, activation of NMDARs enhances intracellular reactive oxygen species (ROS) produced by Nox2 enzyme as a messenger of receptor signaling pathway (Girouard et al. 2009). Therefore, extensive NMDAR activation can lead to neuronal oxidative damage mediated by overproduced superoxide radicals. However, neural cells are protected against oxidative damage by enzymatic and non-enzymatic antioxidant systems (Naziroglu 2012). In particular, catalase is known as a common enzymatic defense biomarker. It has been shown that catalase activity in serum of autistic children is significantly higher than control subjects (María et al. 2013). It could be a compensatory intracellular mechanism to neutralize reactive oxygen species.

In the present study, we mainly focused on the role of NMDA receptor in caudate putamen and investigated the potential effects of optogenetic stimulation of anterior cingulate cortex on the expression of this receptor and Nox2 enzyme in an autistic-like animal model induced by neonatal isolation.

Materials and Methods

Animals

All stages of this study are in accordance with the National Institutes of Health (NIH), bioethics. In addition, the ethics committee of Shahid Beheshti University confirmed the validity of this project. Female and male Wistar rats were

mated (obtained from animal department of Shahid Beheshti University (SBU) neuroscience research center). Pregnant females were housed individually per cage with unlimited access to water and food under controlled laboratory conditions (a 12-h light/dark cycle with conditions of constant temperature at 21 ± 3 °C). The male offspring of these matings served as subjects. Male litters were randomly assigned as either maternal deprivation (MD) or control (Ctrl). All experiments were carried out in adult male offspring from PND 50 to PND 80.

Groups and Experimental Design

Litters' birth was considered as zero postnatal day (PND 0). The next day (PND 1), two-thirds of male offspring were randomly assigned as maternal deprivation. Neonatal isolation was performed for 10 days (PND 1-PND 10) (3 h/day, between 8 and 12 am). During the deprivation procedure, the neonates separated from their mothers and transferred to an individual plastic chamber (10 cm in diameter, 12 cm deep) with suitable distance, so that contact between them would not be possible (Rees et al. 2008; Wu et al. 2014). Other pups, as a control subject, spent the entire day with their mothers. After that, all of the pups remained with their mothers from PND 11 to PND 21. At the end of the infancy in PND 21, mothers were separated from their children and 24 mature male rats were divided into three groups equally (with 8 members in each group): two groups included maternal deprived rats (MD), and one was considered as the control group (Ctrl). They were kept under standard condition until PND 50.

Measures

Optogenetic Intervention

In PND 50, half of MD rats were randomly selected. After being anesthetized with ketamine/xylazine (80/20 mg/kg i. p.), virus carrying pLenti-CaMKIIa-hChR2 (H134R)-mCherry-WPRE (2.5 µl, 10^8–10^9 Tu/ml) was infused in to their ACC in each hemisphere of the brain (AP: + 1.9 mm, ML: ± 0.9 mm, DV: − 3 mm) using a Hamilton syringe (0.05 µl/min) under stereotaxic surgery (Guang-yan Wu and Sui 2015). This group with *channelrhodopsin* was named MD (ChR2+). The members of the other two groups received (plenti-CaMKIIa-mCherry-WPRE) virus, without (ChR2) gene, in the same regions of the brain to investigate the interventional variable effects.

After 3 weeks, we used anti-mCherry antibody (abcam, Cat# ab167453) ensuring the expression of external genes in neurons of ACC by immuno-histochemical detection of mCherry protein (Fig. 1). After that, all of animals in the three groups were anesthetized with ketamine/xylazine.

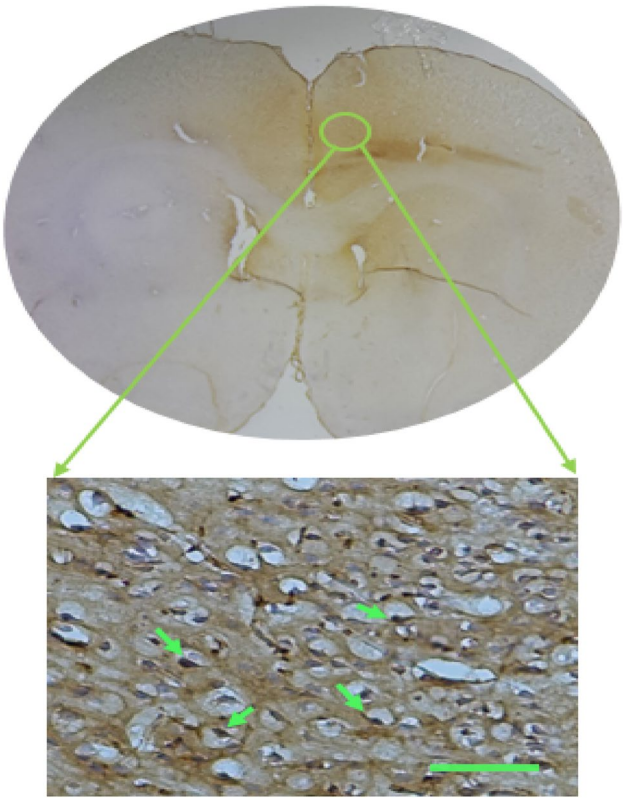


Fig. 1 Selective regions of the virus injection via stereotaxic surgery and example of mCherry expression in the ACC (21 days after virus injection); arrows pointed to mCherry positive neurons. Scale bars indicate 100 µm

Flowingly, dual LC fiber optic cannula was implanted in the brain coordinates (AP: + 1.9 mm, ML: ± 0.9 mm, DV: − 3 mm) through stereotaxic surgery and secure that with dental cement to the skulls (Guang-yan Wu and Sui 2015). Implantable optical fibers were constructed based on standard protocols (Sparta et al. 2012). The animals were allowed to rest in a recovery cage after surgical implantation. The surgery was conducted according to established animal care guidelines and protocols.

One day post surgery, ACC neurons in both hemispheres of the brain were exposed to blue laser (473 nm, ~ 9–11 mW output at the tip of a 200-µm fiber, $f = 10$ Hz, number of pulses = 120, duty = 10%) in all the groups. This process performed during six consecutive days for 15 min per day light was delivered from the 473-nm laser (CNI laser) via optical fiber through a rotary joint patch cable (RJPSF2, Thorlabs) in order to enable the rat to rotate freely in the chamber. Then, the optical commutator was connected to a bifurcated multimode fiber optic cable (BFYL2LF01, Thorlabs) for simultaneous in vivo optical stimulation of two targets in brain areas, also known as Y-cables. Two ports of Y-cables were connected with a ceramic sleeve (ADAL1, Thorlabs) to surgically

implanted LC ceramic ferrule. Rats were lightly anesthetized by isoflurane for connecting of the Y-cables to the dual LC fiber optic cannula.

In each experiment, the laser was turned on for approximately 40 min to stabilize. Subsequently, the photo-stimulation process began.

Behavioral Test Recording

One day after completing optogenetic stimulation period, in PND 80, adult rats were subject to estimation of repetitive behavior and stranger phobia as autistic-like behavior (Crawley 2007, 2012). All of behavioral tests were performed after an hour of adaptation to laboratory environment.

Measurement of Social Interaction

A three-chamber apparatus was used to investigate the rats' social interaction deficit. This maze contained three glass compartments with the same measurements (length–width–height = 40–35–20 cm for each) connected to each other with two gates.

First, each experimental rat was kept in central chamber. It could freely move about in three spaces in order to recognize the setting for 10 min. Then, an empty cage was kept as an object in one of the compartment, as well as keeping cage containing a strange rat as a subject in another compartment. Subject was homogenous in age and gender with experimental rat. Ten minutes were given to each experimental rat to go to the object or subject compartment, and two people recorded the time blindedly. The attendance index in each chamber was the 4-feet rat's entry to each compartment.

Measurement of Stranger Phobia

Immediately after social interaction test recording, when experimental rat was present in central chamber, two gates closed and the empty cage was replaced with another cage containing a new stranger rat. Then, the gates were opened and the time that subjects spent in each compartment near a familiar or a newly stranger rat was recorded during 10 min by two people blindedly. The chambers were cleaned with 70% alcohol and water between two tests (Kaidanovich-Beilin et al. 2011).

Measurement of Repetitive Behavior

In this test, rats were placed in the center of an open-field apparatus [40 × 40 cm] and after 10 min of habituation, two people recorded the time that rats spent in self-grooming as a repetitive behavior for 10 min blindedly. Between the two tests, the chambers were cleaned with 70% alcohol and water (Wu et al. 2014).

Immuno-histochemistry Staining

Immediately, four rats from each group were subjected to anesthesia by ketamine/xylazine after behavioral tests. Besides, they were perfused transcardially with saline solution followed by 4% paraformaldehyde (in phosphate buffer 100 mM, pH 7.4) as a fixative solution. The brains, dehydrated in 30% sucrose solution for 2 days, were removed from the skull after overnight post-fixed in the same fixative. Then, the whole brain was embedded in OCT compound and was quickly frozen at − 80 °C. Four coronal sections (20 µm thickness) from each brain containing CPu regions were produced by a cryomicrotome apparatus (Sci Lab). Two sections were used for NMDAR1 subunit staining using anti-NMDA receptor 1 (5: 1000, Abcam, Cat # 17345), and the other half was evaluated for measuring the level of $gp91^{phox}$ catalytic subunit of Nox2 enzyme using anti-Nox2-$gp91^{phox}$ antibody (1: 1000, Abcam, Cat # 31092) in the CPu region using immuno-histochemistry technique (IHC). Frozen IHC staining has been done according to the previous protocol with a little modification (Jin et al. 2014).

Brain sections were incubated in cold acetone for 10 min, and after three times they were washed up with PBS, treated with 3% H_2O_2 saluted in methanol (10 min) for eliminating endogenous peroxidase activity. Non-specific connections were blocked with 30-min incubation with blocker solution (Dako, Denmark) and primary antibody was added overnight to each brain section at 4 °C. The next day, brain slices were exposed to Biotin and Avidine solutions (ABC, Abcam), respectively, for 25 min. After that, 3,3'-diaminobenzidine (DAB) solution (Dako, Denmark) was added to brain sections (for 10 min) in dark condition at room temperature and cell nuclei were stained with hematoxylin. Finally, the number of cells which expressed high level of NMDAR1 or Nox2-$gp91^{phox}$, as positive cells for each, was counted in two similar regions of CPu in each hemisphere of the brain sections by the light microscope (Nikon, E200) at the objective power of 10×. The average number of positive cells was then counted in eight areas of CPu in each animal.

Western Blotting

After behavioral testing, a set of animals ($n = 4$) was anesthetized with ketamine/xylazine and the whole blood in the vessels was replaced by saline solution via transcardially perfusion. Besides, the brains were rapidly removed and caudate putamen (from both left and right hemisphere) of each animal were collected. The tissue was homogenized in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% TritonX-100, 0.25% sodium deoxycholate, 0.1% sodium

dodecyl sulfate (SDS), 1 mM EDTA, and 1 mM PMSF). Protein level was determined using the Bradford method and 80 µg of protein was loaded on the electrophoresis gel. The samples were then separated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billercia, MA). To block non-specific background, the membranes were incubated with non-fat dry milk (Amersham, Ecl Advance TM) for 1 h at room temperature. Afterwards, the membranes were probed with anti-NMDAR1 antibody (0.5:1000; Abcam, Cat # 17345) and anti-Nox2-gp91^{phox} antibody (2:1000; Abcam, Cat # 31092) overnight at 4 °C. The next day, blots were washed with TBST buffer and probed with HRP-conjugated secondary Goat Anti-Rabbit IgG H&L (HRP) antibody (1:12,000, Abcam, Cat# ab97051) for 1 h at room temperature. Subsequently, the blots were developed by the enhanced chemiluminescence detection system (ECL, BIO RAD, USA). For normalization of protein amounts, β-actin expression was analyzed using β-Actin (13E5) rabbit monoclonal antibody (1:750; Cell signaling, Cat# 4970) as an internal control. Finally, the intensities of bands were quantified by scan of X-ray films and analysis by Image J software.

Catalase Activity

To study catalase activity, the homogenous caudate putamen solution was centrifuged and supernatant was collected. Catalase activity of samples was assayed against catalase buffer contained 30% H_2O_2 in 50 ml PBS compared with blank, the buffer without sample tissue, by spectrophotometer at 240 nm for 2 min in 25°C. The absorption changes at the 240 nm wavelength were related to H_2O_2 substrate decomposition mediated by catalase (Milanizadeh et al. 2018).

Data Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed in 16th version of SPSS. Shapiro–Wilk test was used to evaluate data distribution normality ($p \geq 0.05$). Social interaction and stranger tests were analyzed by repeated-measures ANOVA with condition of the task (object/subject) and (familiar/stranger) as within-subject factors, respectively. The behavioral test and enzymatic and molecular analysis for comparing between three groups were performed using one-way ANOVA followed by Tukey post hoc test. The level of significance was set at < 0.05.

Results

Effects of Periodic Photo-Stimulation of ACC on Autistic-Like Behavior

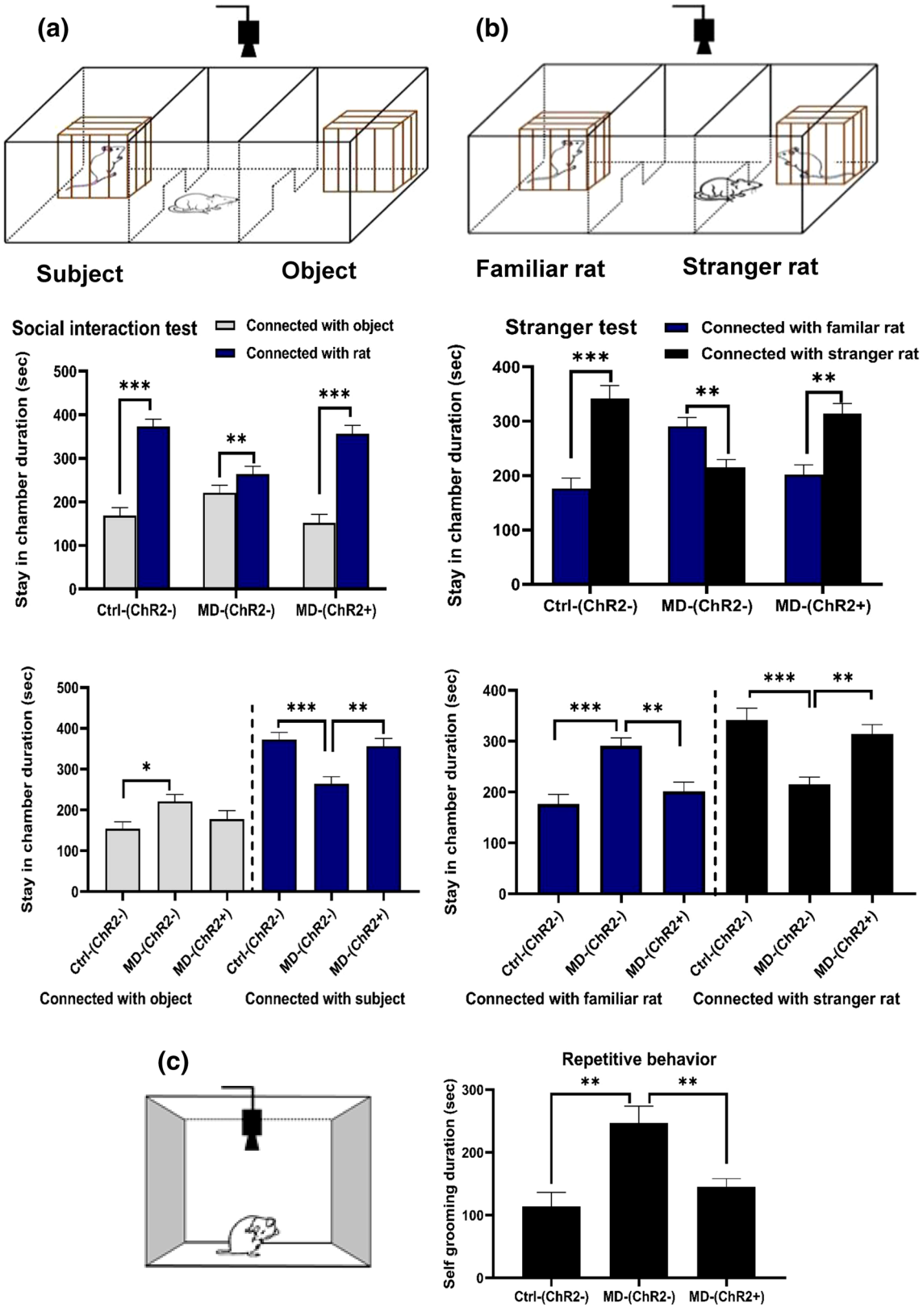
The results showed that neonatal isolation stress leads to long-term disturbances in behavioral performances that are similar to autistic-like symptoms. As is shown in Fig. 2a, during a three-chambered social interaction test, the members of three groups preferred connecting with rat (subject) to empty cage (object). The results of one-way ANOVA analyses of differences between groups represented that the time which MD-(ChR2−) rats spent to connect with subject is less than that was shown in Ctrl-(ChR2−) group ($p = 0.0003$). In contrast, the MD-(ChR2−) spent significantly more time in the chamber with the object compared with Ctrl-(ChR2−) group ($p = 0.023$). Optogenetic treatment led to meaningful increase tendency in MD-(ChR2+) rats to connect with the subject compared with MD-(ChR2−) group ($p = 0.001$).

The result showed that during a three-chambered stranger test, MD-(ChR2−) spent significantly more time in the chamber with the familiar rat compared with opposite chamber with a stranger rat ($p = 0.003$). In contrast, control group meaningfully preferred communication with stranger rats to familiar ones ($p = 0.0001$). Periodic opto-stimulation of ChR2+ neurons in ACC regions of the brain in MD-(ChR2+) group led to significant increase tendency of communication with the stranger rats compared with familiar rats ($p = 0.008$) (Fig. 2b).

Also, the time spent for self-grooming in adult MD-(ChR2−) rats was meaningfully higher than control group ($p = 0.001$). The chronic stimulation of ACC regions caused a significant decrease in self-grooming time as a repetitive behavior indicator in the MD-(ChR2+) groups compared with MD-(ChR2−) rats ($p = 0.009$). However, there was no significant difference in the duration of repetitive behavior between MD-(ChR2+) and Ctrl-(ChR2−) groups ($p = 0.576$) (Fig. 2c).

Effects of Optogenetic Treatment on NMDAR1 and Nox2/gp91^{phox} Protein Density in CPu Regions of the Brain

Maternal deprivation stress in early duration of life led to significant increase in the levels of NMDAR1 and Nox2/gp91^{phox} proteins in the CPu area of the brain in adult MD-(ChR2−) compared with control group. As is shown in Fig. 3, optogenetic treatment for 6 days significantly reduced the density of NMDAR1 and Nox2/gp91^{phox} in the CPu region of the MD-(ChR2+) group compared with MD-(ChR2−) rats.


(a)
Subject
Object
(b)
Familiar rat
Stranger rat
Social interaction test
Connected with object
Connected with rat
Stay in chamber duration (sec)
Ctrl-(ChR2-)
MD-(ChR2-)
MD-(ChR2+)
Stranger test
Connected with familar rat
Connected with stranger rat
Connected with object
Connected with subject
Connected with familiar rat
Connected with stranger rat
(c)
Repetitive behavior
Self grooming duration (sec)

◂**Fig. 2** The effects of optogenetic stimulation of ACC neurons (PND 73–79, 15 min/day) on autistic-like behavior induced by neonatal isolation stress (3 h/day for 10 days) in adult experimental group ($n=8$). **a** Social interaction diagram shows the mean duration of time (±SEM) that members of each group spend in the chamber with the empty cage (as an object) and in the opposite chamber with cage containing rat (as a subject). **b** Stranger test diagram shows the difference of time that each group spends in the chamber with the familiar rat and in the opposite chamber with a stranger rat. **c** This diagram shows the mean self-grooming time (±SEM) that members of each group reveal as a repetitive behavior. Data distribution was normal in each group ($p \geq 0.05$). The level of significance was set at <0.05. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Ctrl-(ChR2−): control group; MD-(ChR2−): maternal deprived group; MD-(ChR2+): channel rhodopsin knock-in maternal deprived group

Effect of Optogenetic Treatment on Catalase Activity in the CPu Regions of the Brain

The mean catalase activation of CPu regions of the brain at MD-(ChR2−) rats was meaningful higher than Ctrl-(ChR2−) group. Under the influence of optogenetic treatment, catalase activity decreased in MD-(ChR2+) rats compared with the MD-(ChR2−) group. However, there was no significant difference between MD-(ChR2+) rats and control group (Fig. 4).

Discussion

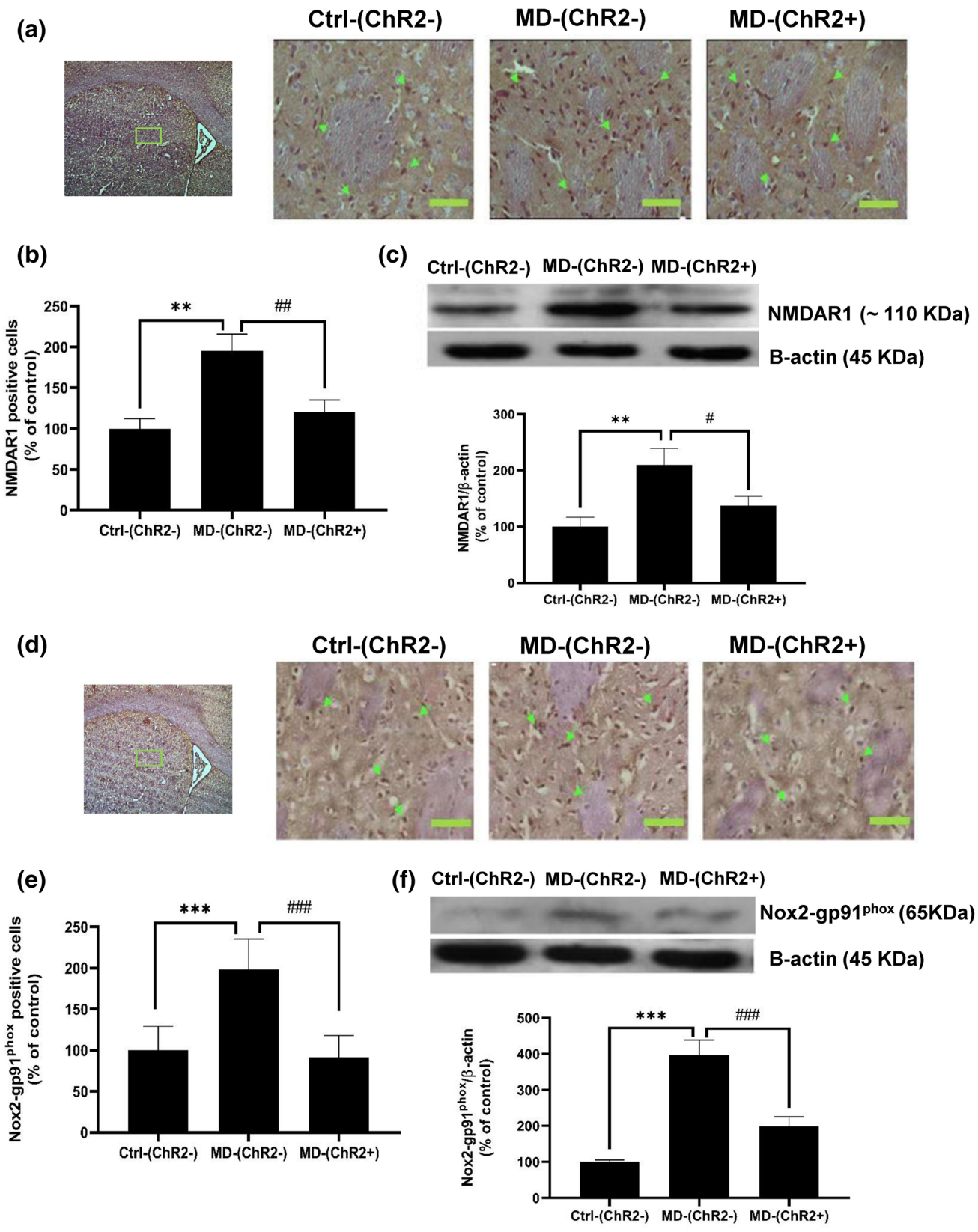
Stress factors during early period of life have been shown to affect brain development and lead to neurobehavioral impairments (Curley et al. 2011; Gudsnuk and Champagne 2012). Though neonatal isolation stress has been shown to increase autism-like phenotypes (Lupien et al. 2009; Tan et al. 2018; Wu et al. 2014), there is no clear evidence of the molecular mechanisms by which MD may bring about such abnormal behavior and striatal functions in adult animals.

The present study shows that MD stress makes changes in offspring behavior as well as increased the NMDAR1 and Nox2-gp91^{phox} levels and also catalase activity in striatum. We identified that although MD rats exhibited increased repetitive/stereotypic-like activity and had a decreased tendency to stranger rats, they only displayed significant preference for subject over an object. Our finding is similar to the result which was previously observed in valproate sodium (VPA)-exposed autistic-like rats (Cho et al. 2017). More importantly, we present results that ACC opto-stimulation for 6 days (PND73-79, 15 min/day) effectively reduces the increased NMDAR1 and Nox2-gp91phox levels in CPu, as well as overcomes the stereotypical behavior and phobia from aliens caused by neonatal isolation in offspring aged 80 days.

Our findings are in agreement with those of other authors who showed that the increased sensitivity of brain structures, after exposing stressful condition, could lie in the fact that stress modifies the effect of dopamine releasing in the prefrontal cortex and striatum regions, which is mediated by NMDA receptors (Rice et al. 2014). This suggests that an appropriate range of NMDAR function is critical for normal behavior, such that, when NMDAR function falls outside this range, behavioral deficit such as ASDs may result (Carvajal et al. 2016; Lee et al. 2015). For instance, the elevation of NMDARs function in ASD has been reported; thus, administration of memantine and amantadine as NMDARs antagonist is shown to improve symptoms associated with ASD (Hosenbocus and Chahal 2013; Rojas 2014). Furthermore, memantine counteracted the increase in NMDAR1 and CaMKIIα levels in the striatum of CTM-exposed mice (which have prenatal exposure to citalopram) and attenuates CTM-induced autistic-like behaviors (Zahra et al. 2018). Therefore, the behavioral impairment seen in our study may be attributed to increased NMDAR-mediated excitation in striatum of MD rats.

In present study, the accurate mechanism of optogenetic stimulation efficacy on NMDAR expression is unknown. Previous studies have shown that optogenetic techniques can affect a variety of proteins that participated in intracellular changes or tissue morphology alterations (Guglielmi et al. 2016). This suggests that elevation of pyramidal cell projection from ACC region, via optogenetic stimulation, leads to excessive Ca^{2+} influx through high-density NMDARs on the CPu cells which can result in neuronal toxicity and in lack of proper functioning of the compensatory mechanisms (Carvajal et al. 2016). It probably can be amended by defensive systems that suppress obligatory NMDAR expression in CPu regions. MicroRNAs could be mentioned as epigenetic factors that affect the expression of genes at the translation levels. For example, miR-204 influences the Ephβ2 expression at the translation level. Ephβ2 is known as a synaptic plasticity modulator that acts via regulation of NMDAR1 subunit expression. A new study has identified that the elevation of miR-204 level followed by aging in the hippocampus can cause the reduction of NMDAR1 level during memory disruption process via the suppression of Ephβ2 expression (Mohammed et al. 2016). It is suggested that this micro-RNA could play critical role in different regions of the brain such as CPu for neural protective aims. Further studies are needed to prove this hypothesis.

In addition, our results indicated that MD stress could elevate Nox2-gp91^{phox} level in CPu, and opto-stimulation of ACC could suppress striatal Nox2-gp91phox overexpression. Previous reports have identified NADPH oxidase type-2 (Nox2) as the primary source of NMDA-induced superoxide production (Brennan et al. 2009; Girouard et al. 2009). Reactive oxygen species (ROS) are key mediators of NMDA receptor signaling (Kishida and Klann 2007). It is likely that the Nox2-gp91^{phox} density, as a catalytic site of Nox2 enzyme,


(a)
Ctrl-(ChR2-)
MD-(ChR2-)
MD-(ChR2+)
(b)
NMDAR1 positive cells (% of control)
250
200
150
100
50
0
**
##
Ctrl-(ChR2-)
MD-(ChR2-)
MD-(ChR2+)
(c)
Ctrl-(ChR2-) MD-(ChR2-) MD-(ChR2+)
NMDAR1 (~ 110 KDa)
B-actin (45 KDa)
NMDAR1/β-actin (% of control)
300
200
100
0
**
#
Ctrl-(ChR2-)
MD-(ChR2-)
MD-(ChR2+)
(d)
Ctrl-(ChR2-)
MD-(ChR2-)
MD-(ChR2+)
(e)
Nox2-gp91phox positive cells (% of control)
250
200
150
100
50
0
***
###
Ctrl-(ChR2-)
MD-(ChR2-)
MD-(ChR2+)
(f)
Ctrl-(ChR2-) MD-(ChR2-) MD-(ChR2+)
Nox2-gp91phox (65KDa)
B-actin (45 KDa)
Nox2-gp91phox/β-actin (% of control)
500
400
300
200
100
0
***
###
Ctrl-(ChR2-)
MD-(ChR2-)
MD-(ChR2+)

◂**Fig. 3** The effects of maternal deprivation stress (PND 1–10, 3 h/day) and optogenetic stimulation of ACC neurons (PND 73–79, 15 min/day) on the expression of NMDAR1 subunit and Nox2 catalytic site in CPu region of the adult experimental rats ($n=4$). Representative images of caudate putamen sections immunohistochemically stained for NMDAR1 (**a**) and Nox2-gp91^{phox} (**d**). Scale bars indicate 50 µm. Histograms showing the number of NMDAR1 (**b**) and Nox2-gp91^{phox} (**e**) positive cells. Representative western blots showing immunoreactivity of NMDAR1 (**c**) and Nox2-gp91^{phox} (**f**) and quantitative analysis of these protein expressions relative to β-actin. $^{**}p<0.01$ and $^{***}p<0.001$ versus control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ versus MD-treated group. Ctrl-(ChR2-): Control group; MD-(ChR2−): maternal deprived group; MD-(ChR2+): channelrhodopsin knock-in maternal deprived group

would be correlated with NMDAR1 accumulation (an obligatory NMDAR subunit). In addition, the synchronic overexpression of NMDAR1 and gp91^{phox}-Nox2 in MD rats can induce oxidative stress via excessive accumulation of ROS. If antioxidant systems do not respond properly, active oxygen radicals can attack vital biomolecules and cause gene damage and lipid peroxidation and reduce cell membrane fluidity and ultimately neuron death as well (McGinnis 2004; Naziroglu 2012). Previous reports have indicated alteration in enzymatic antioxidant activity and oxidant metabolite levels in different cerebral areas of animals under stress of neonatal isolation (Campos-Rangel et al. 2017; Diehl et al. 2014; González-Fraguel et al. 2013; Sogut et al. 2003; Zahra et al. 2018). Based on our finding, maternal deprivation (during PND 1–10, 3 h/day) promoted catalase activity in the CPu of offspring aged 80 days. It can be as an intracellular compensatory mechanism for neutralizing oxygen species, especially produced by Nox2 high activity. It prevents CPu regions against long-term inflammation effects. Optogenetic stimulation of ACC also counteracts the increase in catalase activity in the striatum of MD-(ChR2+) rats. Recently, miRNA-146a has been found as an effective agent that could suppress expression of antioxidant enzyme and inflammatory factors (Ji et al. 2013; Xie et al. 2018). It is probable that alteration in catalase activity in adult MD rats was due to miR-146a reduction. This proposal requires further studies.

These findings indicated that optogenetic stimulation of ACC region may alleviate the symptoms of ASD-like behaviors caused by neonatal isolation through regulating the NMDAR function in CPu in adulthood. However, further experiments examining influence of ACC stimulation of MD rats on the molecular alteration in another region of the brain such as hippocampus, amygdala, and cortex would be carried out in the future.

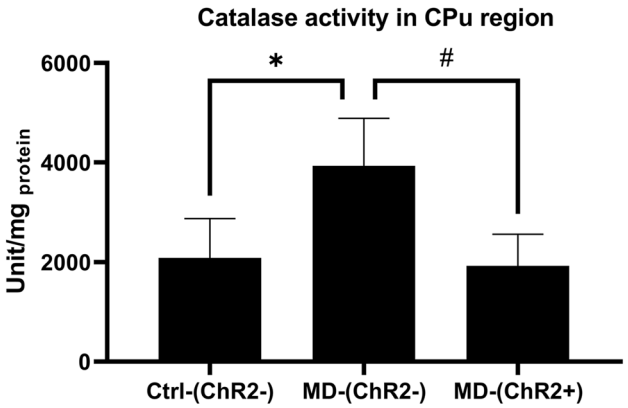


Fig. 4 The effects of optogenetic stimulation of ACC neurons (PND 73–79, 15 min/day) on the catalase activity in CPu regions of the brain in adult maternal deprived rats ($n=4$). $^{*}p<0.05$ versus control group; $^{\#}p<0.05$ versus MD-treated group. Ctrl-(ChR2−): control group; MD-(ChR2−): maternal deprived group; MD-(ChR2+): channel rhodopsin knock-in maternal deprived group

Conclusion

According to our findings, the occurrence of repetitive behavior and social deficit as autistic-like behavior in adult MD rats may be related to overexpression of NMDA receptors and Nox2-gp91^{phox} enzyme, as a NMDAR intracellular messenger, in CPu area. Additionally, neonatal isolation led to elevation of catalase activity in the CPu regions of the adult offspring compared with control group. It could neutralize over oxygen species produced by high level of Nox2 in CPu region. Chronic optogenetic stimulation of ACC neurons containing (ChR2+) led to significant reduction in the appearance of stereotypical behavior and alien-phobia in MD rats. These findings suggested that deviation of NMDAR and NOX2 expression in CPu regions could be related to autistic-like behavior induced by neonatal isolation.

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Compliance with Ethical Standards

Conflict of interest Hereby the authors confirm that there are no conflicts of interest in the present research. All the authors read and confirmed the final paper.

Ethical Approval All the experimental protocols were approved by the Animal Research Ethics Committee at Shahid Beheshti University.

References

Albin, R. L., Makowiec, R. L., Hollingsworth, Z. R., Dure, L. S. T., Penney, J. B., & Young, A. B. (1992). Excitatory amino acid binding sites in the basal ganglia of the rat: A quantitative autoradiographic study. *Neuroscience, 46*(1), 35–48.

Alexander, B., & Poletaev, B. A. S. (2016). Autism: Genetics or Epigenetics? *ARC Journals of Immunology and Vaccines, 1*(2), 7.

Association, A. P. (2013). *Diagnostic and statistical manual of mental disorders*. Washington DC: American Psychiatric Association.

Bahi, A. (2017). Hippocampal BDNF overexpression or microR124a silencing reduces anxiety- and autism-like behaviors in rats. *Behavioural Brain Research, 326*, 281–290. https://doi.org/10.1016/j.bbr.2017.03.010.

Brennan, A. M., Suh, S. W., Won, S. J., Narasimhan, P., Kauppinen, T. M., Lee, H., … Swanson, R. A. (2009). NADPH oxidase is the primary source of superoxide induced by NMDA receptor activation. *Nature Neuroscience, 12*(7), 857–863. https://doi.org/10.1038/nn.2334.

Burguiere, E., Monteiro, P., Feng, G., & Graybiel, A. M. (2013). Optogenetic stimulation of lateral orbitofronto-striatal pathway suppresses compulsive behaviors. *Science, 340*(6137), 1243–1246. https://doi.org/10.1126/science.1232380.

Calabrese, F., Guidotti, G., Molteni, R., Racagni, G., Mancini, M., & Riva, M. A. (2012). Stress-induced changes of hippocampal NMDA receptors: Modulation by duloxetine treatment. *PLoS ONE, 7*(5), e37916. https://doi.org/10.1371/journal.pone.0037916.

Campos-Rangel, A., Saavedra-Molina, L. T.-A., & Manzo-Avalos, A. S. (2017). Effect of maternal separation on oxidative and nitrosative stress in the brain of rat offspring. *The FASEB Journal, 31*(1), 779-7.

Carvajal, F. J., Mattison, H. A., & Cerpa, W. (2016). Role of NMDA receptor-mediated glutamatergic signaling in chronic and acute neuropathologies. *Neural Plasticity, 2016*, 2701526. https://doi.org/10.1155/2016/2701526.

Cho, H., Kim, C. H., Knight, E. Q., Oh, H. W., Park, B., Kim, D. G., & Park, H. J. (2017). Changes in brain metabolic connectivity underlie autistic-like social deficits in a rat model of autism spectrum disorder. *Scientific Reports, 7*(1), 13213. https://doi.org/10.1038/s41598-017-13642-3.

Crawley, J. N. (2007). Mouse behavioral assays relevant to the symptoms of autism. *Brain Pathology, 17*(4), 448–459. https://doi.org/10.1111/j.1750-3639.2007.00096.x.

Crawley, J. N. (2012). Translational animal models of autism and neurodevelopmental disorders. *Dialogues in Clinical Neuroscience, 14*(3), 293–305.

Curley, J. P., Jensen, C. L., Mashoodh, R., & Champagne, F. A. (2011). Social influences on neurobiology and behavior: Epigenetic effects during development. *Psychoneuroendocrinology, 36*(3), 352–371. https://doi.org/10.1016/j.psyneuen.2010.06.005.

Deisseroth, K. (2012). Optogenetics and psychiatry: Applications, challenges, and opportunities. *Biological Psychiatry, 71*(12), 1030–1032. https://doi.org/10.1016/j.biopsych.2011.12.021.

Diehl, L. A., Pereira Nde, S., Laureano, D. P., Benitz, A. N., Noschang, C., Ferreira, A. G., … Dalmaz, C. (2014). Contextual fear conditioning in maternal separated rats: The amygdala as a site for alterations. *Neurochemical Research, 39*(2), 384–393. https://doi.org/10.1007/s11064-013-1230-x.

Fonnum, F., Storm-Mathisen, J., & Divac, I. (1981). Biochemical evidence for glutamate as neurotransmitter in corticostriatal and corticothalamic fibres in rat brain. *Neuroscience, 6*(5), 863–873.

Fuccillo, M. V. (2016). Striatal circuits as a common node for autism pathophysiology. *Frontiers in Neuroscience, 10*, 27. https://doi.org/10.3389/fnins.2016.00027.

Girouard, H., Wang, G., Gallo, E. F., Anrather, J., Zhou, P., Pickel, V. M., & Iadecola, C. (2009). NMDA receptor activation increases free radical production through nitric oxide and NOX2. *Journal of Neuroscience, 29*(8), 2545–2552. https://doi.org/10.1523/JNEUROSCI.0133-09.2009.

González-Fraguel, M. E., Hung, M. L. D., Vera, H., Maragoto, C., Noris, E., Blanco, L., ... & Robinson, M. (2013). Oxidative stress markers in children with autism spectrum disorders. *British Journal of Medicine & Medical Research, 3*(2), 10.

Guang-yan Wu, G. L., Zhang, H., Chen, C., Liu, S., Feng, H., & Sui, J. F. (2015). Optogenetic stimulation of mPFC pyramidal neurons as a conditioned stimulus supports associative learning in rats. *Scientific Reports, 5*, 11.

Gudsnuk, K., & Champagne, F. A. (2012). Epigenetic influence of stress and the social environment. *ILAR Journal, 53*(3–4), 279–288. https://doi.org/10.1093/ilar.53.3-4.279.

Guglielmi, G., Falk, H. J., & De Renzis, S. (2016). Optogenetic control of protein function: From intracellular processes to tissue morphogenesis. *Trends in Cell Biology, 26*(11), 864–874. https://doi.org/10.1016/j.tcb.2016.09.006.

Hauber, W., & Sommer, S. (2009). Prefrontostriatal circuitry regulates effort-related decision making. *Cerebral Cortex, 19*(10), 2240–2247. https://doi.org/10.1093/cercor/bhn241.

Hosenbocus, S., & Chahal, R. (2013). Memantine: A review of possible uses in child and adolescent psychiatry. *Journal of the Canadian Academy of Child and Adolescent Psychiatry, 22*(2), 166–171.

Ji, G., Lv, K., Chen, H., Wang, T., Wang, Y., Zhao, D., … Li, Y. (2013). miR-146a regulates SOD2 expression in H_2O_2 stimulated PC12 cells. *PLoS ONE, 8*(7), e69351. https://doi.org/10.1371/journal.pone.0069351.

Jin, W., Chung, J.-H. S., Baek, S.-B., Kim, C.-J., & Kim, T.-W. (2014). Treadmill exercise inhibits hippocampal apoptosis through enhancing N-methyl-D-aspartate receptor expression in the MK-801-induced schizophrenic mice. *Journal of Exercise Rehabilitation, 10*(4), 7.

Kaidanovich-Beilin, O., Lipina, T., Vukobradovic, I., Roder, J., & Woodgett, J. R. (2011). Assessment of social interaction behaviors. *Journal of Visualized Experiments*. https://doi.org/10.3791/2473.

Karreman, M., & Moghaddam, B. (1996). The prefrontal cortex regulates the basal release of dopamine in the limbic striatum: An effect mediated by ventral tegmental area. *Journal of Neurochemistry, 66*(2), 589–598.

Kim, H., Lee, Y., Park, J. Y., Kim, J. E., Kim, T. K., Choi, J., … Han, P. L. (2017). Loss of adenylyl cyclase type-5 in the dorsal striatum produces autistic-like behaviors. *Molecular Neurobiology, 54*(10), 7994–8008. https://doi.org/10.1007/s12035-016-0256-x.

Kim, H., Lim, C. S., & Kaang, B. K. (2016). Neuronal mechanisms and circuits underlying repetitive behaviors in mouse models of autism spectrum disorder. *Behavioral and Brain Functions, 12*(1), 3. https://doi.org/10.1186/s12993-016-0087-y.

Kishida, K. T., & Klann, E. (2007). Sources and targets of reactive oxygen species in synaptic plasticity and memory. *Antioxidants & Redox Signaling, 9*(2), 233–244. https://doi.org/10.1089/ars.2007.9.ft-8.

Lee, E. J., Choi, S. Y., & Kim, E. (2015). NMDA receptor dysfunction in autism spectrum disorders. *Current Opinion Pharmacology, 20*, 8–13. https://doi.org/10.1016/j.coph.2014.10.007.

Lee, P. R., Brady, D., & Koenig, J. I. (2003). Corticosterone alters N-methyl-D-aspartate receptor subunit mRNA expression before puberty. *Molecular Brain Research, 115*(1), 55–62.

Lee, Y., Kim, H., Kim, J. E., Park, J. Y., Choi, J., Lee, J. E., … Han, P. L. (2018). Excessive D1 dopamine receptor activation in the dorsal striatum promotes autistic-like behaviors. *Molecular*

Neurobiology, 55(7), 5658–5671. https://doi.org/10.1007/s12035-017-0770-5.

Luo, W., Zhang, C., Jiang, Y. H., & Brouwer, C. R. (2018). Systematic reconstruction of autism biology from massive genetic mutation profiles. *Science Advances, 4*(4), e1701799. https://doi.org/10.1126/sciadv.1701799.

Lupien, S. J., McEwen, B. S., Gunnar, M. R., & Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature Reviews Neuroscience, 10*(6), 434–445. https://doi.org/10.1038/nrn2639.

Markovic, B., Radonjic, N. V., Jevtic, G., Stojkovic, T., Velimirovic, M., Aksic, M., … Petronijevic, N. D. (2017). Long-term effects of maternal deprivation on redox regulation in rat brain: Involvement of NADPH oxidase. *Oxidative Medicine and Cellular Longevity, 2017*, 7390516. https://doi.org/10.1155/2017/7390516.

Martin, K. P., & Wellman, C. L. (2011). NMDA receptor blockade alters stress-induced dendritic remodeling in medial prefrontal cortex. *Cerebral Cortex, 21*(10), 2366–2373. https://doi.org/10.1093/cercor/bhr021.

McGeer, P. L., McGeer, E. G., Scherer, U., & Singh, K. (1977). A glutamatergic corticostriatal path? *Brain Research, 128*(2), 369–373.

McGinnis, W. R. (2004). Oxidative stress in autism. *Alternative Therapies Health and Medicine, 10*(6), 22–36 (**quiz 37, 92**).

Milanizadeh, S., Zuwarali, K. N. N., Aliaghaei, A., & Bigdeli, M. R. (2018). Therapeutic potential of pretreatment with allograft sertoli cells transplantation in brain ischemia by improving oxidative defenses. *Journal of Molecular Neuroscience, 64*(4), 533–542. https://doi.org/10.1007/s12031-018-1054-x.

Mohammed, C. P., Rhee, H., Phee, B. K., Kim, K., Kim, H. J., Lee, H., … Kim, K. (2016). miR-204 downregulates EphB2 in aging mouse hippocampal neurons. *Aging Cell, 15*(2), 380–388. https://doi.org/10.1111/acel.12444.

Naziroglu, M. (2012). Molecular role of catalase on oxidative stress-induced Ca(2+) signaling and TRP cation channel activation in nervous system. *Journal of Receptors and Signal Transduction, 32*(3), 134–141. https://doi.org/10.3109/10799893.2012.672994.

Rees, S. L., Akbari, E., Steiner, M., & Fleming, A. S. (2008). Effects of early deprivation and maternal separation on pup-directed behavior and HPA axis measures in the juvenile female rat. *Developmental Psychobiology, 50*(4), 315–321. https://doi.org/10.1002/dev.20292.

Rice, M. W., Smith, K. L., Roberts, R. C., Perez-Costas, E., & Melendez-Ferro, M. (2014). Assessment of cytochrome C oxidase dysfunction in the substantia nigra/ventral tegmental area in schizophrenia. *PLoS ONE, 9*(6), e100054. https://doi.org/10.1371/journal.pone.0100054.

Rojas, D. C. (2014). The role of glutamate and its receptors in autism and the use of glutamate receptor antagonists in treatment. *Journal of Neural Transmission (Vienna), 121*(8), 891–905. https://doi.org/10.1007/s00702-014-1216-0.

Sogut, S., Zoroglu, S. S., Ozyurt, H., Yilmaz, H. R., Ozugurlu, F., Sivasli, E., … Akyol, O. (2003). Changes in nitric oxide levels and antioxidant enzyme activities may have a role in the pathophysiological mechanisms involved in autism. *Clinica Chimica Acta, 331*(1–2), 111–117.

Sorce, S., & Krause, K. H. (2009). NOX enzymes in the central nervous system: from signaling to disease. *Antioxidants & Redox Signaling, 11*(10), 2481–2504. https://doi.org/10.1089/ARS.2009.2578.

Sparta, D. R., Stamatakis, A. M., Phillips, J. L., van Zessen, R., & Stuber, G. D. (2012). Construction of implantable optical fibers for long-term optogenetic manipulation of neural circuits. *Nature Protocols, 7*(1), 12.

Standaert, D. G., Friberg, I. K., Landwehrmeyer, G. B., Young, A. B., & Penney, J. B. Jr. (1999). Expression of NMDA glutamate receptor subunit mRNAs in neurochemically identified projection and interneurons in the striatum of the rat. *Brain Research Molecular Brain Research, 64*(1), 11–23.

Tan, T., Wang, W., Xu, H., Huang, Z., Wang, Y. T., & Dong, Z. (2018). Low-frequency rTMS ameliorates autistic-like behaviors in rats induced by neonatal isolation through regulating the synaptic GABA transmission. *Frontiers in Cell Neuroscience, 12*, 46. https://doi.org/10.3389/fncel.2018.00046.

Tsuda, M. C., & Ogawa, S. (2012). Long-lasting consequences of neonatal maternal separation on social behaviors in ovariectomized female mice. *PLoS ONE, 7*(3), e33028. https://doi.org/10.1371/journal.pone.0033028.

Vertes, R. P. (2006). Interactions among the medial prefrontal cortex, hippocampus and midline thalamus in emotional and cognitive processing in the rat. *Neuroscience, 142*(1), 1–20. https://doi.org/10.1016/j.neuroscience.2006.06.027.

Wu, X., Bai, Y., Tan, T., Li, H., Xia, S., Chang, X., … Dong, Z. (2014). Lithium ameliorates autistic-like behaviors induced by neonatal isolation in rats. *Frontiers in Behavioral Neuroscience, 8*, 234. https://doi.org/10.3389/fnbeh.2014.00234.

Xie, Y., Chu, A., Feng, Y., Chen, L., Shao, Y., Luo, Q., … Chen, Y. (2018). MicroRNA-146a: A comprehensive indicator of inflammation and oxidative stress status induced in the brain of chronic T2DM rats. *Frontiers in Pharmacology, 9*, 478. https://doi.org/10.3389/fphar.2018.00478.

Yizhar, O. (2012). Optogenetic insights into social behavior function. *Biological Psychiatry, 71*(12), 1075–1080. https://doi.org/10.1016/j.biopsych.2011.12.029.

Zahra, A., Jiang, J., Chen, Y., Long, C., & Yang, L. (2018). Memantine rescues prenatal citalopram exposure-induced striatal and social abnormalities in mice. *Experimental Neurology, 307*, 145–154. https://doi.org/10.1016/j.expneurol.2018.06.003.