BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 30(3), 232-237 (2022)

Autism-Like Behavioral Phenotypes in Mice Treated with Systemic N-Methyl-D-Aspartate

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Abstract

Autism spectrum disorder (ASD) having core characteristics of social interaction problems and repetitive behaviors and interests affects individuals at varying degrees and comorbidities, making it difficult to determine the precise etiology underlying the symptoms. Given its heterogeneity, ASD is difficult to treat and the development of therapeutics is slow due to the scarcity of animal models that are easy to produce and screen with. Based on the theory of excitation/inhibition imbalance in the brain with ASD which involves glutamatergic and/or GABAergic neurotransmission, a pharmacologic agent to modulate these receptors might be a good starting point for modeling. N-methyl-D-aspartic acid (NMDA) is an amino acid derivative acting as a specific agonist at the NMDA receptor and therefore imitates the action of the neurotransmitter glutamate on that receptor. In contrast to glutamate, NMDA selectively binds to and regulates the NMDA receptor, but not other glutamate receptors such as AMPA and kainite receptors. Given this role, we aimed to determine whether NMDA administration could result in autistic-like behavior in adolescent mice. Both male and female mice were treated with saline or NMDA (50 and 75 mg/kg) and were tested on various behavior experiments. Interestingly, acute NMDA-treated mice showed social deficits and repetitive behavior similar to ASD phenotypes. These results support the excitation/inhibition imbalance theory of ASD and that NMDA injection can be used as a pharmacologic model of ASD-like behaviors.

Key Words: Autism spectrum disorder, NMDA, E/I imbalance, ASD animal model, Social deficit, Repetitive behavior

INTRODUCTION

One of the most challenging neurodevelopmental disabilities to date is autism spectrum disorder (ASD). Factors such as heterogeneity, numerous etiologic features, and complex pathophysiology among individuals with ASD are attributes to the difficulty in overcoming this disorder (Masi *et al*., 2017; Cheroni *et al*., 2020; Baranova *et al*., 2021). Large-scale studies have identified that genetic defects largely contribute to the development of ASD (Bailey *et al*., 1995; Smalley, 1997)–which could be hereditary or in part by *de novo* mutations (Sebat *et al*., 2007; Sanders *et al*., 2012; Iossifov *et al*., 2014). The latter is also thought to be caused by environmental factors during early prenatal development from various chemical exposures (Constantino and Todd, 2003).

Based on these risk factors, various animal models using either single-genetic mutation or environmental triggers, or both, have been developed to check and understand autistic pathophysiology (Frye and Llaneza, 2010; Patterson, 2011; Bristot Silvestrin *et al*., 2013; El-Kordi *et al*., 2013; Schroeder *et al*., 2015; Kim *et al*., 2016; Kim *et al*., 2018). These models reveal that various pathways are indeed mingled in the manifestation of autism-like behavior defects. Interestingly, some of these animal models have opposite mechanisms and diverse pathways especially on whether they enhance or suppress the function of the excitatory and inhibitory neurotransmission (Bey *et al*., 2018; Lazaro *et al*., 2019; Zerbi *et al*., 2019; Mohammadi *et al*., 2020; Saitow *et al*., 2020).

Accordingly, E/I imbalance theory is being introduced as the "final common pathway" which could explain the pathophysiology of ASD through glutamatergic and GABAergic dysregulations in key brain areas including the hippocampus, amygdala, neocortex, and cerebellum (Rubenstein and Merzenich, 2003; Kim *et al*., 2016; Uzunova *et al*., 2016). Autistic individuals

Open Access **https://doi.org/10.4062/biomolther.2021.133**

Received Aug 9, 2021 **Revised** Sep 15, 2021 **Accepted** Sep 17, 2021
Published Online Oct 27, 2021

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and animal models of ASD exhibit abnormalities in either glutamatergic or GABAergic (or both) development and transmission which leads to social impairments, repetitive behaviors, and other variable symptoms (Kim *et al*., 2016; Horder *et al*., 2018; Gandhi and Lee, 2021). E/I imbalance could also be attributed to malfunctions of other molecules in the brain such as neuropeptides (oxytocin) (Lopatina *et al*., 2018), synaptic proteins (neuroligins) (Hines *et al*., 2008), and immune regulators (Hughes *et al*., 2018). Thus, E/I imbalance highlights abnormal brain information processing and behavior regulation. It also introduces the idea that the maintenance of E/I homeostasis is important for the regulation of normal behaviors especially related to autism (Lee *et al*., 2017).

Given the potential mechanisms of E/I imbalance in the development of ASD, we aimed to pharmacologically induce an aspect of E/I imbalance in the brain and to determine whether it can impact the core conditions of ASD. In particular, we investigated the systemic effects of NMDA, an amino acid analog similar to glutamate acting solely on the NMDA subtype of receptors, in the manifestations of autistic symptoms. Furthermore, this study aims to develop a simple and reliable model of ASD, which can be scrutinized behaviorally and at the molecular level to help the deeper and broader understanding of ASD.

MATERIALS AND METHODS

Animals

Both male and female ICR mice were obtained from Orient Bio (Seoul, Korea) and were acclimated to the animal facility for a week before experiments. These mice were kept in a same-sex group of six mice per cage and maintained under standard conditions (12 h/12 h circadian cycle, light off time at 14:00, 24 ± 2 degrees Celsius temperature, and 55+15% humidity) with food and water freely available. All animal care and procedures were conducted following the Principle of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985) and approved by the Institutional Animal Care and Use Committee of Konkuk University, Seoul, Korea (KU18054).

Drug preparation and administration

N-methyl-D-aspartic acid (NMDA) was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). The drug was dissolved in physiological saline (0.9% w/v NaCl) at varying doses (50 or 75 mg/kg) and either saline or NMDA was administered via the intraperitoneal (i.p.) route.

Behavioral experiments

Behavioral experiments were performed starting from postnatal day 28 (P28). Each experiment was performed under weak lighting (<5 lux) and subjects were habituated in the experiment room for 1 h before the actual experiment. The experiments were tracked and recorded using the EthoVision XT 24 software (Noldus, Wageningen, the Netherlands). Separate batches of mice were used for each behavioral experiment.

Home cage social test

Home cage social test is used to check the social behavior of mice in a natural and less stressful setting (Kim *et al*., 2019). Mice were housed in a large cage (26×42×18 cm) for at least a week to habituate in the home cage environment. Prior to the actual test, an empty wire cage was placed in the middle of the cage for 30 min of object familiarization. After which, a novel stimulus mouse was introduced inside the wire cage to examine the social interaction of the subject mice. The experiment was recorded using an overhead video recorder and the sniffing duration of each mouse to the stranger was manually assessed by a 'blind' observer (Kim *et al*., 2019).

Self-grooming test

Self-grooming is a behavior used to assess complex repetitive, self-directed, and sequentially patterned behaviors (Kalueff *et al*., 2016). The experiment was performed in a plastic cage measuring 27×22×13 cm. The mice were initially placed individually in an empty cage for 10 min as a habituation period. The test session followed immediately after by video-recording for another 10 min. Each self-grooming behavior was manually scored by a blind observer.

Open field test

The open field test assesses the spontaneous locomotor activity of subject mice. Mice were individually allowed to explore an open box (40×40×40 cm) for 25 min. Three boxes were used to test three animals simultaneously per trial and movements were tracked using the EthoVision software (Noldus). The total distance moved, movement duration, and time spent in the center area (20×20 cm area) were extracted for analysis. The surfaces of each box were cleaned and wiped with 70% ethanol at the end of each trial.

Rotarod test

Rotarod was performed to check the locomotive domain of the mice, particularly balance and coordination, which could be affected by NMDA treatment. A rotating rod in accelerating speed was used as a platform for balancing during the test (Morgan *et al*., 2008). Mice were placed on the top surface of the rotating rod which slowly accelerated from 5 RPM to 25 RPM over 5 min. Mice were induced to walk or balance themselves to keep from falling. The latency to fall was recorded, and each mouse had 3 trials separated by at least 20 min intervals.

Statistics

All data were analyzed using one-way ANOVA for column comparisons or two-way ANOVA for multiple variable comparisons and both followed by Tukey's post hoc test in the GraphPad Prism software (CA, USA). Data were expressed as the means ± the standard error of the mean (SEM).

RESULTS

NMDA injection induced social deficits and increased repetitive behavior in mice

Social interaction using a home-cage test was analyzed to confirm whether the activation of excitatory neurotransmission by NMDA is involved in the regulation of social behaviors. Sociability was checked by measuring the sniffing time of the subject mice to a novel mouse. Administration of NMDA showed a statistical difference when compared to the control mice implying social deficit in both male and female NMDA-induced mice. (Fig. 1). Male mice exhibited reduced sniffing time by NMDA treatment in a dose-dependent manner [F (2,

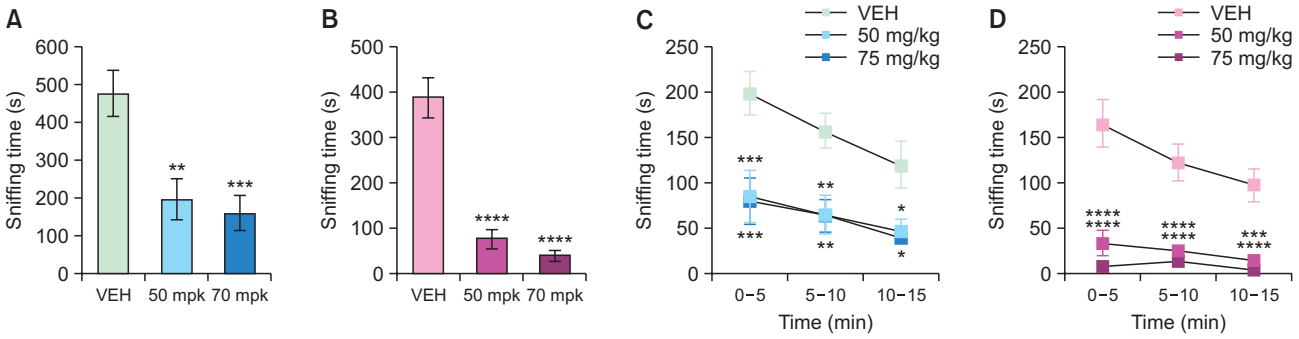

Fig. 1. Home cage social test in male and female mice injected with NMDA. Sniffing time of male (A) and female (B) mice injected with 50 or 75 mg/kg of NMDA. The sniffing time was divided into three 5-min time bins for male (C) and female (D) groups. Bars or symbols indicate the mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001; n=10 per group.

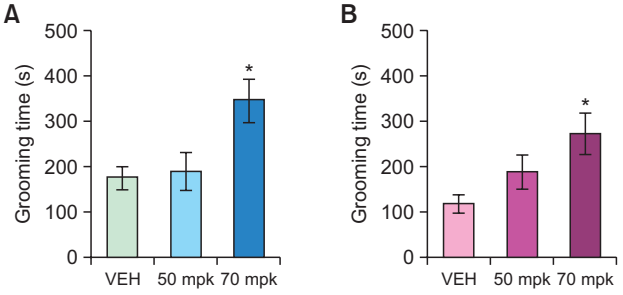

Fig. 2. Self-grooming test in male and female mice injected with NMDA. Grooming time of male (A) and female (B) mice injected with 50 or 75 mg/kg of NMDA. Bars indicate the mean ± SEM. **p*<0.05; n=10 per group.

27)=10.27, *p*=0.0005] (Fig. 1A) and at 5-min time bins (Time, $F_{2,81}$=19.69, *p*<0.0001; treatment, $F_{2,81}$=4.449, *p*=0.0147; interaction $_{time}$×$_{treatment}$, $F_{4,81}$=0.2867, *p*=0.8858; Fig. 1C). A reduced sniffing time was also confirmed in female mice treated with NMDA [F (2, 26)=41.66, *p*<0.0001] (Fig. 1B) even at 5-min time bins (Time, $F_{2,78}$=61.43, *p*<0.0001; treatment, $F_{2,78}$=3.386, *p*=0.0389; interaction time×treatment, $F_{4,78}$=1.474, *p*=0.2180; Fig. 1D). These results indicate that systemic NMDA elicits social deficit in both male and female mice.

In the self-grooming test, NMDA treatment significantly increased the grooming time in male [F (2, 21)=5.823, *p*=0.0097] (Fig. 2A) and female mice, in which NMDA has a dose-dependent effect [F (2, 21)=4.712, *p*=0.0204] (Fig. 2B). Taken together, single NMDA treatment induces social interaction problems and repetitive behaviors in both genders affecting the core symptoms of autism spectrum disorder.

NMDA induced impairment of locomotive functions.

In the open field test, NMDA injection did not affect the distance moved for both male [F (2, 43)=1.206, *p*=0.3095] (Fig. 3A) and female mice [F (2, 26)=0.8462, *p*=0.4405] (Fig. 3B). In contrast, the NMDA injected male mice exhibited reduced movement duration [F (2, 42)=4.196, *p*=0.0218] (Fig. 3C) but not the female mice [F (2, 26)=0.1195, *p*=0.8878] (Fig. 3D). Similarly, the time spent in the center was significantly decreased in NMDA-treated male mice [F (2, 41)=22.55, *p*<0.0001] (Fig. 3E), and only a decreasing trend in NMDA-treated female mice [F (2. 26)=0.7099, *p*=0.5010] (Fig. 3F).

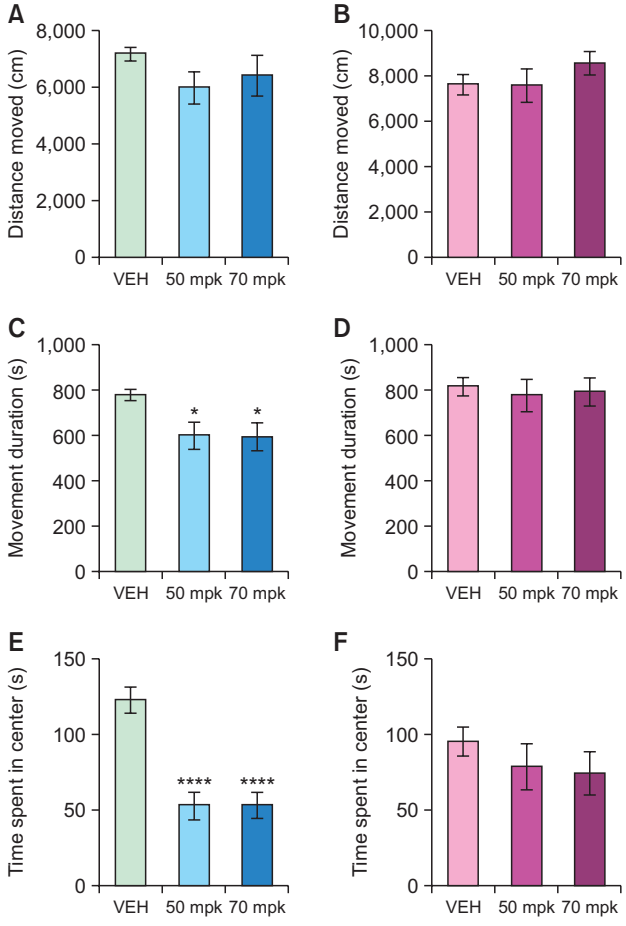

Fig. 3. Open field test in male and female mice injected with NMDA. Distance moved of male (A) and female (B) mice injected with 50 or 75 mg/kg of NMDA. The movement duration (C) and time spent in the center area (E) of NMDA-treated male mice were reduced but not of the NMDA-treated females (D & F, respectively). Bars indicate the mean ± SEM. **p*<0.05 and *****p*<0.0001; n=10 per group.

Thus, NMDA treatment may induce anxiety-like behavior in male mice.

To confirm whether the mice's lack of sociability was not

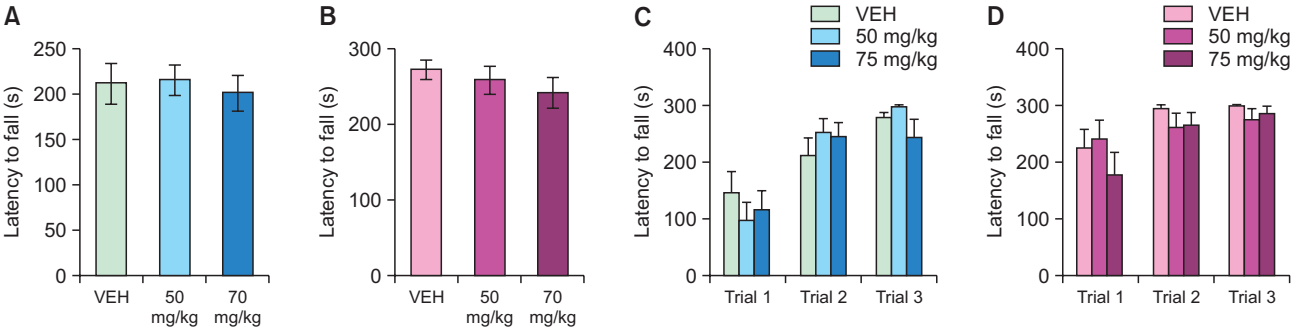

Fig. 4. Rotarod test in male and female mice injected with NMDA. The average latency to fall in male (A) and female mice (B) injected with 50 or 75 mg/kg of NMDA. The latency to fall in each of the three trials was depicted for male (C) and female (D) groups. Bars indicate the mean ± SEM. n=10 per group.

due to impairments in its locomotive functions and motor coordination, rotarod test was employed. The assessment of motor coordination in both male and female NMDA-injected mice demonstrated no significant difference within groups implying that the motor coordination was not affected by NMDA (Fig. 4) based on the average latency to fall [Male: F (2, 27)=0.145, p=0.8657, Fig. 4A; Female: F (2, 27)=0.7679. p=0.4738, Fig. 4B]. Male mice exhibited increased trial performance from the first trial regardless of treatment (Treatment, $F_{2,81}$=0.2046, p=0.8154; trial, $F_{2,81}$=23.66, p<0.0001; interaction treatment×trial, $F_{4,81}$=1.042, p=0.3909; Fig. 4C), whereas female mice have higher baseline performance in the first trial and increased rapidly in the second and third trials (Treatment, $F_{2,81}$=1.027, p=0.3626; trial, $F_{2,81}$=6.66, p=0.0021; interaction treatment×trial, $F_{4,81}$=0.6265, p=0.6450; Fig. 4D).

DISCUSSION

The current study used NMDA to emulate the action of glutamate to activate the NMDA receptors in adolescent mice. Glutamatergic NMDA receptors have been implicated in neurodevelopmental disorders including autism spectrum disorder (Burgdorf *et al*., 2013). The social behaviors of both NMDA-injected male and female mice were decreased compared to control mice wherein a decreased total sniffing time was more prominent in female than male mice, and the effect was dose-dependent. The assessment of repetitive behaviors in a self-grooming test showed that NMDA injection increased the grooming time in both males and females at the higher (75 mg/kg) dosage. These results demonstrate that systemic NMDA injection produced an autism-like response in male and female mice without affecting the motor coordination at the dosages employed in this study. This data suggests that NMDA can be used as a hyper glutamatergic signaling model of ASD that can be employed for early prescreening of therapeutic candidates.

NMDA is a prototype agonist of the ionotropic glutamate receptors called the NMDA receptors which are expressed in many regions of the brain such as the hippocampus, cerebral cortex, cerebellum, striatum, and other regions with differential expressions according to the receptor subtypes (Conti, 1997; Goebel and Poosch, 1999; Law *et al*., 2003). NMDA easily dissolves in saline but its blood-brain barrier penetration is believed to be low from systemic injection so that a higher dosage (>100 mg/kg) is required to have magnified effects, such as seizures or death from excitotoxicity. NMDA was previously used to model seizures in both immature and mature rats (Pitkänen *et al*., 2005), where NMDA-induced seizures in an immature brain cause long-term spatial learning and memory impairment as well as increased seizure susceptibility during adulthood (Stafstrom and Sasaki-Adams, 2003). In this study, we found that the lower systemic dosage of NMDA at 50 mg/kg induced behavioral deficits reminiscent of ASD without affecting the gross motor activity nor seizure phenotypes, suggesting that the protocol used in this study may be adopted for the study of ASD-like behaviors and ASD neurobiology in adolescent mice.

Based on clinical and preclinical evidence, the excitation/inhibition (E/I) imbalance draws much attention as a leading theory underlying the pathophysiology of ASD (Kim *et al*., 2016; Lee *et al*., 2017). This theory is conceived as a disturbance in the equilibrium between the glutamatergic and the GABAergic inputs (Gonçalves *et al*., 2017). In a study, Rubenstein and Merzenich (2003) postulated that an increased ratio of E/I in sensory, mnemonic, social, and emotional systems causes some forms of autism. Increased E/I ratio in the prefrontal cortex has been shown to mimic ASD's social deficits and behavioral tendencies, while a decreased E/I ratio can be found in Rett syndrome (Uzunova *et al*., 2016). Rubenstein and Merzenich (2003) further state that the excitatory/inhibitory imbalance can be caused by a combination of genetic and environmental variables that affect a neural system. This study's results indicate that increasing the excitatory neurotransmission through pharmacological induction (a systemic NMDA injection) will cause social behavior deficits and repetitive behavior in mice. By elucidating this causal relationship, the study can help expound the role of balanced synaptic neurotransmission in regulating the social and repetitive behaviors.

In view of the higher male prevalence found in individuals with ASD (Werling and Geschwind, 2013) and in many animal models (Jeon *et al*., 2018), we compared whether the effect of NMDA in mice would show a sex-difference. Although similar autistic-like symptoms of social deficit and increased grooming behavior were found in both sexes of NMDA-treated mice, a male-specific anxiety-like behavior was observed during the open field test. This could be complemented by the lower movement duration of NMDA-treated males although the distance moved were similar to control levels. Many studies

in rodents have shown sex differences in anxiety-like behaviors which are affected by multiple innate and external factors (Donner and Lowry, 2013). Thus, a variable sex-specific response to NMDA treatment in the neural circuits, whether genetic or hormonal, that drive the exploratory behavior in the open field test could underlie the more anxious males and unaffected females. This result indicates the physiological differences between sexes in response to pharmacologic agents as an environmental factor together with genetic interplay that may lead to or protect from neurobehavioral conditions. As an anxiety-like behavior model, NMDA treatment may only be used for males. Nevertheless, we determined that the use of the NMDA as a pharmacologic model of ASD in mice can be used for both sexes and it would be interesting to determine whether the screening of potential therapeutic candidates may have gender-specific effects.

Since our study is limited to behavioral experiments, further in-depth investigation on the cellular and molecular aspect, as well as experiments on other behavioral domains, should be performed in the future. Moreover, the pharmacokinetic and pharmacodynamics profile of low dose systemic injection of NMDA in the brain should be investigated further to obtain better information of NMDA receptor involvement on the regulation of ASD-like behaviors. These would be valuable to uncover new information in the understanding of NMDA receptors and the brain in general. For now, the NMDA injection model would be of good use as an efficient and fast screening tool for drugs with a potential therapeutic application such as enhancing social interaction and alleviating repetitive behavior.

ACKNOWLEDGMENTS

This work was supported by the KIST (Grant No. 2E30190-20-060) and the National Research Foundation of Korea (NRF) grant funded by the Korean government (NRF-2016R1A5A2012284).

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