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Effects of Intraperitoneal N-methyl-D-aspartate (NMDA) Administration on Nociceptive/Repetitive Behaviors in Juvenile Mice

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Abstract

Dysregulation of excitatory neurotransmission has been implicated in the pathogenesis of neuropsychiatric disorders. Pharmacological inhibition of N-methyl-D-aspartate (NMDA) receptors is widely used to model neurobehavioral pathologies and underlying mechanisms. There is ample evidence that overstimulation of NMDA-dependent neurotransmission may induce neurobehavioral abnormalities, such as repetitive behaviors and hypersensitization to nociception and cognitive disruption, pharmacological modeling using NMDA has been limited due to the induction of neurotoxicity and blood brain barrier breakdown, especially in young animals. In this study, we examined the effects of intraperitoneal NMDA-administration on nociceptive and repetitive behaviors in ICR mice. Intraperitoneal injection of NMDA induced repetitive grooming and tail biting/licking behaviors in a dose- and age-dependent manner. Nociceptive and repetitive behaviors were more prominent in juvenile mice than adult mice. We did not observe extensive blood brain barrier breakdown or neuronal cell death after peritoneal injection of NMDA, indicating limited neurotoxic effects despite a significant increase in NMDA concentration in the cerebrospinal fluid. These findings suggest that the observed behavioral changes were not mediated by general NMDA toxicity. In the hot plate test, we found that the latency of paw licking and jumping decreased in the NMDA-exposed mice especially in the 75 mg/kg group, suggesting increased nociceptive sensitivity in NMDA-treated animals. Repetitive behaviors and increased pain sensitivity are often comorbid in psychiatric disorders (e.g., autism spectrum disorder). Therefore, the behavioral characteristics of intraperitoneal NMDA-administered mice described herein may be valuable for studying the mechanisms underlying relevant disorders and screening candidate therapeutic molecules.

Key Words: Stereotypy, Tail Licking/Biting, Nociception, Repetitive Behavior, E/I Imbalance

INTRODUCTION

Most neurons in the brain are connected to each other through excitatory or inhibitory synapses. Dysregulation of excitatory/inhibitory neurotransmission (E/I imbalance) has been implicated as an underlying pathophysiological mechanism of psychiatric disorders, including schizophrenia and autism spectrum disorder (Kehrer *et al*., 2008; Gao and Penzes, 2015; Nelson and Valakh, 2015; Uzunova *et al*., 2016). NMDAR antagonists such as ketamine and phencyclidine (PCP) induce a schizophrenia-like psychosis in both humans and animal models (Javitt *et al*., 2012). Likewise, MK801 (dizocilpine), which inhibits NMDA receptors by binding to the receptor in its activated state, has been used to generate a pharmacologic model of psychosis.

The behavioral abnormalities induced by MK801 include behavioral inflexibility (Svoboda *et al*., 2015), impaired spatial memory (van der Staay *et al*., 2011), social withdrawal (Rung *et al*., 2005), and repetitive behaviors (Nozari *et al*., 2014). Acute or chronic intraperitoneal injection of MK801 has been reported to dysregulate some structural and behavioral features of brain function both in rats and mice in ways reminiscent of psychiatric disorders, including schizophrenia and autism spectrum disorder (Moy *et al*., 2013; Nozari *et al*., 2014;

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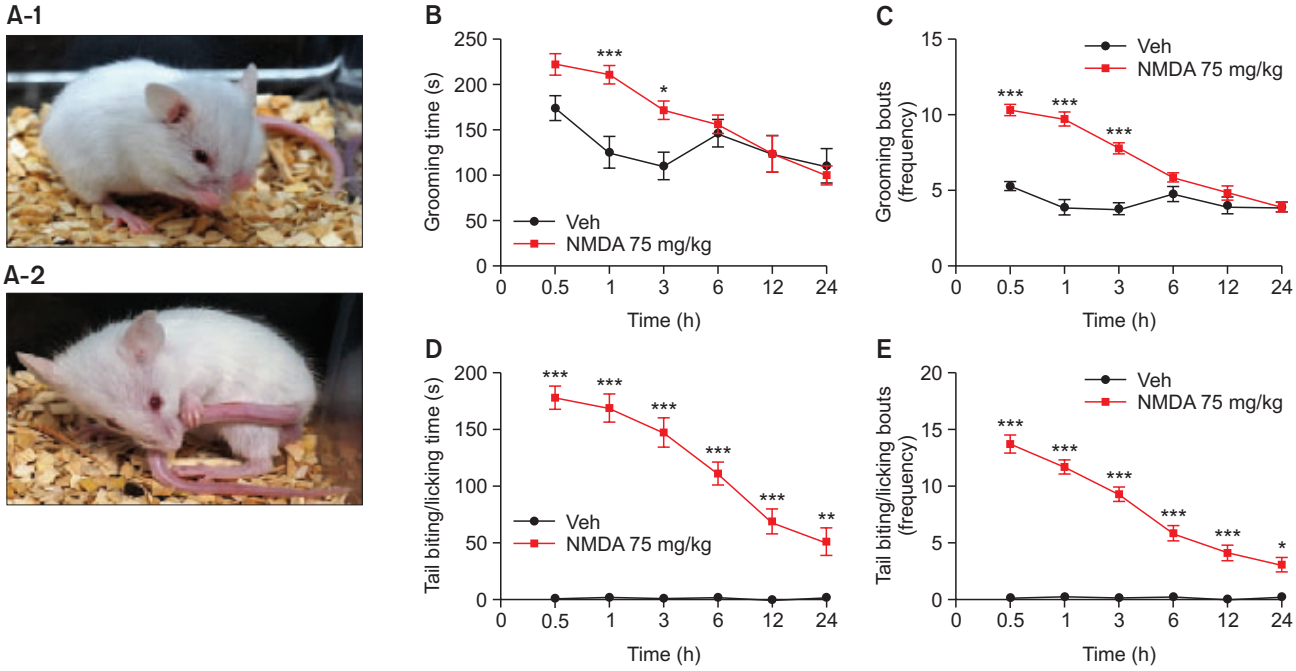


Fig. 1. Intraperitoneal NMDA administration increased nociceptive/repetitive behaviors, especially tail biting/licking behaviors. Nociceptive/repetitive behaviors were analyzed after intraperitoneal administration of saline or NMDA at 75 mg/kg to 3-4 weeks old mice (P24-P26). (A) Representative pictures of NMDA-induced nociceptive/repetitive behaviors. (A-1) Grooming behaviors. (A-2) Tail biting/licking behaviors. (B) Duration of grooming behaviors. (C) Frequency of grooming behaviors. (D) Duration of tail biting/licking behaviors. (E) Frequency of tail biting/ \licking behaviors. The number of animals per condition was as follows: Vehicle group with saline (n=8) and 75 mg/kg NMDA group (n=12). Mouse behavior was recorded for 20 min at each time point. All data are expressed as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. Vehicle. Veh=Saline-injected mice (Vehicle group), NMDA 75 mg/kg=NMDA-exposed mice in the 75 mg/kg group.

Wu *et al*., 2016). Accordingly, compounds such as glycine, D-serine, or D-cycloserine, which activate NMDAR by binding to the glycine site on the receptor, convey significant therapeutic effects in animal models of schizophrenia.

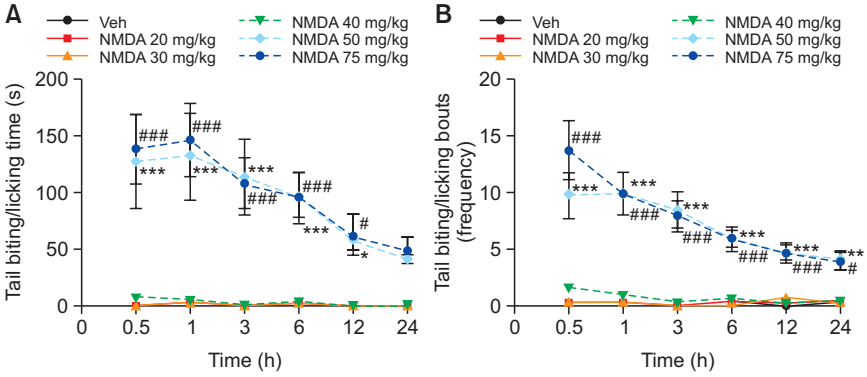
In spite of the wide use of MK801 injection to model psychiatric disorders and recent reports suggesting etiological and therapeutic roles of NMDARs in relieving autism-like symptoms in preclinical studies (Won *et al*., 2012; Kim *et al*., 2014; Lee *et al*., 2015; Kim *et al*., 2017), few studies have examined the effects of peripheral administration of NMDA on neurobehavioral and neurobiological features in experimental animals. More recently, we systemically treated ICR mice with either AMPAR agonists or antagonists and observed social behavioral impairments in mice, adding additional support for the E/I imbalance theory of ASD (Kim *et al*., 2018). Although there are relatively ample behavioral studies using antagonists of NMDARs, few studies have examined the neurobehavioral features of experimental animals after treatment with NMDAR agonists mainly due to adverse outcomes such as induction of seizure at high concentrations. Administration of high concentrations of NMDAR agonists into the CNS can induce neuronal cell death and general toxicity, which may limit the analysis of more subtle behavioral characteristics. It has been suggested that intrathecally administered NMDA can induce nociceptive responses, such as biting directed toward the hind paws (Raigorodsky and Urca, 1990; Sakurada *et al*., 1990), whereas high concentrations of NMDA induce neurotoxic outcomes such as seizure, especially in younger animals (Schoepp *et al*., 1990; Kabova *et al*., 1999).

In the present study, we found that systemic administration of NMDA to juvenile mice induced nociceptive behavior, as well as repetitive tail biting/licking behaviors. These effects lasted at least 24 h in a concentration and animal age-dependent manner without inducing massive BBB breakdown or neuronal cell death. Altered nociceptive sensitivity and repetitive/stereotyped behaviors are frequently observed across many psychiatric conditions both in human and experimental animals. Therefore, inducing nociceptive/repetitive behaviors in systemically NMDA-injected juvenile rodents may provide versatile animal models not only for investigations of the pharmacological and molecular mechanisms of such behaviors, but also as an efficient means to screen therapeutic agents regulating such behavioral abnormalities.

MATERIALS AND METHODS

Animals

Outbred ICR mice were purchased from Orient Bio (Gapyeong, Korea). Animals were maintained in controlled environment at a temperature range of 23 ± 2°C and humidity range of 50 ± 10% on a conditioned circadian cycle (lights on: 2:00 pm, lights off: 2:00 am). Mice were housed in standard polycarbonate cages (20×26×13 cm) and allowed to freely access food and water. All procedures including animal treatments and maintenance were approved by the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and were conducted according to the Animal Care and Use



C

Groups	Percent of wounded mice after 24 h (%)
Veh	0%
NMDA 20 mg/kg	0%
NMDA 30 mg/kg	0%
NMDA 40 mg/kg	0%
NMDA 50 mg/kg	50%
NMDA 75 mg/kg	57.1%

Fig. 2. Dose-dependent effects of systemic NMDA treatment on tail biting/licking behaviors. A range of NMDA doses were administered by i.p. injection and tail biting/licking behaviors were measured. (A) Duration of tail biting/licking behaviors. (B) Frequency of tail biting/licking behaviors. (C) Percentage of wounded mice after 24 h in each group. All data are expressed as the mean ± SEM. The number of animals per condition were n=8 for all groups. *p<0.05, **p<0.01, ***p<0.001 vs. Vehicle, $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 vs. Vehicle. Veh=Saline-injected mice (Vehicle group), NMDA 20, 30, 40, 50, and 75 mg/kg=NMDA-exposed mice in the 20, 30, 40, 50, and 75 mg/kg groups, respectively.

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Treatments

N-methyl-D-aspartate (NMDA, Cat No. M1360) was obtained from Tokyo Chemical Industry (Tokyo, Japan) and Glacial acetic acid (Cat No. 1005-4400) was purchased from Daejung Chemical (Seoul, Korea). NMDA and acetic acid were dissolved and diluted using normal saline (0.6 or 0.7% v/v, respectively). The dosage of injection was decided according to the body weight of mice on the day of experiment (10 ml/kg, i.p.).

Behavioral studies

Behavioral observation: 1 h before starting the experiment, each mouse was put in a transparent polycarbonate cage (20×26×13 cm) with corncob bedding to a depth of 5 cm. The mice were injected according to body weight. Immediately after intraperitoneal NMDA or saline injection, the mice were placed in the transparent cage and each time-course of experiment was recorded. The time and frequency of nociceptive and repetitive behaviors, such as grooming, and tail biting and licking, were measured through video recordings at each time point.

Hot plate test: The hot plate test was conducted according to previous reports (Ogren and Berge, 1984; Tjolsen *et al*., 1991). The hot plate apparatus comprised a heatable metal plate with a temperature adjustable system. The temperature at the edges of the plate was 1°C lower than that at other surfaces. Except for the edges of the plate, the surface temperature was constant within 0.3°C. The animals (n=8) were treated with saline or NMDA (50 or 75 mg/kg; i.p). After 30 min, mice were placed on the hot plate at a temperature below the nociceptive threshold (about 35°C). The heating rate was 1°C/min. Nociceptive responses such as latency of paw licking or jumping were measured by an observer blind to the treatment condition.

Writhing test: Approximately 1 h prior to the writhing test, the mice were habituated to an individual cage which was also used as the observation chamber in the behavioral testing room. The animals (n=8-10) were treated with saline or NMDA (50 or 75 mg/kg). After 30 min, writhing was induced by intraperitoneal injection of 0.6% or 0.7% acetic acid at a volume of 10 ml/kg body weight. We counted the number of writhing behaviors (stretching, extension of hind legs, or contraction of the abdomen) for 20 min.

Tissue preparation

Mice in each group (n=8-9 per group) were sacrificed for tissue preparation. The spinal cord, thalamus, and prefrontal cortices were isolated, immediately frozen in liquid nitrogen, and then stored at –80°C.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

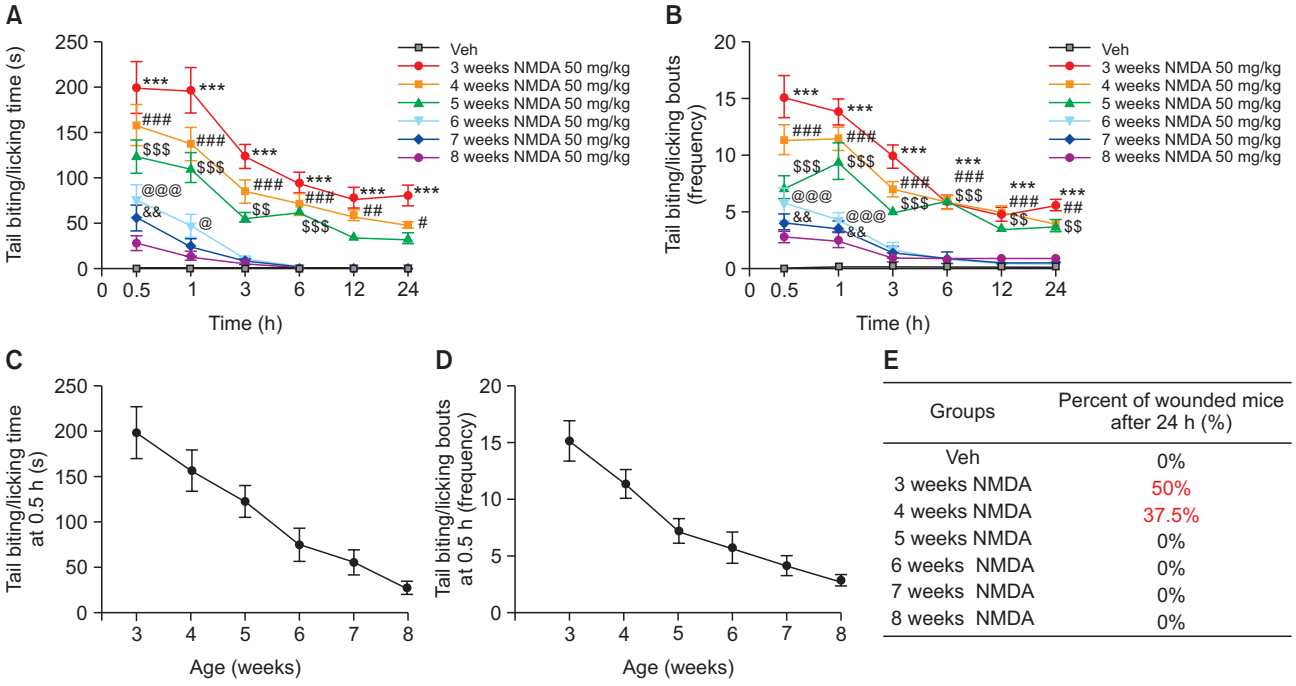
Three mice from each group were sacrificed and the tissue samples were isolated as described above in section 2.4. For the sample analysis, each segment of tissue was homogenized with a 3-fold volume of DDW. Samples were added to 50% acetonitrile containing an internal standard at a ratio of 1:4 and sonicated for 30 min. The tube was centrifuged at 842 g at 4°C for 10 min. The supernatants were analyzed using an API 3200 QQQ LC/MS/MS system with an Agilent 1200 series binary pump. Turbo Spray was utilized for ionization in positive ion mode. The samples were resolved on a YMC triart C18 (2.1×100 nm, 3 μm). The mobile phase was generated by mixing eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile). The flow rate was 0.25 ml/min.

Evans blue assay

At 1 h after saline or NMDA injection, 3 mice from each group were anesthetized with Zoletil and Rompun in saline. Evans blue dye (2% wt/vol in PBS) in a volume of 2 ml/kg was given by tail vein injection and the brain was removed.

Immunohistochemistry

A 26 G needle was inserted into left ventricle of the heart and transcardial perfusion was performed with 0.9% saline followed by cold 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4). The brain was isolated and post-fixed in the same fixative at 4°C and then cryoprotected overnight in 30% (w/v) sucrose in phosphate-buffered saline (PBS). The samples were sectioned at a 20 μm thickness on a cryostat



E

Groups	Percent of wounded mice after 24 h (%)
Veh	0%
3 weeks NMDA	50%
4 weeks NMDA	37.5%
5 weeks NMDA	0%
6 weeks NMDA	0%
7 weeks NMDA	0%
8 weeks NMDA	0%

Fig. 3. Age-dependent effects of systemic NMDA injection on tail biting/licking behaviors. Tail biting/licking duration and frequency were measured after NMDA injection (i.p.) at different ages (3-8 weeks old). (A) Duration of tail biting/licking behaviors. (B) Frequency of tail biting/licking behaviors. (C) Correlation between duration of tail biting/licking and age. (D) Correlation between frequency of tail biting/licking and age. (E) Percentage of mice with wounded tails after 24 h in each group. All data are expressed as the mean ± SEM. The number of animals per condition is as follows: Vehicle, n=4; 50 mg/kg NMDA groups n=8 at all ages. Veh vs. 3 weeks mice (NMDA 50 mg/kg) $^{***}p<0.001$, Veh vs. 4 weeks mice (NMDA 50 mg/kg) $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, Veh vs. 5 weeks mice (NMDA 50 mg/kg) $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$, Veh vs. 6 weeks mice (NMDA 50 mg/kg) $^{@}p<0.05$, $^{@@@}p<0.001$, Veh vs. 7 weeks mice (NMDA 50 mg/kg) $^{\&\&}p<0.01$. Veh=Saline-injected mice (Vehicle group), 3, 4, 5, 6, 7, and 8 weeks NMDA 50 mg/kg=3, 4, 5, 6, 7, and 8 week old mice administered NMDA at 50 mg/kg, respectively.

and then the sections were preserved in 24-well plates with tissue stock solution. Sections were blocked in a solution containing 10% Horse serum, 0.3% Triton X-100 in PBS for 1 h at room temperature. Sections were then incubated overnight at 4°C with the following primary antibodies: rabbit anti-IgG (1/500; abcam, Cambridge, UK), mouse anti-NeuN (1:500; Millipore, Burlington, MA, USA), rabbit anti-E-cadherin (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), or mouse anti-ZO-1 (1:500; Invitrogen, Carlsbad, CA, USA). After washing samples three times in PBS containing 1.5% Horse serum and 0.1% Triton X-100, the sections were incubated for 2 h at room temperature with Alexa 488 and Alexa 546 as the secondary antibodies. Images were visualized and imaged using a confocal microscope (ZEN2009, Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Data were quantified and presented as mean ± standard error of the mean (SEM). Statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by Newman-Keuls analysis. Two-way ANOVA was used to identify the interaction between two factors. Differences were considered statistically significant when the *p* value was less than 0.05 ($p<0.05$). All statistical analyses were conducted using GraphPad prism (version 5) software (San Diego, CA, USA).

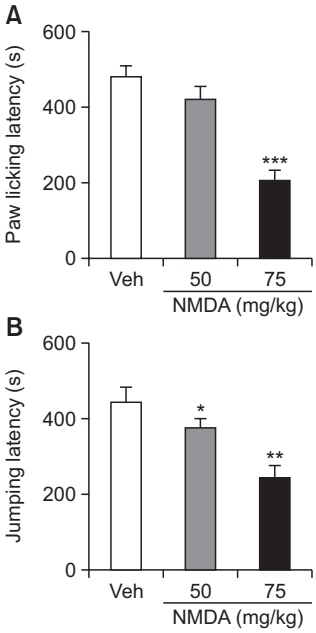
RESULTS

Effects of intraperitoneal NMDA injection on nociceptive/repetitive behaviors

After intraperitoneal NMDA administration, stereotypic behaviors like freezing, hyperactivity, and curling were observed, among which grooming and biting/licking behaviors were particularly noticeable (Fig. 1A-1, 1A-2). Therefore, we measured the duration and frequency of grooming or tail biting/licking behaviors. Note that as tail biting or licking are hard to differentiate upon close observation, they are counted as one behavioral entity.

In behavioral analysis performed using the time bin method, the grooming duration of NMDA-induced mice group was higher than that of the vehicle group in the early time points and gradually decreased up to 24 h (Fig. 1B). Similarly, the number of grooming bouts increased in the NMDA-treated mice group, but it was not significantly different to that in the vehicle group after 6 h (Fig. 1C). However, the group injected with NMDA at 75 mg/kg demonstrated a significantly increased duration of tail biting/licking behaviors compared with the vehicle group at all time points examined (Fig. 1D). Additionally, the number of tail biting/licking bouts were also enhanced in NMDA-exposed mice, but not in the vehicle group (Fig. 1E).

We next examined the dose-dependency of NMDA effects on tail biting/licking behaviors. The doses of NMDA used in this study were 20, 30, 40, 50, and 75 mg/kg. The duration of



Groups	Average of paw licking latency (s) (Mean ± SEM)	Statistical significance
Veh	485.5 ± 28.28	–
NMDA 50 mg/kg	425.8 ± 30.05	–
NMDA 75 mg/kg	211.3 ± 23.19	*** (Veh vs. NMDA 75 mg/kg)

Groups	Average of jumping latency (s) (Mean ± SEM)	Statistical significance
Veh	443.8 ± 41.02	–
NMDA 50 mg/kg	376.8 ± 25.42	* (Veh vs. NMDA 50 mg/kg)
NMDA 75 mg/kg	248.1 ± 30.00	** (Veh vs. NMDA 75 mg/kg)

Fig. 4. High concentrations of NMDA decreased the latency to paw licking or jumping in the hot plate test. Nociceptive heat sensitivity was measured using the hot plate test. (A) Latency to paw licking. (B) Latency to jumping. All data are expressed as the mean ± SEM. The number of animals per condition was n=8 for all groups. **p*<0.05, ***p*<0.01, ****p*<0.001 vs. Veh. Veh=Saline-injected mice (Vehicle group), NMDA 50 and 75 mg/kg=NMDA-exposed mice in the 50 and 75 mg/kg groups, respectively.

tail biting/licking was similar to the vehicle group in the 20, 30, and 40 mg/kg NMDA groups. Interestingly, the 50 and 75 mg/kg NMDA-injected groups displayed statistically significant increases in tail biting/licking duration and frequency compared to the vehicle group (Fig. 2A, 2B). We observed a red skin injury on mouse tails after 24 h in the NMDA 50 and 75 mg/kg groups at a frequency of 50% and 57.1% wounded mice, respectively (Fig. 2C).

In order to determine whether the effect of NMDA is age-dependent, we treated the mice at 3, 4, 5, 6, 7, and 8 weeks of age with 50 mg/kg NMDA. Tail biting/licking behaviors increased at 0.5 and 1 h in all groups, while the tail biting/licking behaviors at 6, 7, and 8 week old mice were similar to those of the vehicle group at 3 h. On the other hand, 3, 4, and 5 week old mice displayed increased tail biting/licking behaviors compared to the vehicle group. The 3 week old mice showed the highest duration of tail biting/licking behaviors (Fig. 3A).

The 3, 4, and 5 weeks old NMDA-injected mice also showed higher tail biting/licking frequency than the vehicle group, while the 6, 7, and 8 week old groups showed no statistical differences when compared with their matched vehicle groups after 3 h (Fig. 3B). The correlation between tail biting/licking behavior and age was inversely proportional to each other (Fig. 3C, 3D), suggesting clear age-dependency of the observed nociceptive/repetitive behavior. As for frequency of wounded tails after 24 h, there were no injured mice in the 5, 6, 7, and 8 week old groups treated with 50 mg/kg NMDA. However, 3 and 4 week old mice were wounded at proportions of 50% and 37.5%, respectively (Fig. 3E).

Effects of intraperitoneal NMDA injection on nociceptive behaviors

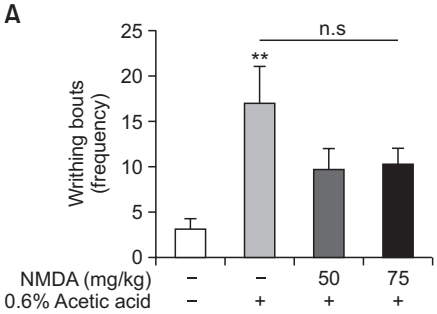
The hot plate test was performed to evaluate possible hypersensitivity to heat after NMDA injection by measuring the latency to lick the paws and to jump from the hot surface. As a result, the latency of paw licking was decreased in the 75 mg/kg NMDA-exposed mice but not in the 50 mg/kg group (Fig. 4A). Further, mice injected with NMDA exhibited a lower latency of jumping than the vehicle group (Fig. 4B).

As another measure of hyperalgesia or hypersensitivity effects of NMDA in nociceptive conditions, the writhing test was performed by counting the number of writhing behaviors (stretching, extension of hind legs, and contraction of the abdomen) recorded after acetic acid injection. Writhing frequency and number were increased by 0.6% and 0.7% acetic acid administration. However, prior systemic treatment with NMDA at either 50 or 75 mg/kg concentrations did not further increase acetic acid-induced writhing (Fig. 5A, 5B). Altogether, these results suggest that systemic administration of NMDA increased global nociceptive reactivity rather than modifying simple pain reflexes responding to peripheral nociceptive stimuli.

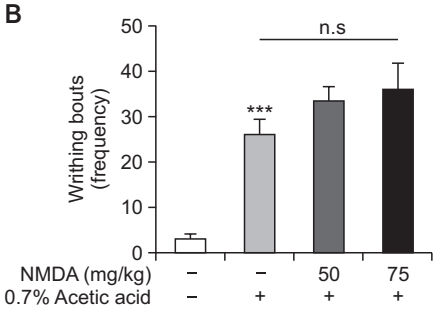
Absence of extensive BBB breakdown and excitotoxicity after systemic NMDA administration

Systemic treatment with 50 mg/kg NMDA resulted in increased NMDA concentrations in the cerebrospinal fluid (CSF), rising from less than limit of quantification (LLOQ, 0.977 ng/ml) to 31.5 ± 28.1 ng/ml. However, we observed only a nonsignificant trend towards an increase in the tissue levels of NMDA in spinal cord, thalamus, and prefrontal cortex homogenates from treated animals. Thus, penetration of NMDA into the CNS appears to be moderate (Table 1).

To unequivocally determine whether the observed stereotypic behaviors were accompanied by excitotoxicity and BBB breakdown, we performed Evans Blue BBB penetration analy-



Groups	Average of writhing bouts (frequency) (Mean ± SEM)	Statistical significance
Saline+Saline (S+S)	3.125 ± 1.076	–
Saline+0.6% Acetic acid (S+0.6A)	17.13 ± 4.142	** (S+S groups vs. S+0.6A groups)
NMDA 50+0.6% Acetic acid (N50+0.6A)	9.750 ± 2.274	–
NMDA 75+0.6% Acetic acid (N75+0.6A)	10.38 ± 1.690	–



Groups	Average of writhing bouts (frequency) (Mean ± SEM)	Statistical significance
Saline+Saline (S+S)	2.889 ± 0.9782	–
Saline+0.7% Acetic acid (S+0.7A)	26.63 ± 4.192	*** (S+S groups vs. S+0.7A groups)
NMDA 50+0.7% Acetic acid (N50+0.7A)	30.60 ± 3.792	–
NMDA 75+0.7% Acetic acid (N75+0.7A)	34.00 ± 7.262	–

Fig. 5. Exposure to NMDA by dose did not affect writhing frequency in the writhing test. Pain responsiveness was measured using the writhing test. (A) The number of writhing behaviors elicited by 0.6% acetic acid. (B) The number of writhing behaviors elicited by 0.7% acetic acid. All data are expressed as the mean ± SEM. The number of animals per condition is as follows: S+S group when using 0.6% acetic acid (n=8), S+0.6A group (n=8), N50+0.6A group (n=8), N75+0.6A group (n=8), S+S group when using 0.7% acetic acid (n=9), S+0.7A group (n=11), N50+0.7A group (n=14), N75+0.7A group (n=14). **p<0.01, ***p<0.001 vs. S+S group. S+S group=mice injected with saline two times, S+A group=mice injected with 0.6 or 0.7% acetic acid after injection of saline, N50+A group=mice injected with 0.6 or 0.7% acetic acid after injection of NMDA at 50 mg/kg, N75+A group=mice injected with 0.6 or 0.7% acetic acid after injection of NMDA at 75 mg/kg.

sis, and immunohistochemical assessments of BBB and neuronal integrity of thalamus and prefrontal cortex in vehicle or NMDA-exposed mice 1 h after NMDA injection. Although the positive control (stereotaxic surgical injury) showed a clear increase in Evans Blue penetration in the brain, the NMDA treated groups did not show a significant increase in Evans Blue penetration across all age groups examined in this study (4, 6 and 8 weeks), suggesting that extensive breakdown of the BBB was not elicited by our experimental conditions (Fig. 6). Similarly, immunohistochemical staining of NeuN, ZO-1, and peripheral IgG showed no differences between the NMDA-treated and the vehicle-treated groups, suggesting absence of both substantial BBB breakdown and excitotoxic cell death in the brain (Fig. 7A, 7B).

DISCUSSION

In this study, we identified behavioral effects of intraperitoneal NMDA administration on stereotypic nociceptive and repetitive behaviors. Most strikingly, we observed that intraperitoneal NMDA administration increased tail biting/licking behaviors along with general grooming. These effects were dosage-dependent and juvenile mice showed the strongest responses. Similar to our results, in experiments using postnatal 12 and 18 days rats, NMDA syndrome was shown to involve several age-dependent specific behaviors such as tail-twisting automatisms and seizures, and in postnatal 60 day-old rats, NMDA induced significantly fewer tail-twisting automatisms than in young rats (Kabova *et al*., 1999).

Most studies involving systemic administration of NMDA focus on its excitotoxic and convulsant effects at high concentrations. In contrast, Giménez-Llort *et al*. (1995) administered relatively low concentration of NMDA to adult Wistar rats and found that NMDA produced an acute short-lasting depressant effect on movement and rearing followed over the next 2 days by a long-lasting increase in fast moving exploratory activity, which was only significant during the dark period (Gimenez-Llort *et al*., 1995). In the present study, we found that systemic administration of NMDA induced stereotypic nociceptive/repetitive behaviors only in juvenile mice without inducing seizure-like behavior or massive damage to the BBB and brain. Whether the same or lower concentrations of NMDA (50 mg/kg or less) modulates other psychiatric behaviors in juvenile rodents should be investigated further in future studies.

In general, the blood brain barrier in adult animals makes it difficult for NMDA to penetrate the central nervous system. However, several studies have shown that immature rodents are particularly sensitive to systemic injection of excitatory amino acids (Chung *et al*., 2000). Schoepp and colleagues reported that NMDA administered systemically to immature rats led to motor convulsions that were reduced by pretreatment with DL-2-amino-5-phophonovaleric acid or (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, a competitive antagonist of NMDA receptors (Schoepp *et al*., 1990). Brace and colleagues have shown that both NMDA and kainite not only damage neurons and myelin, but also impair the integrity of the blood-brain barrier (Brace *et al*., 1997). These findings suggest that although penetration is strongly limited by the blood-brain barrier system, NMDA injected systemically

Table 1. The effects of intraperitoneal NMDA administration on the endogenous NMDA levels in CNS tissue of experimental mice

	Weight (mg)	Vehicle group			Mean ± SEM
		#1	#2	#3	
Spinal cord	635.8	21.4	32.7	22.2	25.4 ± 3.6 (ng/g)
Prefrontal cortex	257.3	4.8	3.6	7.4	5.3 ± 1.1 (ng/g)
Thalamus	1,742.8	12.8	11.6	7.0	10.5 ± 1.8 (ng/g)
Cerebrospinal fluid		<LLOQ	<LLOQ	<LLOQ	
	Weight (mg)	NMDA group			Mean ± SEM
		#1	#2	#3	
Spinal cord	630.8	30.2	18.8	26.7	25.2 ± 3.4 (ng/g)
Prefrontal cortex	831.7	15.9	4.4	4.7	8.3 ± 3.8 (ng/g)
Thalamus	2,627.8	26.9	10.3	10.1	15.8 ± 5.7 (ng/g)
Cerebrospinal fluid	-	16.7	63.9	13.8	31.5 ± 16.2 (ng/ml)

Endogenous NMDA levels were measured (ng/g) using LC-MS/MS. The number of animals per condition was n=3 for each. Vehicle group=saline-injected mice, NMDA group=NMDA-exposed mice at 50 mg/kg group.

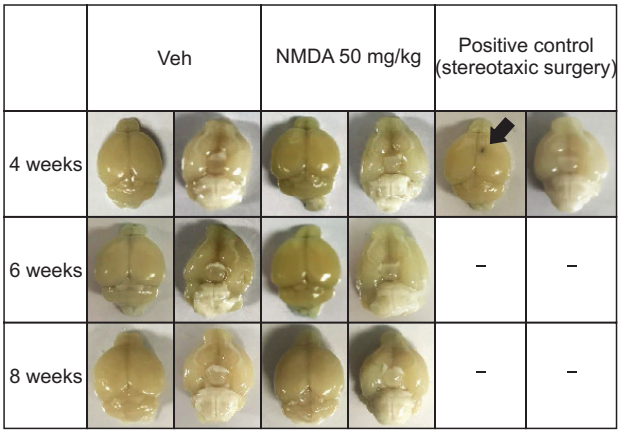


Fig. 6. Intraperitoneal NMDA injection did not induce substantial BBB breakdown. BBB permeability was assessed using Evans blue assay. The number of animals per condition was follows: Vehicle group (n=3), NMDA 50 mg/kg group (n=3). Veh=Saline-injected mice (Vehicle group), NMDA 50 mg/kg=NMDA-exposed mice at 50 mg/kg group.

acts directly on particular receptors located in the brain after penetration through the blood-brain barrier in young rodents (Chung *et al*., 2000).

In the present study, we found significantly elevated levels of NMDA in CSF, while no significant alterations in tissue level of NMDA were demonstrated in thalamus and prefrontal cortex. Similarly, we did not observe significant Evans Blue penetration or IgG, NeuN, and ZO-1 immunoreactivity changes in the brain. Altogether, these results suggest that systemically administered NMDA in juvenile mice penetrated into brain and spinal cords at levels insufficient to change BBB integrity and excitotoxic damage to brain tissue. Therefore, our method may be beneficial for examining the behavioral effects of NMDA receptor activation, separate from the extensive damage higher doses cause across the nervous system.

Several studies have reported that intrathecal (i.t.) injection of NMDA or non-NMDA receptor agonists produce behavioral responses including hindlimb scratching, and biting and/or licking of the hind paw and the tail (Urca and Raigorodsky, 1988; Mjellem *et al*., 1993; Brambilla *et al*., 1996; Brace *et al*., 1997). These behaviors resemble those induced by neurokinin-1 (NK-1) receptor agonists such as substance P (Hylden and Wilcox, 1981, 1983; Sakurada *et al*., 1989, 1994a, 1994b). In addition, i.t. administration of spermine, an endogenous polyamine, induced nociceptive behavior mainly consisting of biting and/or licking of the hind paw along with a slight increase in hindlimb scratching in mice. The effect of spermine is mediated through the polyamine recognition site on the NMDA receptor ion-channel complex (Tan-No *et al*., 2000). Further, it has been found that different classes of glutamate receptor antagonists including MK801 and memantine cause antinociception in animal models (Cahusac *et al*., 1984; Murray *et al*., 1991; Kristensen *et al*., 1994), as well as tonic pain (Dickenson and Sullivan, 1990; Dickenson and Aydar, 1991; Yamamoto and Yaksh, 1992; Brace *et al*., 1997). Considering the similarity of central sensitization in pain and itch pathways and the effectiveness of NMDA receptor antagonists on the inhibition of itch (Jinks and Carstens, 1998; Tan-No *et al*., 2000), the stereotypic behavior observed in this study might represent augmented and/or compulsive neuropathic itch responses. In any case, the stereotypic nociceptive/repetitive behaviors induced by systemic administration of NMDA in juvenile mice, may help to understand the mechanism underlying repetitive behaviors such as stereotypies, compulsions, obsessions, and self-injurious actions in human and animal models of neurodevelopmental and neuropsychiatric disorders (Kim *et al*., 2016).

At present, it is unclear how intraperitoneal injection of NMDA modulates complex nociceptive/repetitive behaviors. Considering the limited penetration of NMDA into CNS and CSF, intrathecal sensitization effects of NMDA and the existence of peripheral NMDA receptors participating in nociceptive response (Raigorodsky and Urca, 1990; Schoepp *et al*., 1990; McRoberts *et al*., 2001; Cairns *et al*., 2003), it is a reasonable assumption that the observed behavioral response against NMDA in juvenile mice may be modulated by both CNS and peripheral mechanisms. In any case, it is important to note that in several neuropsychiatric disorders, such as

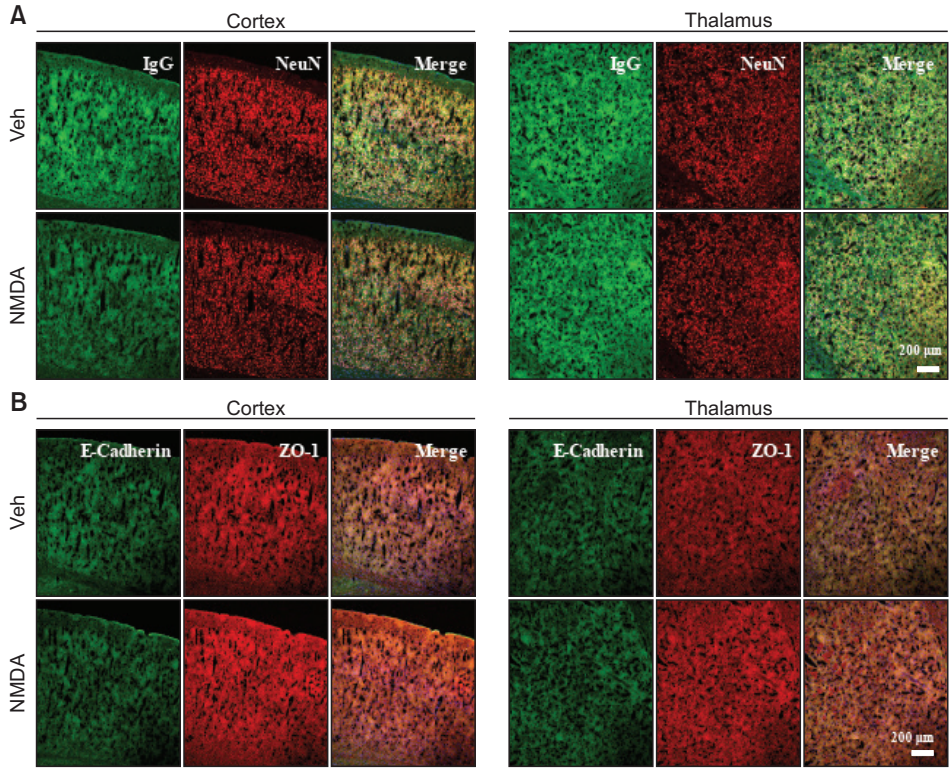


Fig. 7. Intraperitoneal NMDA injection did not affect excitotoxic cell death. Excitotoxic cell death and BBB breakdown were evaluated using immunohistochemistry. The number of animals per condition was as follows: Vehicle group (n=3), NMDA 50 mg/kg group (n=3). Veh=Saline-injected mice (Vehicle group), NMDA 50 mg/kg=NMDA-exposed mice at 50 mg/kg group.

schizophrenia and autism spectrum disorder. In these contexts, there is presumed to be an imbalance in glutamatergic neurotransmission which is especially prominent in NMDA signaling pathways. A prevailing hypothesis is these glutamatergic neurotransmission imbalances underlie altered sensory gaiting, nociceptive responses, and repetitive behaviors. The nociceptive and repetitive behavior changes in this experiment therefore support this model of sensory-motor dysregulation and may help to understand and devise strategies to control particular behavioral features of relevant neuropsychiatric disorders.

This study demonstrated that intraperitoneal NMDA administration increased self-grooming and nociceptive and repetitive behaviors, especially tail biting/licking behaviors, in juvenile mice. The effects were age- and dose-dependent and were unaccompanied by massive BBB breakdown and excitotoxic damage to neural tissue. While the results from the present study may add additional support for the widely accepted E/I imbalance hypothesis of neuropsychiatric disorders and demonstrate mechanistic usefulness of our approach as a model, careful characterizations by in depth behavioral profiling as well as investigation of the detailed molecular mechanism underlying the observed phenomena should be carried out going forward. In addition, a rigorous delineation of the human relevance of the observed behavioral manifestations is warranted.

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