Disruption of ultrasonic vocalization with systemic administration of the non-competitive N-methyl-d-aspartate receptor antagonist MK-801 in adult male mice

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Abstract

Vocal communication is essential for survival in many animals, including humans. Mice produce ultrasonic vocalizations (USVs) in various social contexts. N-methyl-d-aspartate (NMDA) receptors are widely distributed in the central nervous system and play essential roles in synaptic plasticity, learning, and behavior. However, the role of NMDA receptors in USVs remains unclear. Here, we examined the effects of systemic administration of a noncompetitive NMDA antagonist, MK-801, on USVs during courtship and in other social and nonsocial behaviors. First, we investigated the USVs emitted by male mice. After the successful classification of USVs into 11 categories using VocalMat, an open-source computational vision and machine-learning technique, we analyzed the relationship between USV properties and approaching behavior toward female mice. Male mice produced USVs when they were close to female mice, which confirmed that USVs were associated with social signals. Second, we examined the effects of intraperitoneal injection of MK-801 on USVs and approach behavior. Intraperitoneal injection of MK-801 dose-dependently decreased the number of USVs and increased locomotor activity, but did not affect the time spent with female mice. In additional experiments, we confirmed that the injection of MK-801 decreased the time spent with a novel same-sex individual in the social preference task, increased locomotor activity, and inhibited excretion in the open-field task. Taken together, these results demonstrate that NMDA receptors play important roles in vocal communication and social behavior.

(226 words)

Keywords

- Ultrasonic vocalizations
- N-methyl-D-aspartate receptors
- MK-801
- Social behavior
- Schizophrenia

Abbreviations

N-methyl-d-aspartate (NMDA); ultrasonic vocalizations (USVs)

1. Introduction

In many animals, including humans, vocal communication is essential for survival, has been observed across numerous animal species, and is a fundamental research topic in the evolution of language and communication (Marler, 1961; Seyfarth et al., 1980). Rodents communicate via both audible and ultrasonic sound frequencies (Ehret and Bernecker, 1986; Zippelius and Schleidt, 1956). Mice use ultrasonic vocalizations (USVs), at frequencies ranging from 30 to 110 kHz, to communicate with each other (Holy and Guo, 2005) in diverse social contexts, including parent–infant, male–female, and same-sex interactions. During courtship and mating, male mice produce spectrally complex USVs, which are thought to attract females and increase male reproductive success (Marconi et al., 2020; Portfors & Perkel, 2014). The presentation of female mice or their urine to adult male mice induces USVs (Nyby et al., 1979; Tschida et al., 2019; Ziobro et al., 2024). Furthermore, female mice preferentially select males that produce USVs over those that do not, which suggests that male USVs attract females (Pomerantz et al., 1983; Tschida et al., 2019). Therefore, USVs are a reliable behavioral assay for examining vocal communication and social interactions in rodents.

N-Methyl-d-aspartate (NMDA) receptors, which are prolifically distributed in the central nervous system, play essential roles in synaptic plasticity, learning, and memory, and are ionotropic glutamate receptors (Nakanishi, 1992). The systemic administration of MK-801 (dizocilpine maleate), a noncompetitive, selective NMDA receptor antagonist, causes learning and memory deficits in various behavioral tasks in rodents (Åhlander et al., 1999; Benvenga & Spaulding, 1988; Janus et al., 2023; Kawabe et al., 1998; Manahan-Vaughan et al., 2008; Ohno & Watanabe, 1996; Ozawa et al., 2021; Ward et al., 1990; Whishaw & Auer, 1989; Wozniak et al., 1990). Moreover, MK-801 dose-dependently induces symptoms of motor dysfunction such as hyperactivity, stereotypies, and ataxia (Carey et al., 1998; Ford et al., 1989; Koek et al., 1988; Mabunga et al., 2019). However, the causal relationship between NMDA-receptor blockade by MK-801 and USVs is unelucidated.

Based on the glutamate hypofunction theory of schizophrenia (Olney and Farber, 1995), animal studies using NMDA antagonists, such as phencyclidine, MK-801, and ketamine, have been conducted extensively. In particular, the acute application of NMDA antagonists in animals successfully mimics both the positive and negative symptoms of schizophrenia, such as abnormal motor function (Andine et al.,1999; Carey et al., 1998; Ford et al., 1989; Mabunga et al., 2019), alterations in prepulse inhibition (Bast al., 2000),

and impaired social behavior (Sams-Dodd, 1996; Rung et al., 2005). Furthermore, the administration of NMDA antagonists in humans elicits symptoms of schizophrenia, such as hallucinations, delusions, and affective blunting (Javitt & Zukin, 1991). These findings support the usefulness of acute MK-801 treatment as a preclinical assessment tool for investigating the neurobiological mechanisms of schizophrenia (Manahan-Vaughan et al., 2008).

In this study, we examined the effects of acute systemic administration of MK-801 on USVs in the social context as well as on other social and non-social behaviors in male mice. First, we investigated the USVs emitted by female male mice. Using open-source computational vision and machine-learning techniques, we classified USVs into 11 categories and analyzed the relationship between the properties of USV and approach behavior in female mice. Second, we examined the effects of intraperitoneal injection of MK-801 on USVs and approach behavior. Third, we ascertained whether the intraperitoneal injection of MK-801 affects the preference and time spent by novel same-sex individuals in the social preference task. Fourth, we determined whether the intraperitoneal injection of MK-801 increases locomotor activity and examined its effects on excretion in an open-field task.

2. Material and methods

2.1 Subjects

All data were collected from 3- to 6-month-old adult male wild-type C57BL/6J mice that were maintained on a 12:12 light cycle, and all the experiments were conducted during the dark phase of the light cycle. The mice had unrestricted access to food and water in their cages. Their weights were monitored daily. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Research Committee of Keio University. This study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

2.2 Drug

The NMDA receptor antagonist (+)-MK-801 hydrogen maleate (MK-801; Sigma-Aldrich, St Louis, Missouri, USA) was dissolved in saline, divided into small aliquots, and stored in a refrigerator at 4°C until further use. Prior to all the experiments, we administered the MK-801 solution intraperitoneally at a dose of 0.1, 0.2, or 0.4 mg/kg. For the habituation and control conditions, saline was administered intraperitoneally, as in the drug condition. The concentration of MK-801 used in this study (0.1, 0.2, and 0.4 mg/kg, was determined from the the results of previous studies in mice and rats (Bradford et al., 2010; Ozawa et al., 2021; Kawabe et al., 1998; Mabunga et al., 2019).

2.3 Experimental procedure

2.3.1 Experiment 1: Social interaction task

In Experiment 1, we used a social interaction task to examine the effects of the intraperitoneal injection of MK-801 on USVs and the approach behavior of male mice toward female mice (Fig. 1). Before starting the social interaction task, we paired the subject and female mice in a normal cage for 1 week. This procedure provided a sex experience for male mice prior to the social interaction experiment. After pairing with a female mouse, the male mice were habituated to the experimental box for more than 3 days, and the social interaction test commenced after the pairing and housing procedures. Mice were intraperitoneally injected with 0.1, 0.2, or 0.4 mg/kg MK-801 or saline, 5 min before the experiment. We conducted the experiments for 4 days per week and repeated the schedule for 3 weeks. Given the relative daily variations in the number of USVs, especially on the first day of the week, we undertook practice sessions on the first and fourth days of the week. We then obtained stable saline data on the second day of the week, and reliable drug

manipulation data on the third day. The order of MK-801 0.1, 0.2, and 0.4 mg/kg injections was randomized. The social interaction test consisted of habituation and test phases. During the habituation phase, a male mouse was inserted into the experimental box and allowed to move freely for 15 min. After the habituation phase, the subject male mouse was temporarily removed from the box, and a novel female mouse in the basket was placed in the corner of the box. A 500-mL empty glass bottle was placed on top of the pen stand to prevent the mouse from climbing. During the social interaction phase, the male mouse was inserted into the experimental box again and allowed to freely explore the box for 10 min. Above the experimental box, we set a microphone (CM16/CMPA, Avisoft) to record the USVs and a camera (C980GR, Logicool) to capture the location of the subject male mouse. To mask external noise, the experiments were conducted in a sound-attenuating box (ENV-018V, Med Associates Inc.). After completing the experiment, we wiped the inside of the box, basket, and bottle with 70% alcohol and waited for 30 min for them to air-dry.

2.3.2 Experiment 2: Social preference task

To examine whether the NMDA antagonist, MK-801, affects social preference in male mice, we used a social preference task (Fig. 2) in 32 adult male C57BL/6J mice (3- to 6-month old) as subjects and 2 adult male C57BL/6J mice (3-month old) as stimuli. All mice were group-housed with 2–4 individuals in their home cages. In this study, we used a between-subjects design; therefore, we used each subject only once. A custom-made white vinyl chloride box (25 cm width × 50 cm long × 30 cm, height) was used for the test. Male mice were placed in a white cylindrical pen stand (8 cm diameter × 10 cm height). Pen stands containing the stimulus individuals were placed at the center of both ends of a 50-cm-wide box. A 500-mL empty glass bottle was placed on top of the pen stand to prevent the mouse from climbing. Two partition walls (5-cm long × 5-mm thick × 30-cm high) were attached to both sides of the center of a 50-cm-wide box to separate the spaces on the left and right sides. Cameras (C920r, Logicool) were placed 80 cm above the bottom of the box. The mice performing the social preference test were videorecorded on a Windows PC. Mice were intraperitoneally injected with 0.1, 0.2, or 0.4 mg/kg MK-801 or saline and then inserted into the box 5 min after the injection. After 15 min of habituation, the mice were temporarily removed from the box, and the stimulus novel male C57BL/6J mice and a novel object were placed in the basket. The mice were then inserted into the center of the box and allowed to freely explore for 10 min. White noise (75 dB) was presented throughout the

experiment to mask external noise. After running the experiment, we wiped the inside of the box, baskets, and bottles with 70% alcohol and waited for 30 min for it to air-dry.

2.3.3 Experiment 3: Open-field task

To examine the effects of MK-801 on spontaneous locomotor activity, we conducted an open-field task using 12 adult male C57BL/6J mice (2- to 4-months old). The procedure for the open-field task has been described previously (Kaneko et al., 2022). Habituation of the apparatus and saline injection were performed for 60 min/day for 3 days prior to commencing the experiment. The apparatus consisted of a custom-made white vinyl chloride box measuring 50-cm long × 50-cm wide × 50-cm high. The cameras (Logicool HD Webcam C920r, Logicool) were placed 106 cm above the bottom of the box. Videos were recorded on a Windows PC. During habituation, the animals were inserted into an open field immediately after receiving an intraperitoneal injection of saline, and were allowed to freely explore for 60 min. The order of the saline and MK-801 0.1, 0.2, and 0.4 mg/kg injections was randomized, with a >2-day interval between each session of the open-field experiments to avoid the effect of the MK-801 injection continuing until the next session. The duration of the experiment was 60 min. White noise (75 dB) was presented throughout the experiment to mask external noise. After running the open-field experiment, the box was wiped with 70% alcohol and allowed to air-dry for 30 min.

2.4 Analysis

For all experiments, RStudio (version 2022.02.0; RStudio PBC, MA, USA) and GraphPad Prism (version 10.3.0; GraphPad, CA, USA) were used for data analysis. In Experiment 1, we used an open-source USV analysis framework, VocalMat (Fonseca et al., 2021), to detect and categorize USVs. The start times for the video and audio files were synchronized using a custom-made electric circuit consisting of a button, LED light, and small speaker. In all the experiments, we used an open-source visual programming framework, Bonsai (Lopes et al., 2015) to track the activities of the mice using computer vision analysis. The videos were converted into grayscale, smoothed, and monochrome inverted. Mice were identified by setting a contrast threshold. The locomotor activity of the mice in the box was quantified by measuring changes in their central coordinates. In Experiment 1, we defined mice as spending time with the stimulus if they were located within 4.0 cm of the edge of the box that contained the stimulus mice. In Experiment 2, we defined the mice as spending time with the stimulus if they were located within 7.5 cm of the edge of the box that

contained the stimulus mice. In Experiment 3, we defined the center area as the 25 cm × 25 cm inner portion of the box.

3. Results

We used a social interaction task to examine the effects of intraperitoneal injection of MK-801 on the USVs and approach behavior of male mice toward female mice (Fig 1A). First, we analyzed the USVs using VocalMat, an open-source computational vision and machine-learning technique (Fonseca et al., 2021). We successfully classified USVs into 11 categories, including chevron, reverse chevron, down-frequency modulation, up-frequency modulation, flat, short, complex, step-up, step-down, two steps, and multiple steps, and then discriminated them from noise (Fig 1B). Second, we analyzed the relationship between the properties of USVs and the approaching behavior toward female mice. We used an open-source visual programming framework, Bonsai, to track the activities of the mice by using computer-vision analysis (Lopes et al., 2015). The videos were converted into grayscale, smoothed, and monochrome inverted. The location of each mouse was determined by setting a contrast threshold. The locomotor activity of the mice in the box was quantified by measuring changes in their central coordinates. We intraperitoneally injected saline (MK-801 0.1, 0.2, or 0.4 mg/kg 5 min before the social interaction experiment. Under all drug conditions, male mice produced USVs when they were close to female mice, which confirmed that USVs are associated with social signals (Fig. 1C). Intraperitoneal injection of MK-801 dose-dependently decreased the number of ultrasonic vocalizations (Fig. 1D, left panel, F (3, 38) = 11.53, p < 0.0001, one-way ANOVA; saline vs. MK-801 0.2 mg/kg conditions, p = 0.0023, post hoc Tukey test; saline vs. MK-801 0.4 mg/kg conditions, p < 0.0001, post hoc Tukey test; MK-801 0.1 mg/kg vs. MK-801 0.4 mg/kg conditions, p = 0.0258, post hoc Tukey test), and increased locomotor activities (Fig. 1D, central panel, F (3, 38) = 3.61, p = 0.022, one-way ANOVA; saline vs. MK-801 0.4 mg/kg conditions, p = 0.0379), but did not affect the time spent with female mice (Fig. 1D, right panel, F (3, 38) = 0.55, p =0.654, one-way ANOVA). The highest dose of MK-801, 0.4 mg/kg, almost completely muted the USVs (Fig. 1D). There was no obvious tendency for a specific category of USVs to be emitted at a specific location in the box (Fig. 1E).

To examine the effects of intraperitoneal injection of MK-801 on social preference for a same-sex social stimulus, we used a social preference task (Fig. 2). Mice were intraperitoneally injected with saline or 0.1, 0.2, or 0.4 mg/kg MK-801, and then placed in a box at 5 min after the injection. After 15 min of habituation to the experimental setup, the mice were temporarily removed from the box, and social and non-social stimuli, comprising a novel male C57BL/6J mouse and a novel object, were placed in each basket. The mice were then placed in the center of the box and allowed to explore freely for 10 min (Fig. 2A).

We found no impairment in the preference of male mice after the intraperitoneal injection of MK-801 (Fig. 2B). There was a significant difference in the preference for males in the saline group (t (7) = 7.046, p = 0.0002, paired t-test), MK-801 0.1 mg/kg group (t (7) = 2.930, p = 0.0022, paired t-test), MK-801 0.2 mg/kg group (t (7) = 2.769, p = 0.0267, paired t-test), and MK-801 0.4 mg/kg group (t (7) = 5.067, p = 0.0015, paired t-test). However, injections of MK-801 dose-dependently and significantly decreased the time spent with a social stimulus at the highest dose (0.4 mg/kg of MK-801; Fig. 2C left panel, F (3, 28) = 6.173, p = 0.0023, one-way ANOVA; saline vs. MK-801 0.4 mg/kg conditions, p = 0.001, post hoc Tukey test). The injection of MK-801 did not affect the time spent with the non-social object (Fig. 2C, right panel, F (3, 28) = 0.3721, p = 0.7737, one-way ANOVA).

To examine the effects of intraperitoneal injection of MK-801 on locomotor activity and excretion, we performed an open-field task. After 60 min of habituation to the box for 3 days, we performed an open-field test. In this test, mice were intraperitoneally injected with saline or 0.1, 0.2, or 0.4 mg/kg of MK-801 and then placed in a box at 5 min after the injection. The spontaneous locomotor activity of the mice increased after the injection of MK-801 (Fig. 3A, left panel, F (2.177, 21.77)□=□7.817, p□=□0.0023, repeated measures one-way ANOVA). We found a significant increase in locomotor activity in all the doses of MK-801 (0.1, 0.2, and 0.4 mg/kg) compared to the saline condition (saline vs. MK-801 0.1 mg/kg, p□<□0.0001, post hoc Tukey test; saline vs. MK-801 0.2 mg/kg, p□=□0.0184; saline vs. MK-801 0.4 mg/kg, p□=□0.0367; post hoc Tukey test). Time course of locomotor activity during the open-field task indicated that the effect of intraperitoneal injections of MK-801 on locomotor activity was observed to commence approximately 15 min after injection and continue for more than 60 min (Fig. 3A, right panel).

Furthermore, we examined the time spent in the center of the box while the mice performed the open-field task. A significant increase of the time spent in the center box of the mice was observed after the injection of MK-801 0.4 mg/kg condition (Fig. 3B; F (2.573, 25.73)□=□37.42, p□<□0.0001, repeated measures one-way ANOVA; saline vs. MK-801 0.4 mg/kg, p□<□0.0001, post hoc Tukey test; MK-801 0.1 mg/kg vs. MK-801 0.4 mg/kg, p□=□0.0003; MK-801 0.2 mg/kg vs. MK-801 0.4 mg/kg, p□=□0.0002; post hoc Tukey test). The time spent in the center during the open-field task indicated that the effect of intraperitoneal injections of MK-801 on locomotor activity was observed soon after injection and continued for more than 60 min (Fig. 3B, right panel).

To assess the effect of MK-801 on excretion, which is an indirect measurement of autonomic nervous system function, feces and urine samples were collected and quantified

after the mice performed an open-field task. The effect of MK-801 on fecal output was significant in both feces (Fig. 3C, right panel, F (2.086, 20.86)□=□5.555, p□=□0.0109, repeated-measures one-way ANOVA) and urine (Fig. 3C, right panel, F (1.740, 17.40)□=□12.82, p□=□0.0006, repeated-measures one-way ANOVA). MK-801 injection dose-dependently decreased feces and urine output (feces: saline vs. MK-801 0.4 mg/kg conditions, p□=□0.0093; MK-801 0.1 mg/kg vs. MK-801 0.4 mg/kg conditions, p□=□0.0090; MK-801 0.2 mg/kg vs. MK-801 0.4 mg/kg conditions, p□=□0.0224; urine: saline vs. MK-801 0.4 mg/kg conditions, p□< 0.0001; MK-801 0.1 mg/kg vs. MK-801 0.4 mg/kg conditions, p□=□0.0196; MK-801 0.2 mg/kg vs. MK-801 0.4 mg/kg conditions, p□=□0.0024).

4. Discussion

To clarify the role of NMDA receptors in USVs, we examined the effects of an intraperitoneal injection of the NMDA receptor antagonist MK-801 in male mice. First, we investigated the USVs emitted by female male mice. After we successfully classified USVs into 11 categories and discriminated them from noise using open-source computational vision and machine learning techniques (Fonseca et al., 2021), we analyzed the relationship between the properties of USVs and approach behavior in female mice. Male mice produced USVs when they were close to female mice, and thereby confirmed that USVs were associated with social signals. Second, we examined the effects of intraperitoneal injection of the NMDA antagonist MK-801 on USVs and approaching behavior. Injection of MK-801 dose-dependently decreased the number of USVs and increased locomotor activity but did not affect the time spent with female mice. Third, we confirmed that injection of MK-801 decreased the time spent with individuals of the same sex in the social preference task. Fourth, we demonstrated that MK-801 injection increased locomotor activity and time spent in the center and inhibited excretion in the open-field task.

We successfully classified USVs into 11 categories and discriminated them from noise using VocalMat, an open-source computational vision and machine-learning technique (Fonseca et al., 2021). By integrating the results obtained from the video and audio data, we revealed that male mice emitted USVs when they were close to female mice. This result confirms that USVs are associated with courtship signals in male mice. In our experimental setup, it was impossible to detect whether the male or female mice emitted USVs. However, almost no USV was recorded after the highest dose of MK-801. This result ruled out the possibility that female mice emitted USVs in response to the presence of male mice. Furthermore, there was no clear tendency for male mice to emit a specific category of USVs at a specific distance from the stimulus female mice, which suggested that specific categories of USVs may not be important in this courtship situation. Although accumulating evidence suggests that different ultrasonic call categories exist and have already been defined in many ways (Arriaga et al., 2012; Chabout et al., 2015; Holy and Guo, 2005), an understanding of the meaning of the different categories of USVs in mice is still lacking (Premoli et al., 2021). Advances in sophisticated analyses of USVs have made it possible to analyze and quantify USVs emitted by rodents more easily and accurately (Coffey et al., 2019; Fonseca et al., 2021; Van Segbroeck et al., 2017; Neunuebel et al., 2015; Tachibana et al., 2020). Further analyses that combine pose estimation or behavioral classification techniques utilizing computer vision analysis (Lauer et al., 2022; Mathis et al., 2018; Nath et

al., 2019; Weinreb et al., 2024; Wiltschko et al., 2020) with auditory feature analysis in a free-access social interaction situation (John et al., 2023; Matsumoto et al., 2022) would constitute an important step in understanding the biological relevance of specific categories of USVs.

The intraperitoneal injection of MK-801 dose-dependently decreased USVs in the social interaction test and decreased excretion in the open-field task. To the best of our knowledge, no study has reported the effects of MK-801 on excretion, and this research provides the first evidence that MK-801 inhibits fecal output and urination. Excretion is modulated by the balance of activities of the autonomic system, which comprises the sympathetic and parasympathetic nervous systems. Evidence suggests that USVs critically depend on the precise control of laryngeal muscles in coordination with ongoing respiration (MacDonald et al., 2024; Park et al., 2024), which is known to be strongly modulated by the pre-Bötzinger complex, which controls heart rate and blood pressure by modulating both sympathetic and parasympathetic activities (Menuet et al., 2020). Accordingly, there is evidence that shows administrations of MK-801 affect respiration (Fouts et al., 1988). Autonomic nervous system dysfunction in the form of increased sympathetic activity and decreased parasympathetic activity has been implicated in schizophrenia and its treatment (Stogios et al., 2021). Understanding the autonomic modulations evoked by MK-801 injections is useful for understanding the role of autonomic modulation in the mechanisms of vocalization and schizophrenia.

Another possibility is that the MK-801-induced decrease in USVs affects cortical or subcortical vocalization-related brain regions. As NMDA receptors are highly expressed in the cerebral cortex, the blockade of NMDA receptors might disrupt the neuronal activities that generate motor commands or coordinate the vocalization implicated in the prefrontal cortex. The anterior cingulate cortex plays an important role in action sequences, social behavior, and vocalization. In humans, non-human primates, and rodents, (1) stimulation of a limited region of the anterior cingulate cortex produces vocalization (von Cramon & Jürgens, 1983; Gan-Or et al., 2023), (2) lesions in the anterior cingulate cortex impair vocalization (Nagarajan et al., 2024), and (3) neuronal activity in the anterior cingulate cortex is associated with vocalization (Gavrilov et al., 2019; Gan-Or et al., 2023; Jafari et al., 2023). In addition, the ventrolateral prefrontal cortex plays an important role in the volitional control of vocalization (Hage & Nieder, 2013; Gavrilov et al., 2019). In subcortical areas, the midbrain periaqueductal gray (PAG) is a key region that regulates vocal production (Adametz and O'Leary, 1959; Tschida et al., 2019; Ziobro et al., 2024). Future studies that

examine local brain region-specific manipulations of NMDA receptor activity are necessary for a circuit- and receptor subtype-level understanding of vocalization.

Intraperitoneal injection of MK-801 did not affect the time spent by female mice in Experiment 1. We confirmed that injection of MK-801 decreased the time spent by individuals of the same sex in the social preference task. These differences might have been caused by differences in the experimental setups used in Experiments 1 and 2. In Experiment 1, we used a narrow box to effectively record the USVs emitted by male mice, and this was not the best experimental situation to examine social interaction. Considering the obvious effects on USVs, we might be able to obtain more obvious effects of MK-801 on social behavior if we used more natural experimental conditions. Altered sociability is a key feature of schizophrenia and has been reported in animal studies with pharmacological and genetic manipulations of NMDA receptor function. However, existing findings examining the manipulation of NMDA receptor functions in social behavior are controversial (Furuie & Yamada, 2022; Gururajan et al., 2010; Hanks et al., 2013; Moy et al., 2012; Wedzony et al., 2008). As observed in Experiments 1 and 3, the intraperitoneal injection of MK-801 increased locomotor activity. In addition, NMDA receptors are crucial for synaptic plasticity, learning, and memory and are affected by habituation (Gururajan et al., 2010). One of the reasons why the blockade of NMDA receptors produces different results in social behavior in each laboratory may be the confounding factors of the effects on motor control, habituation, learning, memory, and social recognition. To determine the complexity of the effects of blocking NMDA receptors on social behavior, future studies should manipulate the functions of NMDA receptors in a specific location in the brain using a well-controlled but naturalistic social behavior task.

In Experiment 3, we demonstrated that MK-801 injection increased the time spent in the center of the box. Presumably, the increased time spent in the center by the MK-801 injection was not caused by the effect on anxiety, but rather, by the increased stereotyped behavior, such as circulating or jumping movements. This explains why the effects of MK-801 on locomotor activities were not as obvious at the highest dose, 0.4 mg/kg, compared to that at the lower dose, 0.1 mg/kg. This account is supported by the findings of previous studies that showed that stereotyped behavior increased in a dose-dependent manner after the injection of MK-801 (Janus et al., 2023; Mabunga et al., 2019). Blockade of NMDA receptors may affect the state transition of sequential movements. As USVs require motor coordination and sequential motor planning, the mechanism underlying

the effects of MK-801 on USVs and locomotor activity may involve a shared physiological motor-coordination mechanism.

Our study has two major limitations. First, C57BL/6J mice were used. There are species-specific differences in USVs. For example, rats emit USVs in response to appetitive and aversive situations (Premoli et al., 2023) whereas mice do not exhibit obvious valence-related USVs. Comparison of the current results with those of rats and other animals is useful for a translational understanding of the mechanisms of schizophrenia. Second, we did not perform ovariectomies or ovarian hormone administration in the female mice. In the future, it will be interesting to study how the conditions of female mice, such as sex cycle or sex hormonal levels, affect USV emission from male mice.

In conclusion, the present study revealed that the systemic administration of a non-competitive NMDA receptor antagonist, MK-801, disrupts courtship USVs in adult male mice. As acute MK-801 administration imitates the positive and negative symptoms of schizophrenia, our findings have promising potential for understanding the mechanisms underlying schizophrenia.

Glossary

N-methyl-d-aspartate (NMDA); ultrasonic vocalizations (USVs)

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Code availability

The original code for the analyses is available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

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Author contributions: CRediT

RT and KT. designed the experiments. RT collected all social interaction data with the help of KT and KY. DN and KT collected all social preference data with the help of KH. MT, YU, and HK helped arrange the experimental and data analysis environments for the USVs. MY and SM collected all the open-field data with the help of KT. RT, YU, MY, and KT analyzed the data. RT, YU, MY, and KT created figures. KT wrote the manuscript. KT revised the manuscript.

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Figure Captions

Fig. 1. Effect of MK-801 on USVs and other properties in the social interaction task.

A. The apparatus used for the test was a custom-made transparent vinyl chloride box (27-cm long × 8.5-cm wide × 30-cm high), with baskets containing a well-habituated novel female mouse placed at the edge of the 27-cm-long box. Mice were intraperitoneally injected with saline, 0.1, 0.2, or 0.4 mg/kg MK-801 and then placed in a box 5 min after the injection. After 15 min of habituation to the experimental setup without female mouse stimulus (upper left panel), the subject mouse was temporally removed from the box, and the novel female mouse stimulus was placed in the basket. Then, the subject mice were placed in the box and allowed to explore freely for 10 min (upper right panel). Experiments were conducted in a sound-attenuating box to mask the external noise. Experiments were conducted for 4 days per week and the schedule was repeated for 3 weeks (bottom panel). Given the daily variations in the number of USVs, we conducted practice sessions on the first and fourth days of the week. The data from saline and drug injections were obtained on the second and third days of the week, respectively, to generate stable saline data just before the test day with drug manipulation. **B.** USVs were classified into 11 categories, including chevron, reverse chevron, down-frequency modulation, up-frequency modulation, flat, short, complex, step-up, step-down, two steps, and multiple steps, and discriminated from noise using VocalMat, an open-source computational vision and machine-learning technique (Fonseca et al., 2021). **C.** Time spent by male mice in each location from the novel social-stimulus mouse. **D.** Number of the USVs (left panel), time spent with the social stimulus (center panel), and locomotor activities (left panel). E. Number of classified USVs which were aligned with the distance from the female stimulus. Error bars indicate standard error of the mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. $N = 7$.

Fig. 2. Effect of MK-801 on the social preference task.

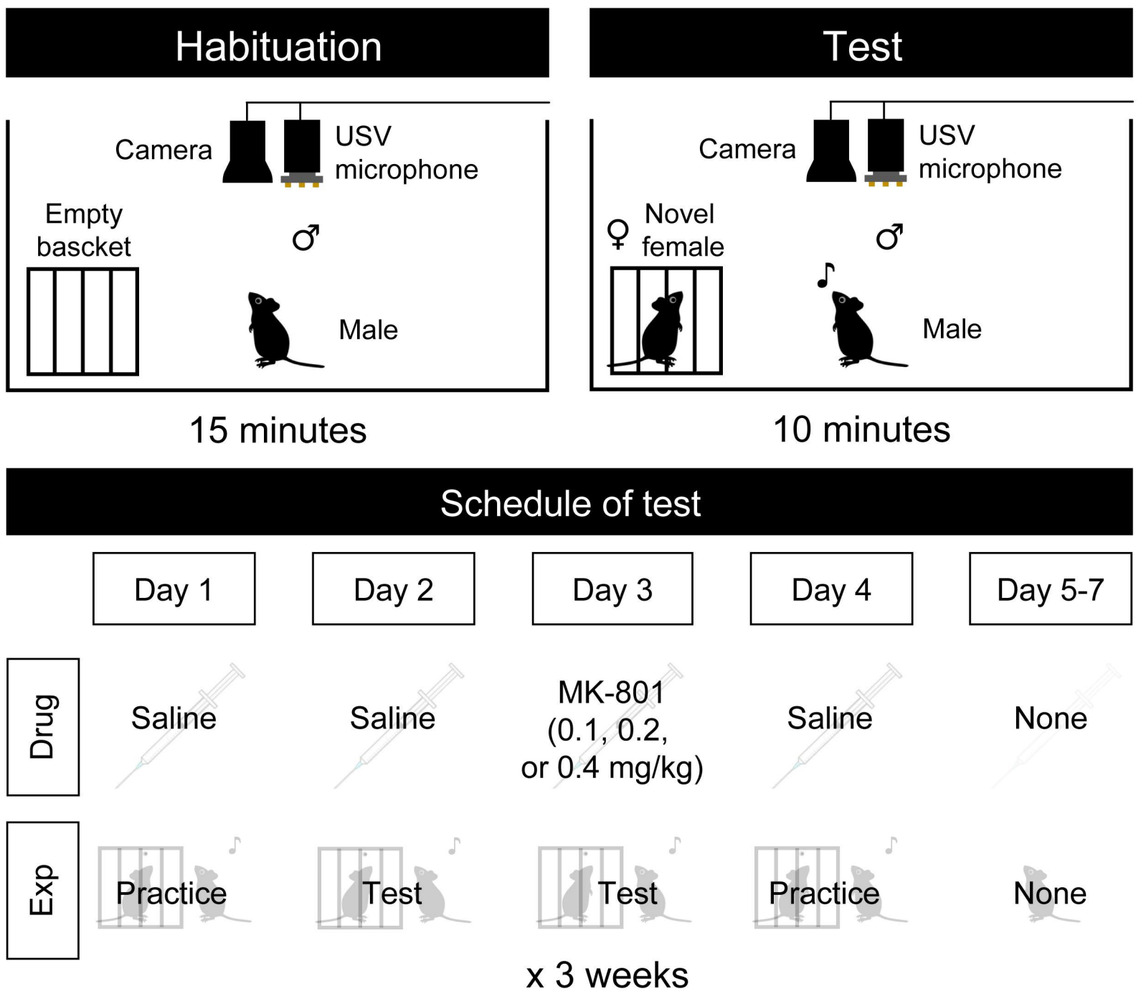
A. The apparatus used for the test was a custom-made white vinyl chloride box (25-cm long × 50-cm wide × 30-cm high), with baskets containing a novel male mouse or a novel object placed at the center of the 25-cm-long box and at both ends of the 50-cm-wide box. A wall was attached to the center of the 50-cm-wide box to separate the space on the left and right sides. Mice were intraperitoneally injected with saline, 0.1, 0.2, or 0.4 mg/kg of MK-801 and then placed in a box 5 min after the injection. After 15 min of habituation, the mice were temporally removed from the box, and the novel male mouse and the novel object were placed in each basket. The subject mice were then placed in the center of the box and allowed to move freely for 10 min. White noise (75 dB) was presented throughout the experiment to mask the external noise. **B.** Time spent by male mice with the novel social and non-social object stimuli . **C.** Time spent with a social stimulus (left) and time spent with a non-social object (right). Error bars indicate standard error of the mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. $N = 8$ in each group.

Fig. 3. Effect of MK-801 on the open-field task.

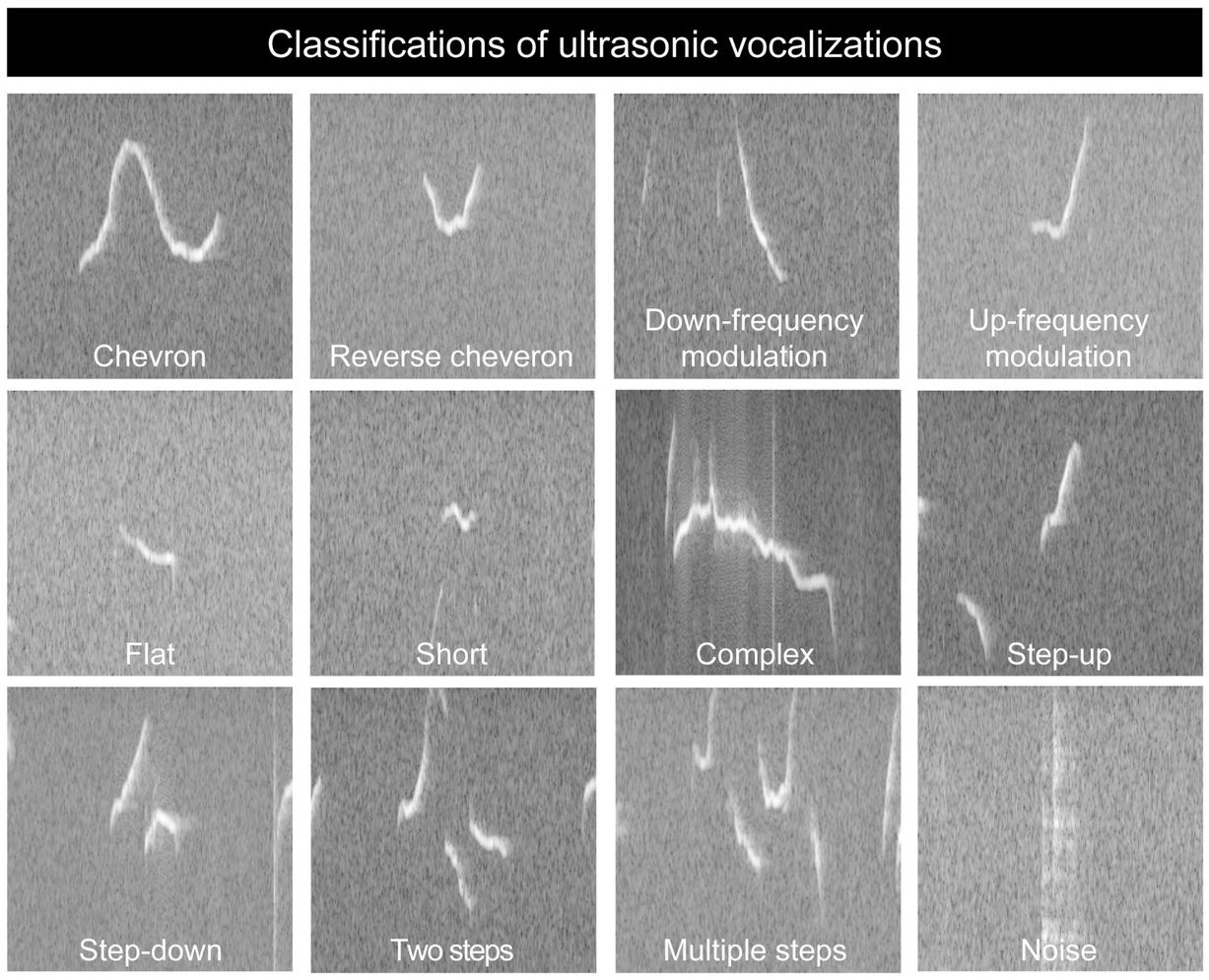
A. Overall locomotor activities from 10 to 60 min after commencing the open-field task following the injection of saline or MK-801 (left panel). Time course of open-field activities (right panel). **B.** Time spent in the central location of the open-field box from 10 to 60 min after commencing the open-field task (left panel). Time course

of the time spent in the central location of the open-field box (right panel). We defined the central area as the 25 cm × 25 cm inner portion of the box. **C.** Amount of feces collected after 60 min of the open-field task (left panel). Amount of feces collected after 60 min of the open-field task. Error bars indicate standard error of the mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. $N = 11$.

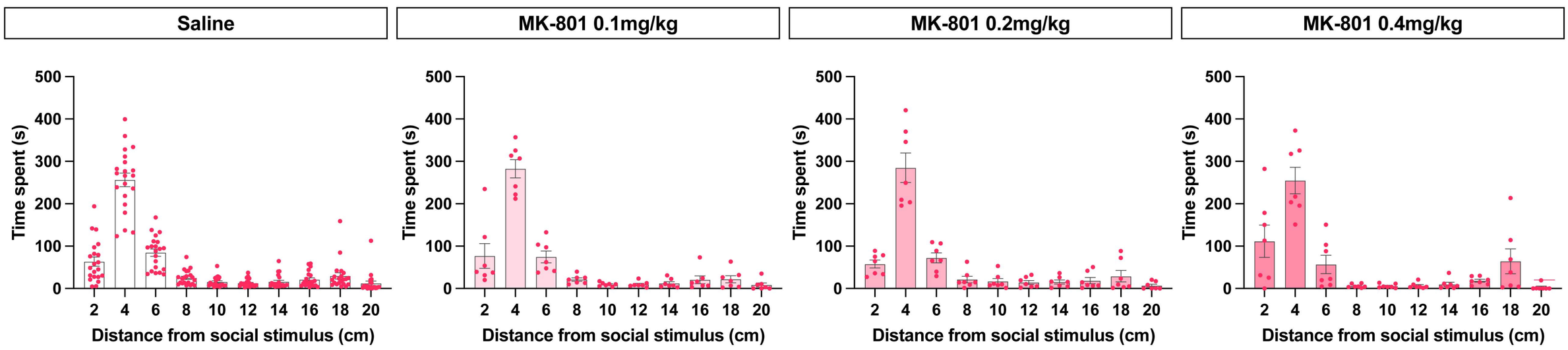
A.


Habituation
Camera
USV microphone
Empty bascket
Male
15 minutes
Test
Camera
USV microphone
Novel female
Male
10 minutes
Schedule of test
Day 1
Day 2
Day 3
Day 4
Day 5-7
Drug
Saline
Saline
MK-801 (0.1, 0.2, or 0.4 mg/kg)
Saline
None
Exp
Practice
Test
Test
Practice
None
x 3 weeks

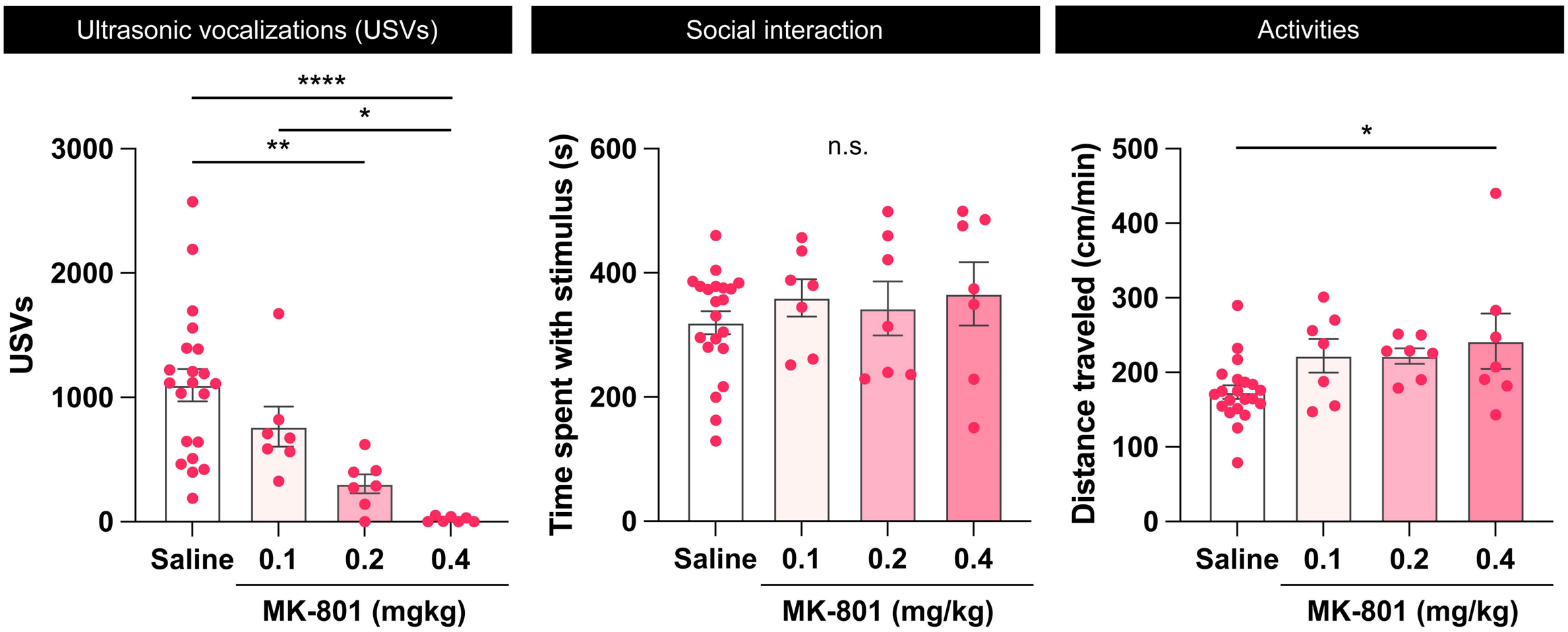
B.


Classifications of ultrasonic vocalizations
Chevron
Reverse cheveron
Down-frequency modulation
Up-frequency modulation
Flat
Short
Complex
Step-up
Step-down
Two steps
Multiple steps
Noise

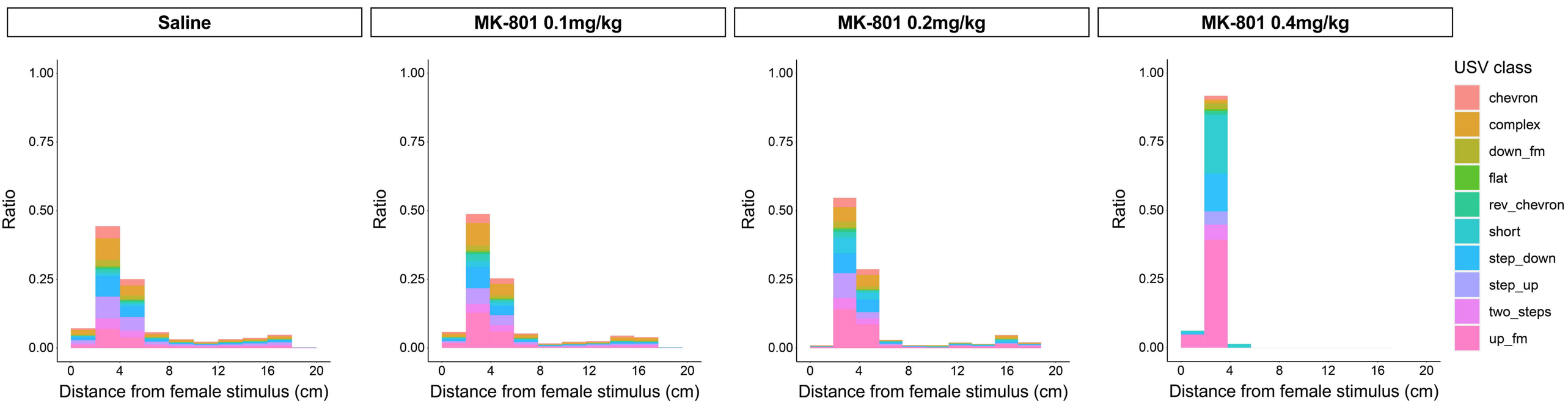
C.


Saline
MK-801 0.1mg/kg
MK-801 0.2mg/kg
MK-801 0.4mg/kg
Time spent (s)
Distance from social stimulus (cm)

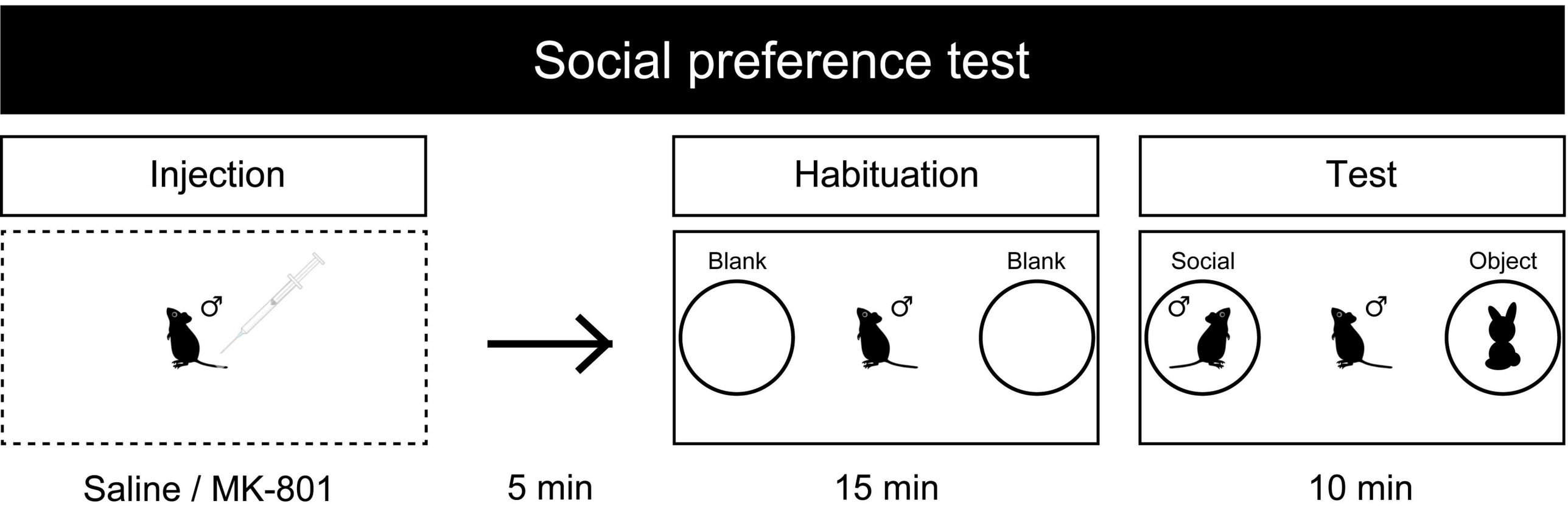
D.


Ultrasonic vocalizations (USVs)
Social interaction
Activities
USVs
Time spent with stimulus (s)
Distance traveled (cm/min)
n.s.
Saline
0.1
0.2
0.4
MK-801 (mgkg)
MK-801 (mg/kg)

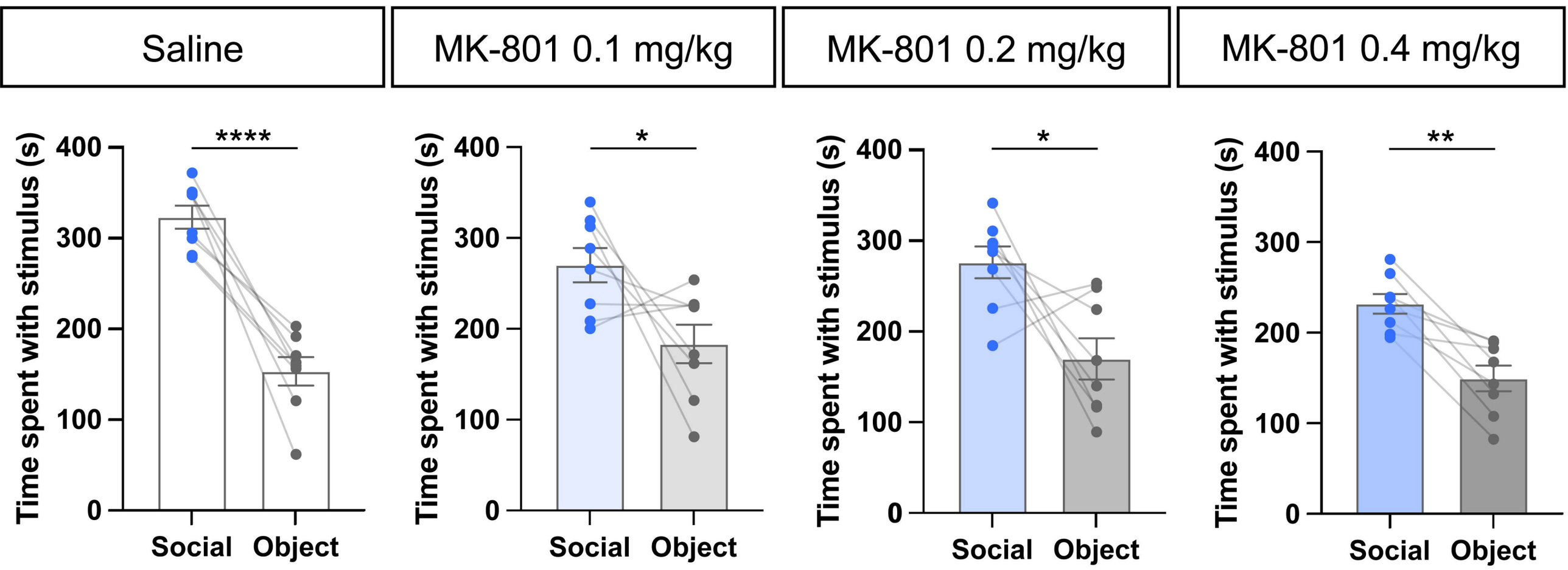
E.


Saline
MK-801 0.1mg/kg
MK-801 0.2mg/kg
MK-801 0.4mg/kg
Ratio
Distance from female stimulus (cm)
USV class
chevron
complex
down_fm
flat
rev_chevron
short
step_down
step_up
two_steps
up_fm

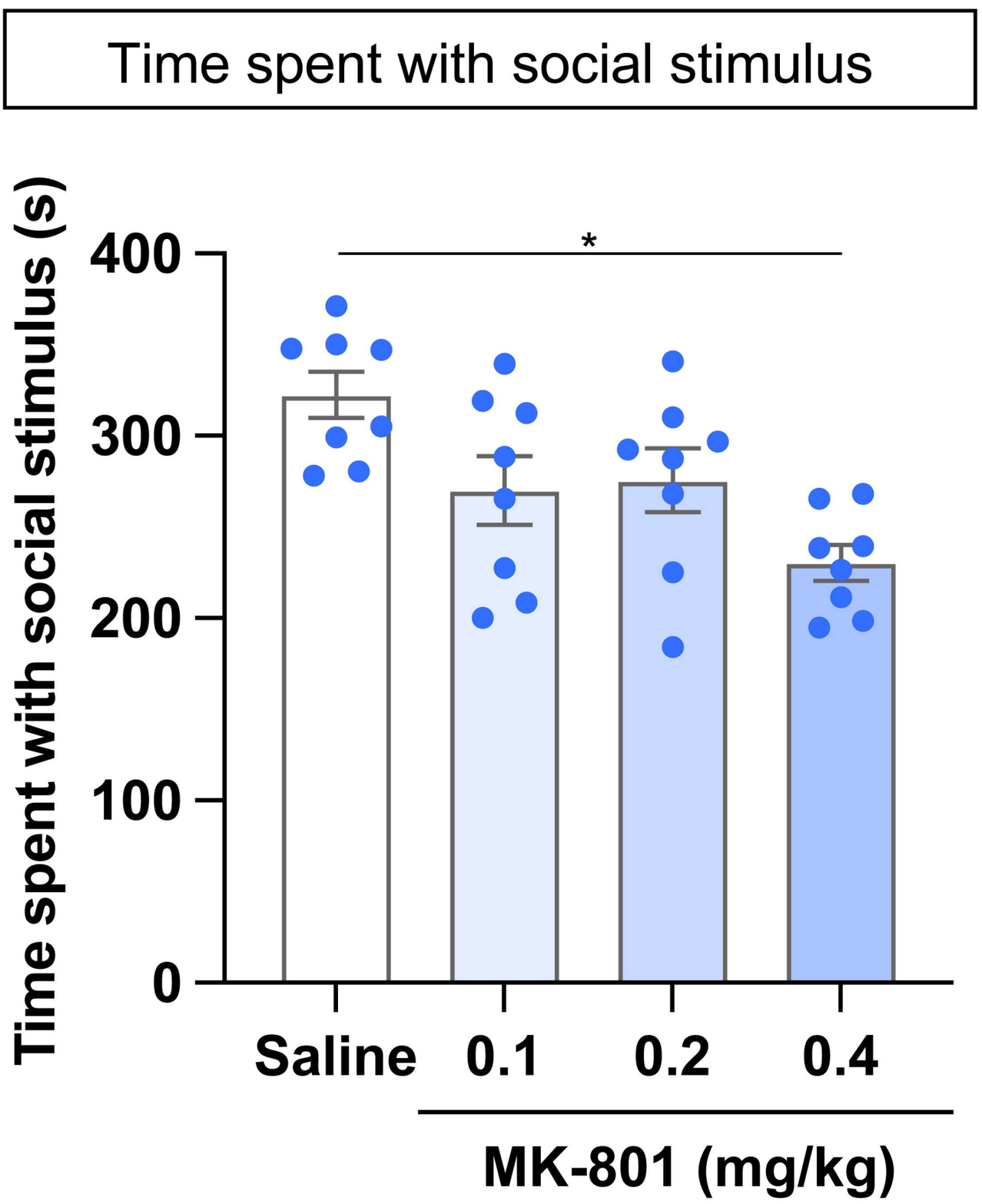
A.


Social preference test
Injection
Habituation
Test
Blank
Blank
Social
Object
Saline / MK-801
5 min
15 min
10 min

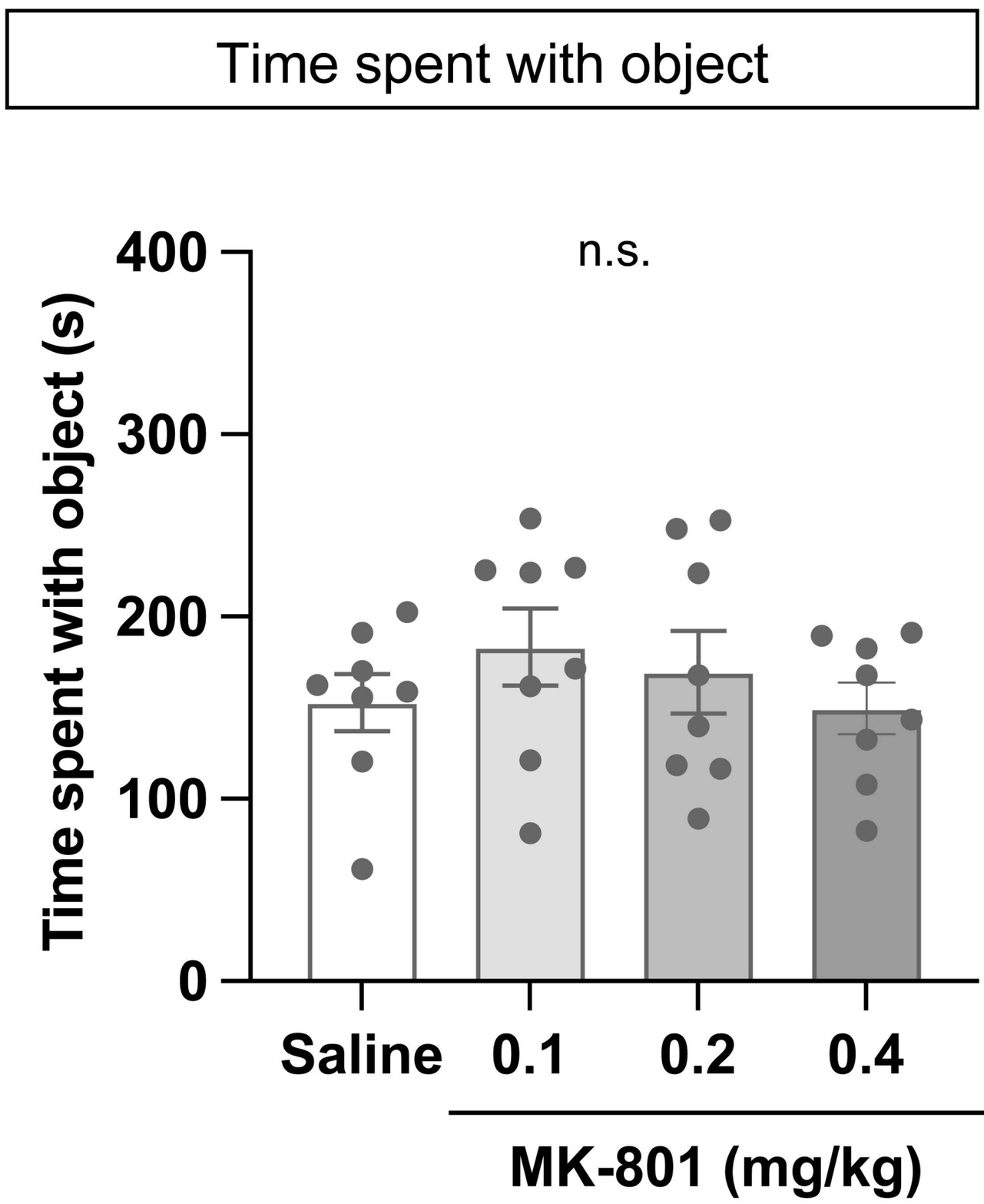
B.


Saline
MK-801 0.1 mg/kg
MK-801 0.2 mg/kg
MK-801 0.4 mg/kg
Time spent with stimulus (s)
400
300
200
100
0
****
*
*
**
Social
Object

C.


Time spent with social stimulus
Time spent with social stimulus (s)
400
300
200
100
0
*
Saline
0.1
0.2
0.4
MK-801 (mg/kg)

D.

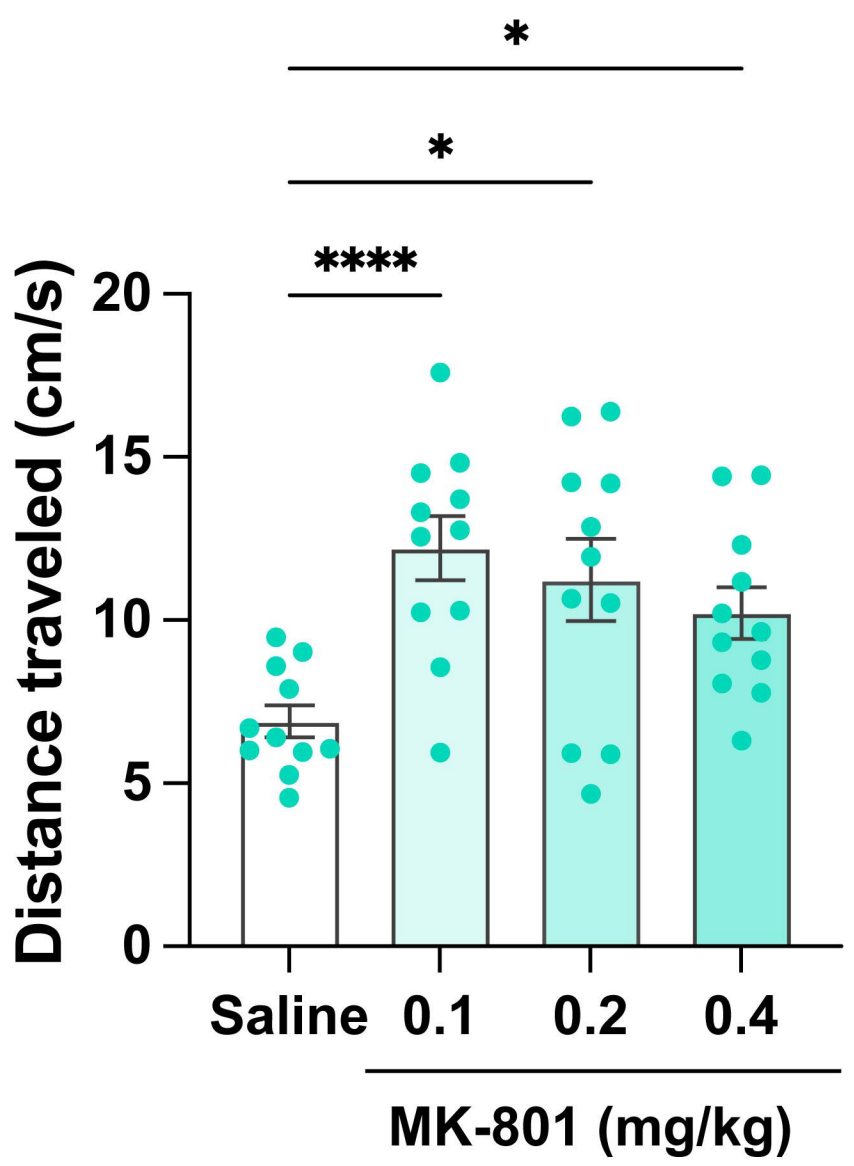

Time spent with object
Time spent with object (s)
400
300
200
100
0
n.s.
Saline
0.1
0.2
0.4
MK-801 (mg/kg)

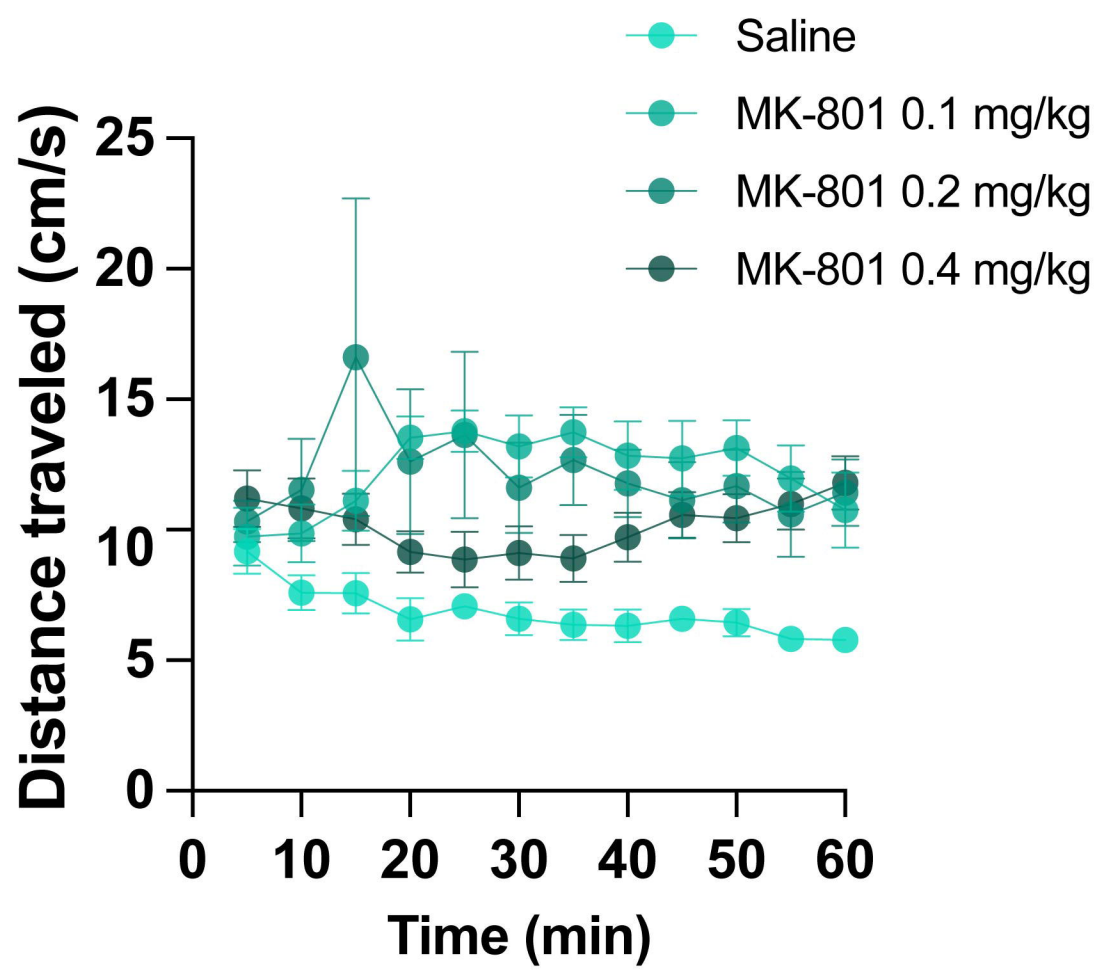
A.

Locomotor activity

Overall

Time course



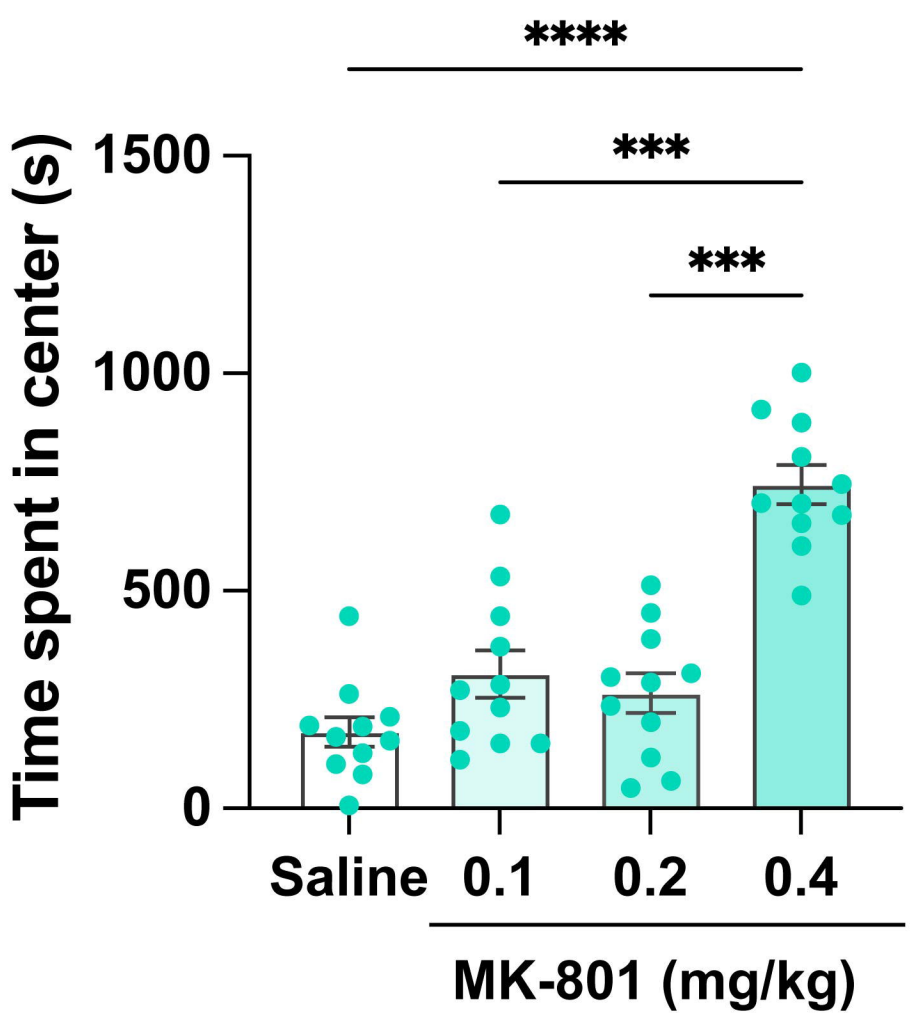


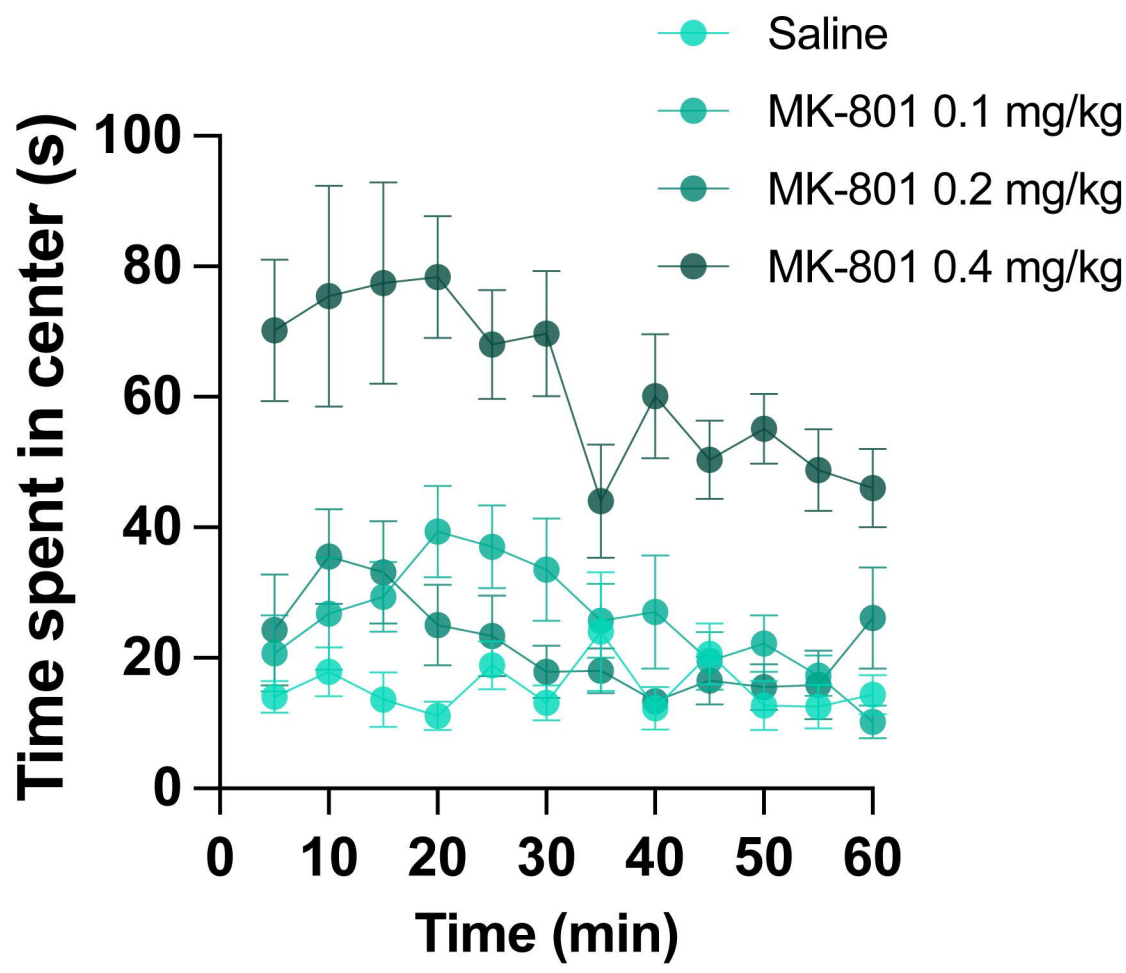
B.

Time spent in center

Overall

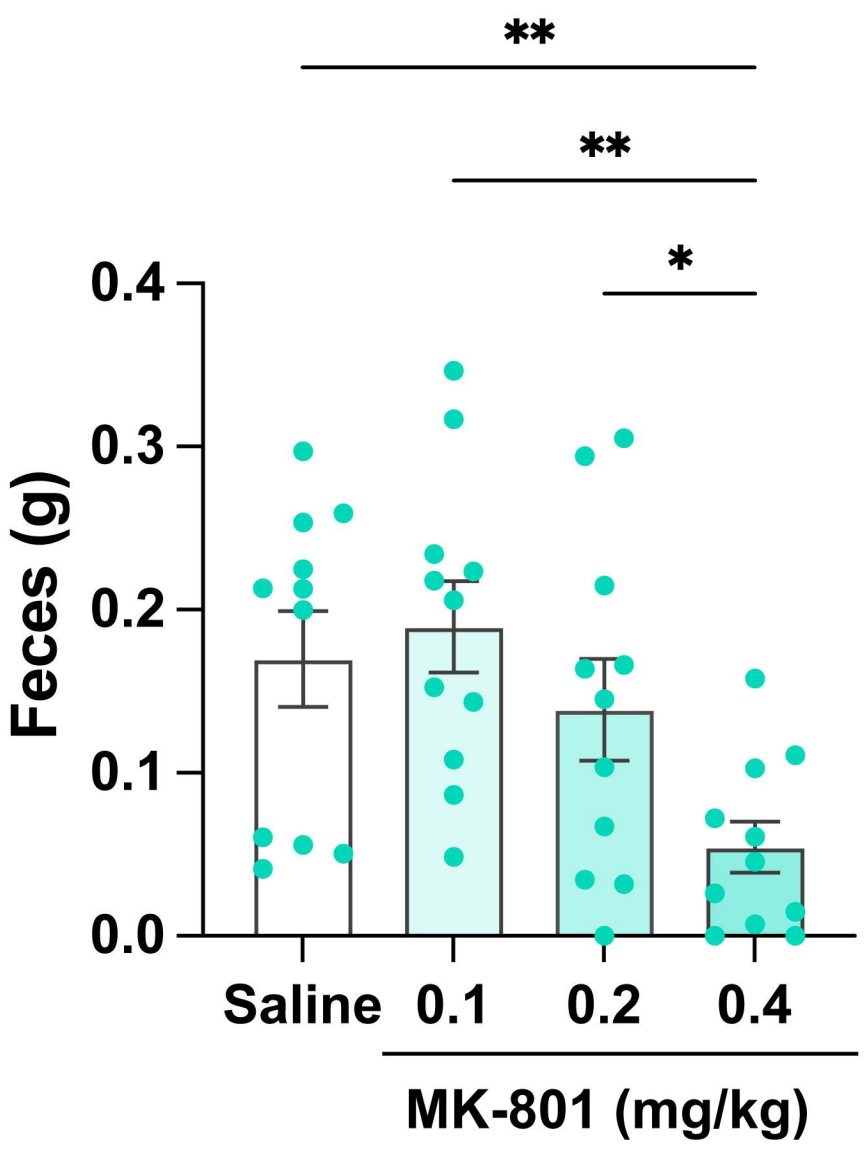
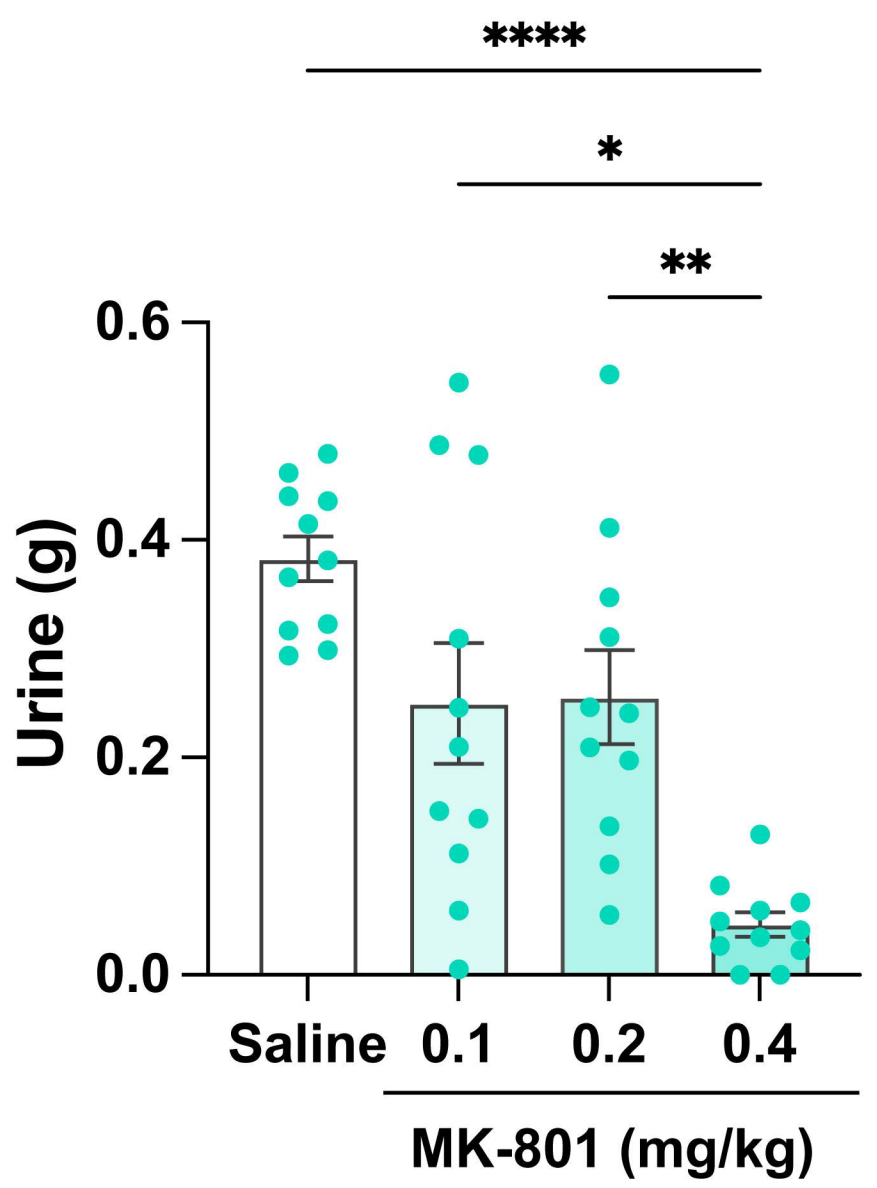
Time course





C.

Excretion

Feces

Urine