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Research report

Ketamine administration during the second postnatal week induces enduring schizophrenia-like behavioral symptoms and reduces parvalbumin expression in the medial prefrontal cortex of adult mice

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HIGHLIGHTS

- Ketamine administration in mice on PND 7, 9 and 11.
- Characterization of schizophrenia-like behavioral phenotype in adulthood.
- Loss of parvalbumin immunoreactivity in the prefrontal cortex.
- Severe persistent deficits in attentional tasks, social and novelty discrimination.

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ABSTRACT

Dysfunctions in the GABAergic system are considered a core feature of schizophrenia. Pharmacological blockade of NMDA receptors (NMDAR), or their genetic ablation in parvalbumin (PV)-expressing GABAergic interneurons can induce schizophrenia-like behavior in animals. NMDAR-mediated currents shape the maturation of GABAergic interneurons during a critical period of development, making transient blockade of NMDARs during this period an attractive model for the developmental changes that occur in the course of schizophrenia's pathophysiology. Here, we examined whether developmental administration of the non-competitive NMDAR antagonist ketamine results in persistent deficits in PFC-dependent behaviors in adult animals. Mice received injections of ketamine (30 mg/kg) on postnatal days (PND) 7, 9 and 11, and then tested on a battery of behavioral experiments aimed to mimic major symptoms of schizophrenia in adulthood (between PND 90 and 120). Ketamine treatment reduced the number of cells that expressed PV in the PFC by ~60% as previously described. Ketamine affected performance in an attentional set-shifting task, impairing the ability of the animals to perform an extradimensional shift to acquire a new strategy. Ketamine-treated animals showed deficits in latent inhibition, novel-object recognition and social novelty detection compared to their SAL-treated littermates. These deficits were not a result of generalized anxiety, as both groups performed comparably on an elevated plus maze. Ketamine treatment did not cause changes in amphetamine-induced hyperlocomotion that are often taken as measures for the positive-like symptoms of the disorder. Thus, ketamine administration during development appears to be a useful model for inducing cognitive and negative symptoms of schizophrenia.

1. Introduction

Glutamatergic hypotheses of schizophrenia are based on the ability of *N*-methyl-D-aspartic acid receptor (NMDAR) antagonists such as ketamine or phencyclidine (PCP) to induce core symptoms of the illness, including cognitive deficits, both in healthy humans [1–3] and in animals [4–7], and to exacerbate symptoms in schizophrenics [8]. Subchronic blockade of NMDARs in animals reduces the levels of the GABA-synthesizing enzyme glutamic acid decarboxylase 67 (GAD67) and the calcium-binding protein parvalbumin (PV) in the prefrontal cortex and hippocampus [6,9–11]. These changes replicate findings from postmortem studies in the brains of schizophrenic patients, suggesting that dysfunction of GABAergic interneurons in these brain areas is a core feature of schizophrenia [12–14]. The PV-expressing interneurons serve a critical role in the induction and maintenance of synchronous

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network oscillations required for the integration of sensory input with stored information [15,16]. Accordingly, in schizophrenia cognitive processes such as attention, strategy shifting, novelty and social recognition which require an intact prefrontal cortex (PFC) or hippocampus are disrupted [13,17–20].

In animals, acute or subchronic blockade of NMDARs in adult subjects has been used extensively to model the behavioral, biochemical and neurophysiological alterations seen in schizophrenia [7,21–23]. However, schizophrenia is a developmental disorder and insults to the developing brain can contribute significantly to the manifestation of the disorder in early adulthood [24,25]. Because GABAergic interneurons profoundly affect the postnatal development of cortical circuitry, the blockade of NMDARs or subunit deletion early in development may persistently affect behavior by disrupting the maturation of PV cell connectivity [26–28]. Accordingly, in contrast to the reversible effects of NMDAR antagonism observed in adult animals, embryonic and repetitive treatment during the second postnatal week can produce enduring behavioral and neurochemical deficits [29–31], thus modeling the neurodevelopmental nature of the disease.

A considerable number of studies have investigated the effects of acute or subchronic administration of ketamine in adult animals. However, so far it has not been tested whether and how subchronic treatment with ketamine during a critical developmental period affects behaviors in adult rodents that are thought to measure positive, negative and cognitive symptoms of schizophrenia. Here, we treated mice with ketamine on postnatal days (PND) 7, 9 and 11 and performed experiments in adult animals (90–120 PND) to study persistent changes in behavior and PV expression in the medial prefrontal cortex (mPFC). Developmental ketamine treatment reduced PV expression consistent with previous findings [32]. Developmental ketamine administration resulted in deficits in attentional set-shifting, impaired latent inhibition and novel object recognition. Ketamine-treated animals also showed signs of deficient social investigation or social amnesia in response to repetitive exposure of a stimulus animal or a novel animal in their home cages. These deficits were not due to generalized anxiety as ketamine-treated animals did not differ from vehicle-treated animals in their exploration behavior on an elevated plus maze. Surprisingly, ketamine-treatment did not lead to enhanced hyperlocomotion in an open field in response to an acute amphetamine challenge across a range of amphetamine doses (1.0, 2.5, and 5.0 mg/kg). Taken together, ketamine administration during development appears to induce several core symptoms of schizophrenia that persist into adulthood, and specifically it has high face validity for inducing cognitive and negative-like symptoms of the disorder.

2. Methods

2.1. Animals and ketamine treatment

Non-carrier/WT adult male mice of the G42 line (CB6-Tg[Gad1-EGFP]G42Zjh/J; Jackson Laboratories, Bar Harbor, ME, RRID:IMSR_JAX:007677) [33] were used for the experiments. Upon weaning, the mice were group housed with ad libitum access to food and water, and maintained on a 12:12 light/dark cycle. Animals were separated and singly housed for (1) experiments involving food restriction and (2) social investigation. All experiments were performed during the light cycle. Saline (SAL) or 30 mg/kg ketamine (Ketathesia HCl, Henry Schein, Dublin, OH) injections were administered subcutaneously at 10 ml/1 kg to pups on PND 7, 9 and 11 [32]. All procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas at Dallas.

2.2. Behavioral testing

Prior to behavioral experiments, mice were handled for 2 weeks for 10 min daily by the experimenters in the room where the experiments were performed.

2.2.1. Attentional set-shifting task

Procedures followed those previously described [34]. Mice were gradually food restricted to 85% of their free-feeding weight. On the first day of habituation, animals were placed into the center of a cross maze. Four reward pellets (Cheerios bits) were placed in each of the arms of the maze, and mice were allowed to freely explore the maze and to consume the food pellets for 15 min. If a mouse consumed all 16 pellets prior to 15 min, it was removed from the maze and placed in a holding cage while the maze was rebaited, then the mouse was placed back in the center of the maze. On the second day of habituation, arms were only baited with two pellets each (in the middle and at the end of the arms). Again, all arms were rebaited whenever a mouse consumed all 8 food pellets and mice were allowed to explore the maze for a total of 15 min. On the third day of habituation, only one food pellet was placed at the end of each arm. To reach habituation criterion, animals were required to consume all four food pellets on the maze at least 4 times within the 15 min period. All animals in this study reached this criterion on the third habituation day. On the following day, the animals' turn bias was determined. Therefore, the cross maze was converted into a t-maze by blocking off one of the arms. Mice were placed in the stem arm and allowed to turn left or right to obtain a food pellet. After the mouse consumed the reward, it was returned to the stem arm and allowed to make another choice. If the mouse chose the same arm as on the initial choice, it was returned to the stem arm until it chose the other arm and consumed the food pellet. The direction (right or left) chosen four or more times over seven trials was considered the turn bias. On the following day (Response Discrimination day), mice were trained on a response discrimination task which required them to always turn in the opposite direction of their turn bias to obtain the food reward. The location of the stem arm was pseudorandomly varied among three arms to discourage mice from using an allocentric spatial strategy to locate the food. A visual clue (vertical black and white stripes on 13 × 10 cm plastic sheet) was placed to the side of one arm opposite the stem arm. Placement of the visual cue into the right or left arm varied pseudorandomly to balance the frequency of occurrences in each arm across blocks of 12 consecutive trials. Similarly, the order of the start arms alternated pseudorandom manner so that the frequency of arms was balanced across blocks of 12 trials. Training continued until mice reached a criterion of 9 correct choices over 10 consecutive trials. After achieving acquisition criterion, a probe trial was administered which began in the previously unused fourth arm. If the mice performed the probe trial correctly, then response discrimination training was completed. If an incorrect turn occurred, response training continued until five consecutive correct choices were made, and another probe trial was administered. This procedure continued until the mouse reached the criterion and also made a correct choice on the probe trial. On the following day (Shift to Visual-Cue Learning day), mice were trained to shift their strategy to now follow the visual cue to obtain a food reward. The location of the visual cue and the position of the start arm were again varied pseudorandomly and their frequency was balanced across blocks of 12 consecutive trials. The training and response criteria on Response Discrimination day and Shift to Visual-Cue Learning day were identical. Training continued until the mouse made a correct choice on the probe trial. For each day, we analyzed the total number of trials to criterion and the number of probe trials required to reach criterion. For the Shift to Visual-Cue Learning day, errors were scored as entries into arms that did not contain the visual

cue, and they were further broken down into three subcategories to determine whether ketamine treatment altered the ability to either shift from the previously learned strategy (perseverative errors) or to maintain the new strategy after perseveration had ceased (regressive or never-reinforced errors). In order to detect shifts in the strategies animals used, trials were separated into consecutive blocks of four trials each. A perseverative error occurred when a mouse made the same egocentric response as required during the Response Discrimination day, but which was opposite to the direction of the arm containing the visual cue. Six of every 12 consecutive trials required the mouse to respond in this manner (i.e. enter the arm opposite of the previously learned turn direction). A perseverative error was scored when the mouse entered the incorrect arm on three or more trials per block of four trials. Once the mouse made less than three perseverative errors in a block, all subsequent errors were now scored as regressive errors (because at this point, the mouse was following an alternative strategy at least half of the time). The third type of errors, termed "never reinforced errors", was scored when a mouse entered the incorrect arm on trials where the visual cue was placed on the same side that the mouse had been trained to enter on the previous day.

2.2.2. Latent inhibition task

In order to test whether developmental ketamine administration impaired latent inhibition in adulthood, we used the conditioned taste aversion (CTA) procedure, modified from [35]. Mice were singly housed in cage racks with no access to water valves. The experiment was performed over 10 days. The mice were water-restricted for a period of 17 h and 45 min every day, with fluids available for consumption during a 15 min testing period in the morning, and again for 6 h in the afternoon. Over a period of 4 days, animals were habituated to drinking out of two water spouts attached to graduated pipettes for 15 min following 12 h of water-restriction overnight. Following the testing phase, the normal water bottles were not immediately restored to the cages so that mice were not able to predict that free access to water would follow the end of the testing phase. This was done to encourage the mice to consume high amounts of fluids during the testing phase. Free access to water was made available again after another 5 h and 45 min. Following 4 days of habituation, mice were randomly assigned to one of three groups: (1) non-pre-exposed + saline injections (NPE-NaCl); (2) non-pre-exposed + LiCl injections (NPE-LiCl); (3) sucrose pre-exposed + LiCl injections (PE-LiCl). During 3 days of pre-exposure, the animals in the NPE-NaCl and NPE-LiCl groups continued to receive water in both pipettes, whereas the PE-LiCl group received 5% sucrose solution in both pipettes. On experiment day 8 (conditioning day), all three groups were given 5% sucrose solutions in both pipettes. After 15 min, the pipettes were removed and animals received an IP injection of either saline or 0.15 M LiCl before they were returned to their home cages. Testing was performed over 2 days, 24 and 48 h after injections, with the positions of the pipettes counterbalanced. On the test days, all the mice were given a choice between water and 5% sucrose and the amounts of each fluid consumed were measured. We averaged the data over the two test days and calculated for each animal the ratio of sucrose vs. water consumed as an index of conditioned taste aversion and thus a measure of learning.

2.2.3. Novel object recognition task

We used procedures modified from [36]. In brief, the test apparatus was a rectangular wooden box with one large exploratory chamber (40 cm × 40 cm × 22 cm) and one adjacent smaller test chamber (40 cm × 20 cm × 22 cm), accessible through a hole in the wall dividing the two chambers. Testing was completed in three stages: (1) animals were habituated to both test chambers for 10 min. (2) Immediately after habituation, animals were confined to the exploratory chamber and they were given 3 min to explore 4 distinct objects. (3) After a 2-min intertrial interval in the home cage, object recognition was assessed in the test chamber. One familiar object from the exploration trial and one novel object were fixed within the test chamber. The time animals spent investigating the objects in both the exploration chamber and the test chamber were video-recorded for offline analysis. Objects were sprayed with 70% ethanol to remove olfactory cues. Object position and novelty were counterbalanced between treatment groups. Investigation time for the objects in the exploration chamber and the test chamber was scored offline by two investigators who were blind to the experimental condition of the animals. Differences in exploration time for the familiar and the novel object within each treatment group were analyzed using Student's *t*-test.

2.2.4. Social interaction task

Experimental mice and two stimulus mice per group were housed individually in their home cages for at least 24 h prior to experimentation. On the day of testing, a stimulus mouse was placed inside a custom built cage (height 20 cm, steel bars separated by 1 cm, acrylic base and lid) and was then introduced into the experimental animal's home cage 4 consecutive times, with each exposure lasting 1 min. Between exposures, the stimulus mouse was returned to its home cage for 10 min. Ten minutes after the fourth exposure, a novel stimulus mouse was introduced into the experimental animal's home cage for 1 min. All sessions were video-recorded and interaction times (defined as sniffing and investigating at close proximity) were scored offline.

2.2.5. Elevated plus maze

Anxiety-related behavior was assessed using an elevated plus maze. The plus-shaped maze (constructed out of gray-painted wood) consisted of two opposing arms enclosed by 15 cm high walls, and two open arms (each 35 cm long and 5 cm wide), and was placed on 40 cm high legs. Each animal was placed at the center of the maze and activity was video recorded for offline analysis. After the animal explored the maze for 5 min, it was removed and returned to its home cage. The time spent by the animals in the open arms, time spent in the closed arms and the number of entries into the open arms were determined by an experimenter blind to the animal's treatment condition. We measured differences between groups with regard to time spent in the open and closed arms, respectively.

2.2.6. Amphetamine-induced hyperlocomotion

Locomotor and exploratory activity were assessed by placing mice in a 60 × 60 cm open field. Animals were allowed to explore the open field freely for a 30 min baseline period. They then received an i.p. injection of either 1.0, 2.5 or 5.0 mg/kg amphetamine (D-amphetamine hemisulfate; Sigma, St. Louis, MO) and were then returned to the open field for another 60 min. Both sessions were video-recorded and the distance traveled was analyzed using custom software written in Matlab (Rodent tracker, Vulintus, Plano, TX).

2.3. Immunohistochemistry

Animals were perfused transcardially with warm 0.9% saline followed by 4% paraformaldehyde in 0.12 M phosphate buffer (PB; 4 °C, pH 7.4). Brains were postfixed in PFA with 30% sucrose for 1 h and were then transferred to 30% sucrose in PB for approximately 18 h at 4 °C. Coronal slices (40 μm) were cut on a freezing microtome and collected in PBS containing 0.01% NaN_3 as a preservative. Free-floating sections were incubated in combinations of the primary antibodies diluted in 0.3% Triton-X in PBS for 36 h at 4 °C. We used the following primary antibodies: rabbit anti-PV (Swant,

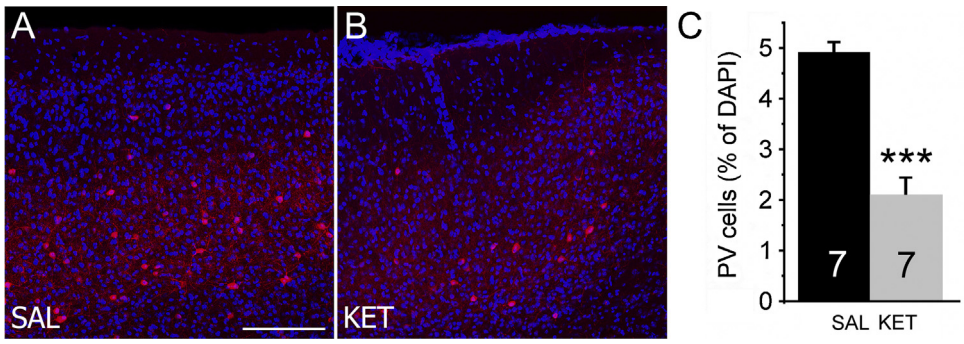

Fig. 1. Developmental ketamine treatment causes loss of parvalbumin (PV) immunoreactivity in adult animals. (A) Confocal images of slices from the medial prefrontal cortex stained for PV (red) and DAPI from saline-treated (A, SAL) and ketamine-treated (B, KET) mice. (C) Quantification of the reduction of PV cells among all cells stained with DAPI. Bars represent mean ± S.E.M., with number of animals in each condition displayed within the bars. Significance indicated as $^{***}p < 0.001$, Student's *t*-test. Scale bars are 250 μm. Number of animals in each group is indicated within the bars.

Switzerland, Cat# PV 25 RRID:AB_10000344; 1:2000 working dilution). Sections were washed at least 3 times for 10 min each in PBS before they were incubated in the secondary antibodies for 2 h at room temperature. We used DyLight 594 Goat Anti-rabbit (Jackson ImmunoResearch, West Grove, PA, Cat# 111-515-144; 1:1000 working dilution) in 0.3% Triton-X in PBS. Sections were again washed three times in PBS before they were mounted and coverslipped using Prolong Gold Antifade with DAPI (Invitrogen, Grand Island, NY). For each animal, a minimum of four sections including the prelimbic and infralimbic regions of the mPFC were imaged on a confocal microscope (Fluoview 1000, Olympus Corporation, Tokyo, Japan) at 20×. The acquired images were converted to TIFF format, and cell counts for PV and DAPI were performed on the 20× images using the thresholding function in Image J (NIH). For the analysis of ketamine-induced changes, the percentages of PV+ cells among all DAPI-stained cells were calculated.

2.4. Data analysis

Differences between the two treatment groups in all experiments were assessed using two-way ANOVAs, repeated-measures ANOVAs and Student's *t*-test as indicated. All data are represented as mean ± SEM, with $p < 0.05$ being considered statistically significant.

3. Results

3.1. Developmental ketamine treatment reduces PV expression in the mPFC of adult mice

A loss of PV expression in the subset of fast-spiking (FS) GABAergic interneurons is a hallmark of the NMDAR-hypofunction model [11,37,38] and several other rodent models of schizophrenia [39,40]. We performed immunohistochemical experiments in the mPFC of adult male mice to quantify the loss of PV expression that results from developmental ketamine (KET) treatment, relative to control littermates that received saline (SAL) injections in the second postnatal week. The number of PV+ interneurons in KET-treated animals was reduced when expressed as the percentage of PV+ cells among all DAPI stained cells (Fig. 1; SAL = 4.91 ± 0.2%, $n = 7$ animals; KET = 2.09 ± 0.3%, $n = 7$; $p < 0.001$).

3.2. Ketamine treatment during development impairs performance in an attentional set-shifting task

We used an attentional set-shifting paradigm to investigate impairments in mPFC function in adult animals that were treated

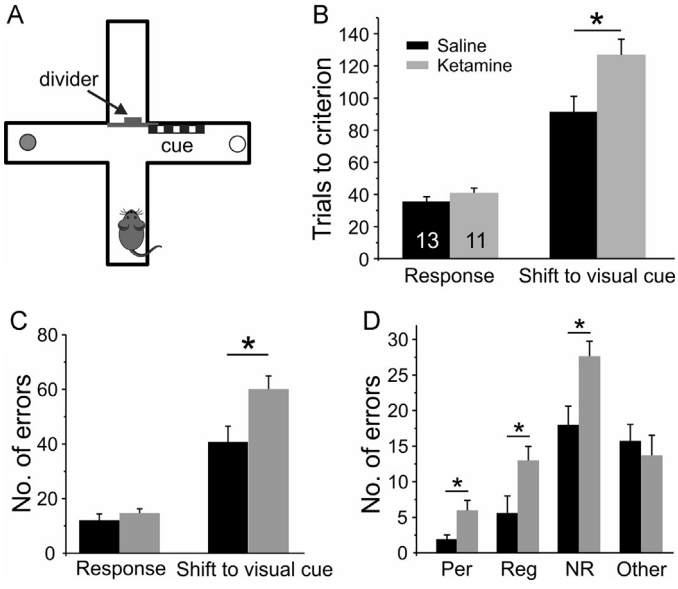

Fig. 2. Developmental ketamine-treatment impaired performance in an attentional set-shifting task. (A) Experimental setup—On Response day, animals were trained to follow an egocentric response strategy (i.e. to turn toward one arm) in a t-maze to obtain food reward. During Response day, animals had to ignore the location of a pseudorandomly placed visual cue. During the Shift-to-Visual Cue day, animals then had to shift their strategy and follow the location of the cue to get the food reward. (B) Ketamine-treated (KET) animals showed no impairment in acquiring the initial response strategy, taking the same number of trials to reach criterion on Response day as SAL-treated animals. On the Shift-to-Visual Cue day, the KET mice took significantly more trials to complete the task indicating treatment-induced persistent deficits in shifting to and maintaining a new strategy. (C) Ketamine mice committed the same number of errors as SAL-treated animals when learning the original response strategy during Response day, however on Shift-to-Visual Cue day KET-treated mice showed a significantly higher number of errors. (D) Errors were categorized as perseverative (Per), regressive (Reg) and never-reinforced (NR), and all other errors which occurred before perseveration began were categorized into a fourth "other" category (see Section 2 for details). The KET-treated mice showed a significantly high number of perseverative, never-reinforced and regressive errors, indicating that they had difficulty switching off a previously relevant but no-longer-relevant strategy, and in maintaining a newly learned strategy. Significance indicated as $^{*}p < 0.05$, Student's *t*-test. Number of animals in each group is indicated within the bars.

with ketamine during development. Saline- ($n = 13$) or ketamine- ($n = 11$) treated animals displayed no differences in the acquisition of the initial response strategy (Response day) when they were required to make an egocentric turning response (cf. Fig. 2A for setup). ANOVA performed with the treatment type as the factor and the number of trials as the dependent variable revealed

that the number of trials to reach criterion on Response day did not differ between the two groups (Fig. 2B, SAL = 34.31 ± 4.17; KET = 40.18 ± 3.53; $F(1,22) = 1.11$; $p = 0.304$), indicating that the two groups of animals performed comparably. ANOVA performed with treatment type as the factor and the number of errors as the dependent variable revealed that the total number of errors committed during the response day was also comparable between the two groups (SAL = 12.15 ± 2.45; KET = 14.64 ± 1.8; $F(1,22) = 0.625$; $p = 0.438$). On the next day (Shift to Visual-Cue Learning day), mice were trained to employ a new strategy that required them to attend to the location of the visual cue to obtain rewards. Ketamine-treated mice were significantly impaired in their ability to employ the new strategy to obtain food pellets (Fig. 2B). Analysis of the number of trials to reach the criterion on Shift to Visual-Cue Learning day revealed a significant main effect of treatment (SAL = 91.08 ± 10.45; KET = 127.09 ± 6.87; $F(1,22) = 7.64$, $p = 0.011$). The total number of errors committed during the shift to visual-cue day was significantly higher in the KET group (SAL = 40.77 ± 5.85; KET = 60.1 ± 4.1; $F(1,22) = 6.826$; $p = 0.016$). The number of probe trials to reach this criterion did not differ between groups (response day: SAL = 1.0 ± 0.0, KET = 1.09 ± 0.09, $p = 0.29$; set-shift day: SAL = 1.46 ± 0.22, KET = 1.18 ± 0.12, $p = 0.29$). A separate analysis conducted on the errors committed during the set-shift with treatment and error type as factors and the number of errors as the dependent variable revealed a significant main effect of treatment ($F(1,88) = 8.71$, $p = 0.004$), a significant effect of error type ($F(3,88) = 24.015$, $p < 0.0001$), but no significant treatment X error type interaction ($F(3,88) = 2.407$, $p = 0.073$). Post hoc comparisons showed that KET-treated mice committed significantly more "Perseverative" errors (SAL = 1.85 ± 0.79; KET = 6 ± 1.46; $p = 0.016$) and "Regressive" errors (SAL = 5.46 ± 2.62; KET = 12.91 ± 2.16; $p = 0.04$), as well as more "Never Reinforced" errors (SAL = 17.85 ± 2.81; KET = 27.54 ± 2.16; $p = 0.014$) compared to saline-treated animals.

3.3. Developmentally ketamine-treated mice show impairment in a latent inhibition task

Latent inhibition (LI) refers to delayed conditioning to a stimulus that has repeatedly been presented without reinforcement [41,42]. In order to determine whether our developmental ketamine treatment model leads to impaired LI in adulthood, we used a conditioned taste aversion (CTA) paradigm and measured changes in sucrose preferences as a consequence of previous exposure (Fig. 3). Analysis of the sucrose preference measured over 2 days following a single pairing with LiCl with treatment type and pre-exposure/non pre-exposure as factors and preference for sucrose as the dependent variable revealed a main effect of treatment ($F(1,48) = 5.81$; $p = 0.02$), a significant main effect of pre-exposure (PE/NPE) ($F(2,48) = 42.31$; $p < 0.0001$) and no treatment × group interaction ($F(2,48) = 1.6$; $p = 0.212$). Post hoc analysis revealed significant differences between the NPE-NaCl and the NPE-LiCl groups ($p < 0.0001$; LSD), but no differences between the NPE-NaCl and the PE-LiCl groups ($p = 0.133$; LSD), indicating that animals that received LiCl injections but were not pre-exposed to sucrose expressed strong taste aversion for sucrose during the test days. Comparisons using Student's *t*-test between the SAL and KET animals in each treatment group revealed that animals within the NPE-NaCl group (SAL = 81.23 ± 2.4%, $n = 9$; KET = 64.23 ± 7.66%, $n = 8$; $p = 0.063$), and those within the NPE-LiCl groups performed comparably (SAL = 16.87 ± 7.85%, $n = 8$; KET = 17.33 ± 5.49%, $n = 9$; $p = 0.96$). However, KET-treated animals in the PE-LiCl group preferred sucrose significantly less than their SAL-treated counterparts (SAL = 74.15 ± 5.32%, $n = 10$; KET = 53.12 ± 7.17%; $n = 10$; $p = 0.03$), indicating impaired latent inhibition in the KET-treated animals.

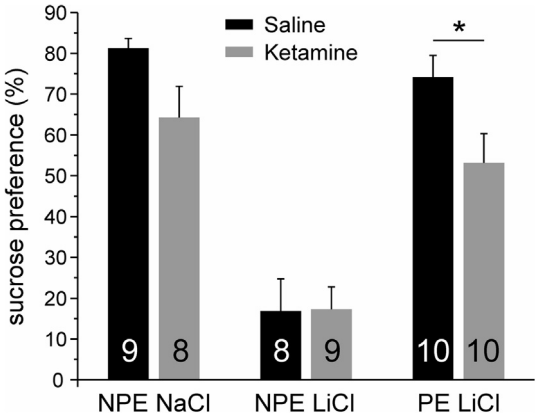

Fig. 3. Ketamine-treated (KET) mice showed impaired latent inhibition in a conditioned taste aversion task. Left: Both SAL or KET mice that were not pre-exposed to a 5% sucrose solution and which received a saline injection during conditioning day do not develop an aversion for sucrose during the test days. Middle: Both SAL and KET mice that were not pre-exposed to sucrose, but were given a 0.15 M LiCl injection after conditioning, express a strong aversion for sucrose during the test days. Right: KET mice pre-exposed to sucrose that received a 0.15 M LiCl injection during conditioning, show impaired latent inhibition when compared to SAL mice. KET mice prefer sucrose less than the SAL controls during the test days, indicating a reduced ability to ignore irrelevant stimuli. Significance indicated as $^*p < 0.05$, Student's *t*-test. Number of animals in each group is indicated within the bars.

3.4. Ketamine treatment impairs novel object recognition

We investigated the effect of developmental ketamine treatment on attention and spatial memory using a novel object recognition task (Fig. 4A). A *t*-test revealed that the SAL-treated mice spent less time investigating the familiar object and more time investigating the novel object (Fig. 4B, left; familiar object 10.43 ± 1.59 s, novel object 17.71 ± 2.78 s; $n = 13$; $p = 0.03$). In contrast, KET-mice spent about the same amount of time investigating both objects (Fig. 4B, right; familiar object 12.14 ± 2.21 s, novel object 15.95 ± 3.07 s; $n = 11$; $p = 0.33$). In order to rule out that the differences in novel object recognition between the SAL and KET groups reflect a generalized reduction of exploratory behavior, potentially resulting from increased levels of anxiety, we measured the time that the animals spent with the familiar object during the exploration phase. We found no differences in the time spent investigating the object in the exploration chamber between our treatment groups (Fig. 4C; SAL: 7.65 ± 1.05 s; KET: 7.43 ± 2.11 s; $p = 0.92$). Therefore, the relative increase in time spent with the familiar object in the KET group appears to indicate deficits in attention and object recognition memory, and not effects of generalized anxiety.

3.5. Ketamine treatment affects social interaction

Next, we tested whether developmental KET treatment leads to persistent social deficits in adulthood, which could serve as an indicator of negative-like symptoms of schizophrenia. Therefore, KET-treated mice ($n = 12$) and SAL-treated littermates ($n = 12$) were tested for their social-recognition abilities in a novel cage (Fig. 5A). The two treatment groups showed marked differences in the duration of their interaction (intense sniffing) with the stimulus mice. A repeated-measures ANOVA, with drug treatment as the between-subjects factor and stimulus mouse presentation as the within-subjects factor revealed a significant main effect of treatment ($F(1,22) = 8.463$, $p = 0.0081$) and a significant interaction between mouse presentation × treatment ($F(4,88) = 2.635$, $p = 0.039$) (Fig. 5B). Comparison of the interaction time between the two groups for each presentation of the first stimulus mouse revealed statistical significance for the first and second

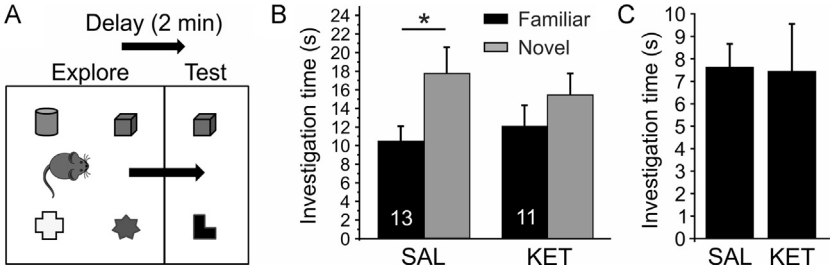

Fig. 4. Ketamine-treated (KET) mice showed deficits in attention to novel stimuli. (A) Novel object recognition task. Mice explored a chamber with 4 objects for 3 min. After a delay of 2 min in their home cages, the mice were introduced into a smaller testing chamber with one familiar object from the previous session and one novel object. (B) Saline-treated (SAL) mice spent significantly more time investigating the novel object, whereas the KET mice spent about equal amounts of time with both objects, indicating persistent attentional deficits and impaired memory of object-recognition. (C) SAL and KET mice both spent equal amounts of time investigating the object to be used as the "familiar object", indicating comparable baseline performance in the exploration chamber. Significance indicated as $^{*}p < 0.05$, Student's *t*-test. Number of animals in each group is indicated within the bars.

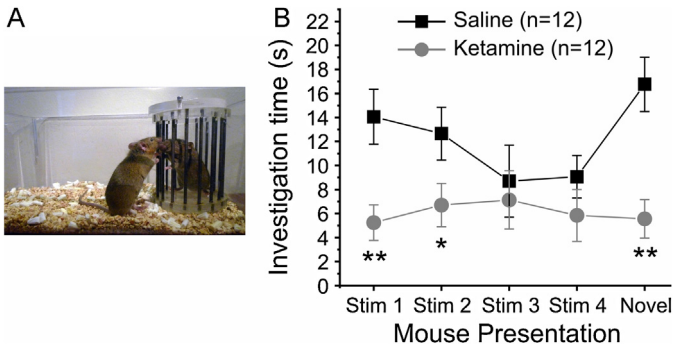

Fig. 5. Ketamine-treated (KET) mice showed impaired performance in a social investigation task. (A) Saline- and KET-treated mice were tested in a social investigation task in which a stimulus mouse was introduced into the test mouse's home cage during four 1-min sessions, each separated by 10 min intervals. During the fifth session, a novel animal was introduced into the test mouse's home cage. (B) The saline-treated animals spent progressively lesser amounts of time investigating the first stimulus mouse during the four sessions. When the novel mouse was introduced, the investigation time increased. The KET mice did not show such a trend, spending less time overall investigating the stimulus mice, indicating that the KET treatment induced deficits in attention to novel stimuli and social amnesia. Significance indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$, Student's *t*-test.

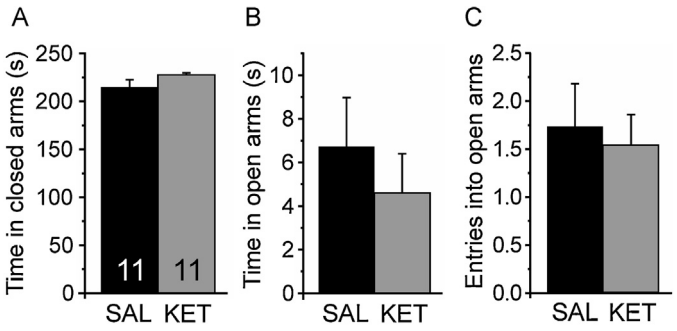

Fig. 6. Ketamine-treated (KET) mice did not show differences in levels of anxiety compared to controls (SAL) in an elevated plus maze. Saline- and KET-treated mice spent equal amounts of time in the open arms (A), equal amounts of time in the closed arms (B), and made comparable number of entries into the open arms (C). Number of animals in each group is indicated within the bars.

presentations (1st presentation SAL 14.06 ± 2.28 s; KET 5.24 ± 1.49 s; $p = 0.004$; 2nd presentation SAL 12.65 ± 2.21 s; KET 6.7 ± 1.81 s; $p = 0.049$). Thus, SAL-treated control mice demonstrated social investigation toward the stimulus male during the first 1-min presentation, but spent progressively less time investigating the same mouse in subsequent presentations, reflecting an intact social short-term memory. In contrast, KET animals did not show this type of social recognition with repeated exposures and overall spent less time interacting with the stimulus mouse. During the last trial, subjects were presented with a novel stimulus male and SAL controls again demonstrated social interaction, suggesting normal social recognition and intact social short-term memory. In contrast, KET mice showed little interaction with the novel stimulus mouse (SAL 16.75 ± 2.25 s; KET 5.56 ± 1.6 s; $p = 0.0005$) which may indicate attentional deficits to novel stimuli and social amnesia.

3.6. Developmental ketamine treatment does not induce anxiety-like behaviors in mice

Because we observed deficits in both social investigation and novel object recognition memory, we next tested behavior on the elevated plus maze task in order to control for whether these deficits were due to altered cognitive processing or reflected increased generalized anxiety. Eleven saline- and 11 ketamine-treated mice were used in the task. The animals were placed in the center of the maze and the time that each animal spent in the open and the closed arms, respectively, during 5 min sessions were measured. Both groups of animals spent more time in the closed arms (Fig. 6A) than in the open arms (Fig. 6B) ($F(1,40) = 1619.55$; $p < 0.001$). There was no group × arm interaction ($F(1,40) = 1.38$; $p = 0.25$), meaning that both treatment groups spent similar amounts of time in the closed and open arms, respectively (open arm SAL = 6.69 ± 2.27 s; KET = 4.59 ± 1.19 s; closed arm SAL = 217 ± 9.25 s; KET = 227.55 ± 4.86 s). Both groups of animals entered the open arms equal number of times (Fig. 6C) (SAL = 1.73 ± 0.45; KET = 1.55 ± 0.31; $p > 0.05$, *t*-test). Thus, unlike developmental PCP treatment, ketamine administration during the second postnatal week does not induce anxiety-like behaviors in adulthood.

3.7. Developmental ketamine treatment does not alter amphetamine-induced locomotor activity

Next, we tested whether developmental KET treatment alters the locomotor activity in response to amphetamine administration in an open field task (Fig. 7). Mice were placed in a 60 × 60 cm open field and allowed to explore for 30 min and the distance traveled was recorded. After this time, animals received i.p. injections of either 1 mg/kg (SAL = 7; KET = 8), 2.5 mg/kg (SAL = 11; KET = 12) or 5.0 mg/kg (SAL = 9; KET = 10) amphetamine. Ten minutes later, they were placed back into the open field and activity was again recorded for an additional 60 min. We first performed an ANOVA with treatment type as the factor and the total distance traveled as the dependent variable. Baseline activity across all treatment groups did not differ significantly (Fig. 7A) (distance traveled: 1 mg/kg SAL $14,757 \pm 1294$ cm; KET $13,603 \pm 959$ cm,

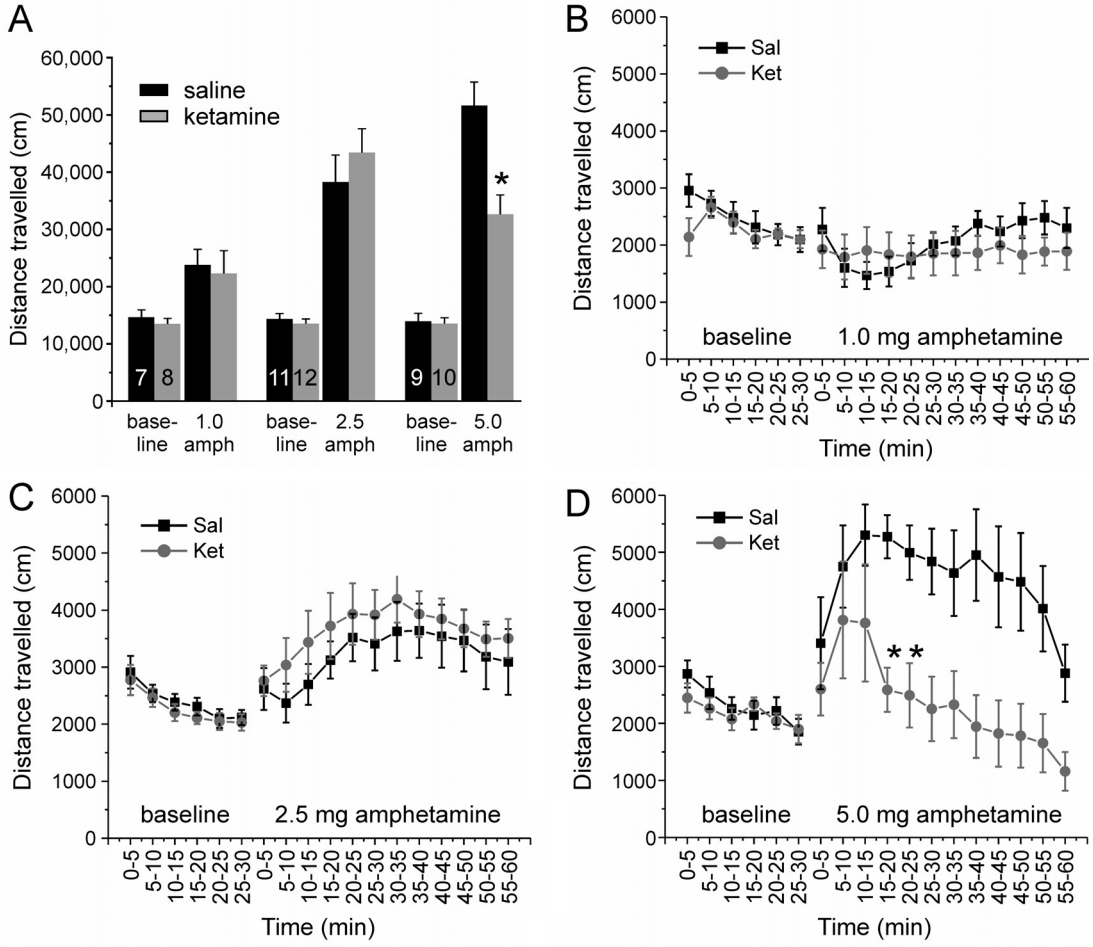

Fig. 7. Ketamine-treated mice showed no relative increase in amphetamine-induced hyperlocomotion in an open field. (A) During baseline conditions, mice from both treatment groups (SAL and KET) traveled approximately the same distance while exploring the chamber. Amphetamine injections induced hyperlocomotion over baseline levels at all concentrations tested (1.0, 2.5 and 5.0 mg/kg). At 1.0 and 2.5 mg/kg, respectively, the relative increase in locomotor activity was comparable between SAL and KET mice. However, at the high dose of amphetamine (5 mg/kg), the increase in locomotor activity in the KET group was significantly lower than in the SAL-treated animals. (B) Both SAL- and KET-treated mice do not show enhancement in locomotor activity at the dose of 1 mg/kg. (C) At the amphetamine dose of 2.5 mg/kg, both groups of mice show similar levels of enhancement in locomotor activity after the amphetamine injection. At 5 mg/kg amphetamine, the SAL-treated animals show a persistent increase in locomotor activity. In the KET group, an initial increase in locomotor activity was followed by a decrease in locomotor activity to baseline levels. (D) Significance indicated as $^*p < 0.05$ in (A) and $p < 0.002$ after Bonferroni correction in (B–D). Number of animals in each group is indicated within the bars in (A).

$p = 0.5$; 2.5 mg/kg SAL 14,355 ± 955 cm; KET 13,545 ± 795 cm, $p = 0.37$; 5.0 mg/kg SAL 13,953 ± 1373; KET 13,514 ± 1010 cm, $p = 0.59$). Amphetamine at 1 mg/kg increased locomotion in both SAL and KET groups (post-injection distance traveled: SAL 23,938 ± 2713 cm; KET 22,425 ± 3969 cm), but the treatment groups did not differ in the magnitude of the effect ($F(1,13) = 0.093$, $p = 0.78$). Amphetamine at 2.5 mg/kg also increased locomotion in both SAL and KET groups (post-injection distance traveled: SAL 38,283 ± 4713 cm; KET 43,440 ± 4152 cm), but the treatment groups did not differ in the magnitude of the effect ($F(1,21) = 0.023$, $p = 0.88$). Similarly, amphetamine at 5 mg/kg increased locomotion in both SAL and KET groups (post-injection distance traveled: SAL 51,679 ± 4118 cm; KET 32,728 ± 3350 cm), however the increase in the KET group was significantly lower than in the controls ($F(1,17) = 17.92$, $p < 0.001$). To analyze potential differences in movement patterns that could contribute to this effect, we binned the 30 min baseline and 60 min post-injection periods into 5 min intervals and performed a repeated-measures ANOVA with treatment group and amphetamine dose as the between-subjects factors, and distance traveled during each of the 5 min bins as the within-subjects factor (Fig. 7). The within-subjects comparison revealed a main effect of time ($F(17,867) = 6.168$; $p < 0.001$), a significant time × amphetamine dose interaction ($F(34,867) = 5.15$; $p < 0.001$), and a time × treatment × amphetamine dose interaction ($F(34,867) = 2.28$; $p < 0.001$). As can be seen in Fig. 7, these differences were mostly due to differences in the responses of the groups in response to the 5 mg/kg amphetamine dose. A repeated-measures ANOVA with treatment group as the between-subjects factors and distance traveled during the 5 min bins as the within-subjects factor revealed a main effect of time ($F(17,289) = 4.959$; $p < 0.0001$) and a significant treatment group × time interaction ($F(17,289) = 2.547$; $p < 0.001$), indicating a difference in the locomotion patterns between the SAL and KET groups after 5 mg/kg amphetamine injection. Both treatment groups showed an initial increase in locomotor activity, which in the SAL group persisted throughout the duration of our observation, but which in the KET group was quickly followed by a reduction in ambulatory activity (Fig. 7D).

4. Discussion

Much of the earlier support for the NMDAR hypofunction theory was based on the effects of acute or subchronic administration of NMDAR antagonists in adult animals and humans [1,21,43]. However, the effects of acute NMDAR antagonism [44–46] often differ

dramatically from those obtained with repeated administration [21,47,48]. Moreover, schizophrenia is a neurodevelopmental disorder and therefore animal models that trigger changes during development have increased construct validity. In this study, we used the non-competitive NMDAR antagonist ketamine to transiently disrupt NMDAR function during the second postnatal week and we characterized persistent changes in behavior, as well as changes in PV expression in the mPFC of adult mice. Our developmental ketamine treatment led to a reduction in the number of PV-expressing cells in the mPFC and changes in a battery of tests aimed to study cognitive functions, as well as correlates of negative and positive symptoms measured in animal models of schizophrenia.

The first two postnatal weeks in rodents correspond to the second trimester of pregnancy in humans, in which transient epigenetic factors or exposure to environmental insults increase the probability of developing schizophrenia as an adult [49,50]. NMDAR blockade or subunit deletion early in development when NMDARs are hypersensitive [51] and FS interneurons express high levels of NMDARs [52,53] can reduce PV expression and may persistently affect behavior into adulthood by disrupting the maturation of FS connectivity [26,27,54]. A loss of PV expression in the mPFC has previously been shown following neonatal ketamine administration [32], and reductions in the number of PV-expressing cells were also seen in the PFC and hippocampus following treatment with other NMDAR antagonists such as PCP and MK-801 [30,55]. Here we replicated the findings from these studies, showing loss of PV expression in the mPFC of mice using subchronic ketamine administration on PND 7, 9 and 11. This loss mimics changes in subsets of PV interneurons observed in postmortem brains of schizophrenic subjects [12–14,56]. The reductions in GAD67 and PV levels that result from subchronic NMDAR blockade are believed to represent maladaptive homeostatic mechanisms in a faulty effort to maintain a balance of excitation and inhibition in the cortical network [37,57]. The synchronous activity of PV cells generates gamma oscillations, which are correlated with performance in a variety of cognitive tasks, including the allocation of attention and working memory. Thus, the loss of PV expression in the prefrontal cortex may underlie the cognitive disturbances associated with schizophrenia [58–60]. Cognitive impairments are the major determinants of long-term social and occupational outcome in schizophrenia [61]. Deficits in executive function have previously been observed in variants of the attentional set shifting task following perinatal PCP or MK-801 treatment [62,63]. Accordingly, our perinatal ketamine treatment produced several enduring behavioral changes that correlate with the symptom dimensions of schizophrenia. Individuals with schizophrenia or with damage to the PFC display marked impairments in tasks used to assess cognitive flexibility [17,64]. Administration of ketamine to adult animals induces attentional deficits [65–68]. Consistent with these earlier reports, we found that perinatally KET-treated animals were not impaired in the acquisition of the original attentional set which required them to use an egocentric turning strategy, but had difficulties performing an extradimensional shift in order to follow a novel strategy based on visual-cue discrimination. Deficits in extradimensional shifts of attention are indicative of changes in medial PFC (i.e. prelimbic and infralimbic cortex) functions in rodents [69,70], or impaired PFC function in primates and humans [71,72]. Specifically, we found that the number of perseverative, never-reinforced and regressive errors was significantly higher in the KET-treated animals. The tendency for perseverative and never-reinforced errors may represent an effect of ketamine's interaction with dopamine D1 and D2 receptors. In a set-shifting task, the PFC engages in multiple strategies, and blockade of dopamine D2Rs disrupts the PFC's ability to disengage from the previous strategy, leading to an increase in perseverative errors [70]. Activation of dopamine D1 receptors stabilizes the newly relevant strategy, and blocking these receptors can then affect the maintenance of the new strategy [73]. Evidence suggests that a D1R/NMDAR interaction in the PFC may be responsible for amelioration of ketamine-induced cognitive deficits by D1R activation [74]. The difficulties in achieving the extradimensional shift that we observed here appear to be unrelated to a potential disinhibition of impulsive behaviors that could be brought on by an anxiolytic effect of ketamine [75–78] as we did not observe differences between our treatment groups in measures of anxiety on the elevated plus maze.

Latent inhibition is another cognitive phenomenon that is related both to learning, and, possibly, attention. In LI, the un-reinforced pre-exposure to a future conditioned stimulus (CS) delays subsequent conditioning of that CS with an unconditioned stimulus (US) [79]. Thus, during pre-exposure, the to-be-conditioned stimulus is irrelevant, and therefore the delayed acquisition of the association between the conditioned stimulus and the unconditioned stimulus reflects the process of overcoming the "learned irrelevance". Consequently, LI is believed to be an ability to ignore irrelevant stimuli [80]. Schizophrenic patients have been reported to display diminished latent inhibition, and this contributes to impaired information processing and an inability to ignore irrelevant stimuli [81,82,41]. Our data show that KET mice that were pre-exposed to sucrose had a diminished capacity to ignore the adverse effects of a LiCl injection during conditioning day, resulting in reduced sucrose preference during the test days. LI is related to learning, because of its effects on associating the CS with the US. The phenomenon can also be attributed to a change in attention [83], or associative learning [84].

Schizophrenic patients also show impairments in declarative memory which are the result of abnormal PFC and hippocampal function [18,85–87]. The novel object recognition task provides a means to evaluate this type of memory in rodents and to evaluate the effects of pharmacological manipulations or changes in cortical function on task performance [88,89]. The task is based on the animals' innate tendency to explore novel objects, which in turn relies on recognition memory [90]. The mPFC plays a role in information retrieval from short-term memory, and together with the perirhinal cortex forms a neural circuit involved in recognition memory [91,92]. Thus, if animals fail to follow their innate tendencies to spend relatively more time with the novel object this is typically considered a sign of impaired recognition memory and mPFC dysfunction. Compared to animals in the SAL-treated group KET animals spent significantly less time exploring the novel object, indicating that they were unable to recognize the object as novel. Our findings are consistent with previous studies that reported impaired attention and recognition memory in adult animals following subchronic administration of ketamine, PCP or MK-801, respectively [23,90,93,94], or following developmental NMDAR blockade using PCP [95].

Deficits in social interaction or social anxiety are common negative symptoms observed in schizophrenic subjects and they have been associated with perturbations in frontocortico-temporal networks [20,96]. Both pharmacological blockade of NMDARs and genetic deletion of NR1 subunits have previously been shown to result in deficits in social interaction, which in animals mimic aspects of the negative symptoms of schizophrenia [27,30,36]. Administration of ketamine or PCP in adult animals [23,97–99], as well as perinatal PCP treatment [100–102] produce impairments in novelty discrimination in a social recognition task. In our study ketamine-treated animals overall showed significantly less interaction with the stimulus mouse and also showed no signs of novelty discrimination when the novel stimulus mouse was introduced in the home cage. Perinatal KET treatment therefore also induces persistent changes in behavior that mimic important aspects of the negative symptoms of schizophrenia.

Differences in social interaction and novelty detection may be a sign of generalized anxiety [89]. Administration of phencyclidine during the second postnatal week has been shown to induce anxiety-like behaviors in male mice [103]. In order to rule this out, we tested the behavior of SAL- and KET-treated animals on the elevated plus maze. The behavior of KET animals did not differ from their littermate SAL controls. Both groups of animals spent similar amounts of time in the open and closed arms and they did not differ in the number of entries made into the open arms. Baseline locomotor activity (before amphetamine administration) was also similar between the groups in a novel environment (open field experiment), in all three groups of animals tested. Thus, this provides strong evidence that the deficits observed in the attentional set-shifting task, the novel object recognition task and the social interaction task are not an unspecific side effect of generalized anxiety in KET-treated animals.

Selective increases in the psychomotor response to an acute amphetamine challenge are widely considered an animal model for dysfunctions in the dopaminergic system and positive symptoms in schizophrenia [104,105]. Somewhat surprisingly, we observed no relative increases in locomotor activity in KET-treated animals in response to an acute amphetamine challenge across a range of amphetamine doses tested. Hyperlocomotion is regarded to mimic dysfunctions in the dopaminergic system, thus serving as an animal model of the psychotic (positive) symptoms in schizophrenia [104]. In NMDAR antagonism models of schizophrenia, a challenge dose of amphetamine or the NMDAR antagonist itself (PCP or ketamine) is used to induce enhanced dopaminergic activity and thus hyperlocomotion [106–108]. It has been speculated that the loss of GABAergic neurons in the PFC and hippocampus may lead to desynchronization and functional reduction of cortical outputs that regulate the dopaminergic system [109,110]. Accordingly, some animal models of schizophrenia that induce dysfunction in the GABAergic neurons in the PFC and hippocampus also show amphetamine- or novelty-induced hyperlocomotion [40,104,107], but not others [27]. The lack of amphetamine-induced psychomotor effects that we describe here versus those previously shown with developmental MK-801 treatment [107], suggests either crucial differences in the mechanisms of action between MK-801 and ketamine, or that ketamine blockade of NMDARs on corticolimbic GABAergic neurons during early postnatal development may permanently induce a brain state akin to that of amphetamine-sensitized mice, thereby resulting in the occlusion of amphetamine-induced hyperlocomotion (cf. [27]). Enhanced activation of the dopamine system can result in a short increase in locomotion that is followed by a decrease in ambulatory activity due to more stereotyped behavior, such as head and limb movements [111]. At the highest amphetamine dose of 5 mg/kg in the KET animals, we found a similar reduction in overall movement; however, our video analysis did not allow us to measure stereotypical behaviors in our animals and therefore we cannot conclude that the decrease in locomotor activity is due to a shift in movement toward stereotypy.

5. Conclusion

Here we provide the first comprehensive characterization of a range of persistent schizophrenia-like symptoms in adult mice following ketamine administration during development. Impairments in PFC function appear to be central to the deficits in executive functions seen in schizophrenic patients, which may result from a loss of PV-expressing interneurons. Although the commonly used NMDAR antagonists MK-801, PCP and ketamine have different pharmacological profiles, they all induce a similar range of schizophrenia-like behavioral deficits in animals. Furthermore, ketamine causes similar deficits regardless of whether it is administered subchronically in adult animals, or during the developmental period. Our results thus add to the growing body of evidence showing NMDAR antagonists' usefulness in studying schizophrenia pathology. However, the neurophysiological mechanisms through which ketamine and other NMDAR antagonists induce these symptoms at various points during development remain largely untested.

Conflict of interest

The authors declare no competing financial interests.

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