

Chronic ketamine impairs fear conditioning and produces long-lasting reductions in auditory evoked potentials

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ABSTRACT

Ketamine is an NMDA receptor antagonist with a variety of uses, ranging from recreational drug to pediatric anesthetic and chronic pain reliever. Despite its value in the clinical setting, little is known about the immediate and long-lasting effects of repeated ketamine treatment. We assessed the effects of chronic administration of a subanesthetic dose of ketamine on contextual fear conditioning, detection of pitch deviants and auditory gating. After four, but not two, weeks of daily ketamine injections, mice exhibited decreased freezing in the fear conditioning paradigm. Gating of the P80 component of auditory evoked potentials was also significantly altered by treatment condition, as ketamine caused a significant decrease in S1 amplitude. Additionally, P20 latency was significantly increased as a result of ketamine treatment. Though no interactions were found involving test week, stimulus and treatment condition, these results suggest that repeated ketamine administration impairs fear memory and has lasting effects on encoding of sensory stimuli.

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Introduction

The *N*-methyl-D-aspartate (NMDA) receptor antagonist ketamine is a pediatric and veterinary anesthetic whose recreational use in the United States has escalated dramatically since the 1990s (DAWN, 2003). Though ketamine-naïve, non-drug abusers enjoy the effects of the drug at a low dose (Morgan et al., 2004a,b), ketamine has repeatedly been shown to elicit and exacerbate psychotic symptoms (Krystal et al., 1994; Malhotra et al., 1997; Newcomer et al., 1999; Morgan et al., 2004a,b). These symptoms, which include auditory hallucinations, blunted affect and withdrawal, are all common to patients with schizophrenia. Therefore, studies verifying the psychotic effects of ketamine not only lend support to the NMDA hypofunction model of schizophrenia but also confirm its ability to replicate behavioral analogues of the disease in non-human studies (Lindsley et al., 2006; Javitt, 2007).

The induction of schizophrenia-related behaviors upon treatment with acute ketamine has been demonstrated in a wide range of rodent studies. In such animals acute ketamine has been shown to

disrupt fear conditioning, mismatch negativity (MMN) and auditory gating, which all serve as endophenotypes for schizophrenia (Brown et al., 2000; Javitt, 2000; Michie, 2001; Winterer et al., 2001; Ong et al., 2005; Maxwell et al., 2006; Pietersen et al., 2007; Ehrlichman et al., 2008). Episodic memory impairments are the most severe cognitive dysfunctions associated with schizophrenia (Heinrichs and Zakzanis, 1998). For this reason, the fear conditioning paradigm, in which an initially neutral (conditioned) stimulus is paired with an aversive (unconditioned) stimulus, is often used to assess memory in animal models of the disease.

In the MMN paradigm, a set of repeated stimuli is followed by a deviant, such as a tone of differing frequency. People with schizophrenia have a decreased ability to detect auditory deviants as measured by changes to the N100 and mismatch negativity (MMN) of their auditory evoked potentials (AEPs) (Javitt, 2000; Michie, 2001; Davalos et al., 2003). The N100 is the negative-most deflection in the human AEP, a modality of event-related potentials (ERPs) occurring approximately 100 milliseconds (ms) post-stimulus whose mouse analog is termed the N40 (Yingling and Nethercut, 1983; Covington and Polich, 1996; Shelley et al., 1999). The MMN generally occurs immediately following the N100 and is manifest as an amplified negative deflection to deviant stimuli (May et al., 1999; Haenschel et al., 2005). Gating refers to a reduction in the second of two successive stimuli. Patients with schizophrenia also exhibit impaired

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auditory gating of the N100, P50 and P200 AEP components (Shenton et al., 1989; Brockhaus-Dumke et al., 2008). The latter two components are positive midlatency ERP constituents occurring 50 and 200 ms post-stimulus whose mouse analogs are the P20 and P80, respectively. The method for assessing auditory gating is very similar in both humans and in mice, making this paradigm a highly translatable measure of schizophrenia-like deficits.

Despite its usage as an anesthetic agent, recreational drug and more recently as a treatment for chronic pain and depression, relatively few studies have addressed the effects of repeated ketamine administration (Berman et al., 2000; Zarate et al., 2006; Okon, 2007; Reuben and Buvarandran, 2007; Cvrcek, 2008; Sinner and Graf, 2008). However, several clinical studies have found that chronic ketamine administration for the treatment of pain has a variety of central nervous system side effects (Kannan et al., 2002; Cvrcek, 2008). In the present study, we use the three different paradigms discussed above in order to assess the immediate and lasting effects of chronic ketamine treatment in mice. A wealth of studies have demonstrated that repeated exposure to subanesthetic doses of ketamine leads to apoptosis, as well as lasting changes in brain physiology, suggesting the presence of irreversible changes in the brain (Scallet et al., 2004; Young et al., 2005; Slikker et al., 2007; Majewski-Tiedeken et al., 2008; Zou et al., 2009). We therefore hypothesized that chronic ketamine would impair contextual fear conditioning, novelty detection and sensory gating at all time points after cessation of treatment by such irreversible changes, rather than acute NMDA receptor (NMDAR) blockade.

Materials and methods

The overall study design is depicted in Fig. 1.

Animals

Twenty-two male C57BL/6J (B6) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) at 5 to 6 weeks of age. Mice were acclimated to the housing facility for approximately one week prior to the commencement of injections. Four test sessions were conducted on mice between the following age ranges: 8 to 9 weeks; 10 to 11 weeks; 13 to 14 weeks; and 15 to 16 weeks. Mice were housed four or five per cage until electrode implantation, after which each mouse was housed singly. One ketamine-treated mouse died between contextual fear conditioning experiments, five saline- and one additional ketamine-treated mouse died before EEG recording began, and 1 ketamine-treated mouse died between EEG sessions. Water and standard rodent chow were available *ad libitum*. All protocols were conducted in accordance with University Laboratory Animal Resources (ULAR) and Institutional Laboratory Animal Care and Use Committee (IACUC) guidelines.

Injections

Following acclimation, mice received a daily intraperitoneal (i.p.) injection of either 0.09% saline ($n=11$) or 5.0 mg/kg ketamine ($n=11$) for fourteen days. Mice received no injections for approxi-

mately 48 h prior to behavioral testing in the contextual fear conditioning paradigm. Forty-eight hours were allowed before test sessions in order to minimize the likelihood that drug was bound to NMDARs during testing. After completion of the first fear conditioning session, mice again received a daily i.p. injection of saline ($n=11$) or ketamine ($n=10$) for an additional fourteen days. Mice received the same treatment of either saline or ketamine during all twenty-eight days of injections. Drug dosage was chosen based on previous studies indicating that repeated exposure of mice to 5 mg/kg ketamine both induces apoptosis and causes significant auditory gating deficits (Maxwell et al., 2006; Majewski-Tiedeken et al., 2008).

Contextual fear conditioning paradigm

In order to minimize the acute effects of ketamine, mice underwent training for contextual fear conditioning 48 h after the last injections on day 14. In training, a 2-s, 1.5-mA scrambled footshock was delivered 2 and 2.5 min after being placed in the chamber. Baseline freezing was measured during the first 2 min (pre-shock), while the immediate, unconditioned freezing response was recorded for 1 min post-shock. Freezing, defined as the absence of visible movement except for respiration, was recorded once every 5 s (Graves et al., 2003). Twenty-four hours after training, mice were placed back into the same chamber (conditioned context) in the absence of footshock. Freezing was recorded for 5 min, for a total of 60 observations. Mice were then returned to their home cages and received fourteen more days of daily injections. Forty-eight hours after the last injection, freezing was measured in the conditioned context for another 5 min. It is important to note that prior to the second testing session, animals did not undergo another training session, thus increasing the difficulty of the task and isolating memory retrieval.

Surgery

Approximately one week after the last injection, animals underwent stereotaxic implantation of electrodes (Plastic Products, Roanoke, VA, USA) for the recording of auditory evoked potentials. Animals were anesthetized with isoflurane prior to and during surgery. Unipolar recording electrodes were placed unilaterally in the right CA3 region of the hippocampus (1.4 mm posterior; 2.65 mm lateral and 2.75 mm deep relative to bregma) and referenced to the ipsilateral frontal sinus. The electrode pedestal was secured to the skull with ethyl cyanoacrylate (Loctite, Henkel KGaA, Duesseldorf, Germany) and dental cement (Ortho Jet, Lang Dental, Wheeling IL, USA). All surgical procedures were consistent with previously published methodologies (Connolly et al., 2003, 2004; Siegel et al., 2003, 2005; Maxwell et al., 2004a,b; Metzger et al., 2007).

Novelty AEPs

Testing in the novelty paradigm occurred three weeks after the last injections of either saline ($n=6$) or ketamine ($n=9$). EEG recordings were conducted in the home cage environment, which was placed in a Faraday cage in order to eliminate signal contamination. Auditory

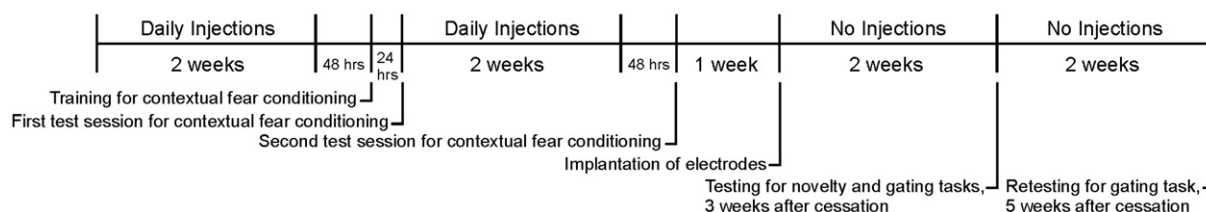


Fig. 1. Schedule of injections and experimental testing.

stimuli were generated by Micro1401 hardware and Spike2, version 6.0 software (Cambridge Electronic Design, Cambridge, UK) and were delivered through speakers attached to the cage top. The stimulus protocol consisted of 24 standard tones (7 kHz) followed by one novel tone that was at a randomly-selected frequency between 5 and 9 kHz in increments of 100 Hz (excluding 7 kHz). Tones were presented at 85 dB compared to a 70-dB white-noise background. These tones were sinusoidal, 50 ms in duration and separated by a 500-ms interstimulus interval. The series of 25 tones was repeated forty times so that half of the deviant tones were of higher and half of lower frequency than the standard tone. Each deviant tone was used only once. Individual waveforms were sampled at 1667 Hz, filtered between 1 and 500 Hz and rejected for movement artifact based on the criterion of two times the root mean squared amplitude per mouse. For each mouse an average standard and novel tone waveform was created, each of which was baseline corrected to 0 μ V. The standard tone waveform consisted of the average response to the 24th standard tone in the series to keep the number of trials constant between novel and standard conditions. These conditions were chosen according to previous results from our lab indicating that MMN and the N1 are sensitive to ketamine treatment under such parameters (Ehrlichman et al., 2008).

Gating AEPs

Testing in the gating paradigm took place both three and five weeks (saline $n = 6$, ketamine $n = 8$) after the last injections. As in the novelty paradigm, recordings were conducted in the home cage environment, which was placed in a Faraday cage. Auditory stimuli were generated by Micro1401 hardware and Spike2, version 6.0 software delivered through speakers on the cage top. The stimulus protocol in the gating paradigm consisted of fifty paired white-noise bursts (10-ms duration, 500-ms intra-pair interval) with a 9-s inter-pair interval presented at 85 dB compared to a 70-dB white-noise background. Each 10-ms white-noise burst was composed of fifty randomly-selected 0.2-ms square waves. Again, individual waveforms were sampled at 1667 Hz, filtered between 1 and 500 Hz and rejected for movement artifact based on the criterion of two times the root mean squared amplitude per mouse. Average waveforms for individual mice were also baseline corrected to 0 μ V. Grand average waves were then produced from 0 to 200 ms following stimulus presentation.

Contextual fear conditioning analyses

The freezing behavior of each subject was measured by calculating the fraction of time that the animal exhibited freezing (ratio of time freezing divided by total observation time). The freezing ratio for one ketamine-treated mouse fell outside two standard deviations of the mean and therefore its data was excluded from analyses. Freezing ratios were analyzed using separate general linear model one-way ANOVAs after two and four weeks of ketamine treatment.

Novelty analyses

Average AEPs were divided into 25-ms bins from 0 to 200 ms for a total of eight sequential epochs as previously described (Umbricht et al., 2005; Ehrlichman et al., 2008). We calculated the difference waveform by subtracting the average AEP in response to standard tones from that of the frequency deviants (Difference = Deviant – Standard). The area under the difference curve was then computed for each epoch. According to this methodology, a value of zero for the difference wave indicates that there was not a deviance-elicited change during a given epoch. A t test was therefore used to compare the difference waves for each treatment condition against a value of zero. We also performed general linear model repeated measures ANOVAs (rmANOVAs) for each epoch in order to determine whether

ketamine disrupted any potential deviance-elicited changes in the AEP. Our hypothesis, based on previously published data using acute ketamine, was that chronic ketamine would attenuate the deviance-elicited AEP response immediately following the N40 component, between 50 and 75 ms (Ehrlichman et al., 2008).

Gating analyses

The amplitudes of the P20 and P80 components of the AEP waveform were chosen by determining the maximum positive, post-stimulus deflection between 10 and 30 ms and 60 and 200 ms, respectively. The amplitude of the N40 component was chosen by determining the maximum negative deflection between 20 and 70 ms post-stimulus. The latency of each of these components was defined as the time post-stimulus at which its maximum deflection occurred. These parameters were chosen based on a previous study published by our group on the immediate and last effects of chronic ketamine treatment on mouse AEPs (Maxwell et al., 2006). Such temporal limits enabled us to identify drug-induced latency shifts. The AEP of one saline-treated mouse was rejected after the second gating recording session due to artifact. Individual rmANOVAs were performed on baseline corrected data for the amplitude of each AEP component in order to identify possible interactions between treatment condition, stimulus and test week. rmANOVAs were also performed on the latencies of each component in response to stimulus 1 (S1) in order to identify potential treatment differences at or between test weeks. Significant interactions were subsequently submitted to Fisher's LSD post hoc analysis. All data analyses were conducted using Statistica 6.1 (Statsoft, Inc., Tulsa, OK).

Results

Contextual fear conditioning

As expected, one-way ANOVAs on pre- and post-shock data gathered during training did not reveal any significant differences between the two treatment groups. There was also no difference between the two groups during the first test session (24 h after training), suggesting that two weeks of daily ketamine treatment did not impair acquisition or initial retrieval of CFC. However, during the second testing session, ketamine-treated mice displayed significantly reduced freezing behavior as compared with saline-treated controls (Fig. 2) [$F(1, 17) = 12.01$, $p = 0.003$]. These data indicate that retrieval of CFC was impaired after a longer interval (17 days as compared to one) between training and testing.

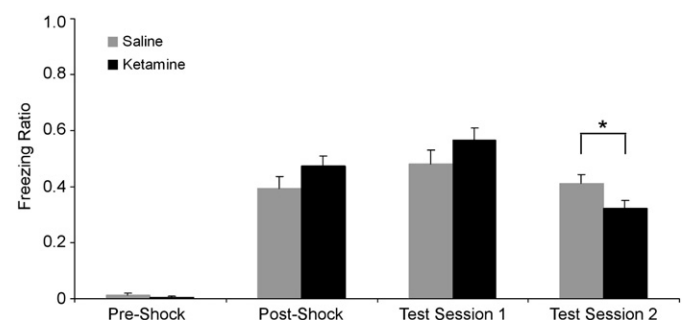


Fig. 2. Freezing ratios of mice chronically treated with either saline (gray) or 5.0 mg/kg ketamine (black) in contextual fear conditioning. Pre- and post-shock data represent baseline and immediate, unconditioned responses before and after footshock, respectively. Freezing behavior in the conditioned context during the first test session (following 2 weeks of ketamine) and second test session (17 days after training) are also shown. Error bars represent standard error of the mean (\pm SEM). Ketamine resulted in a significant impairment of contextual fear conditioning during the second test session (asterisk) but not the first.

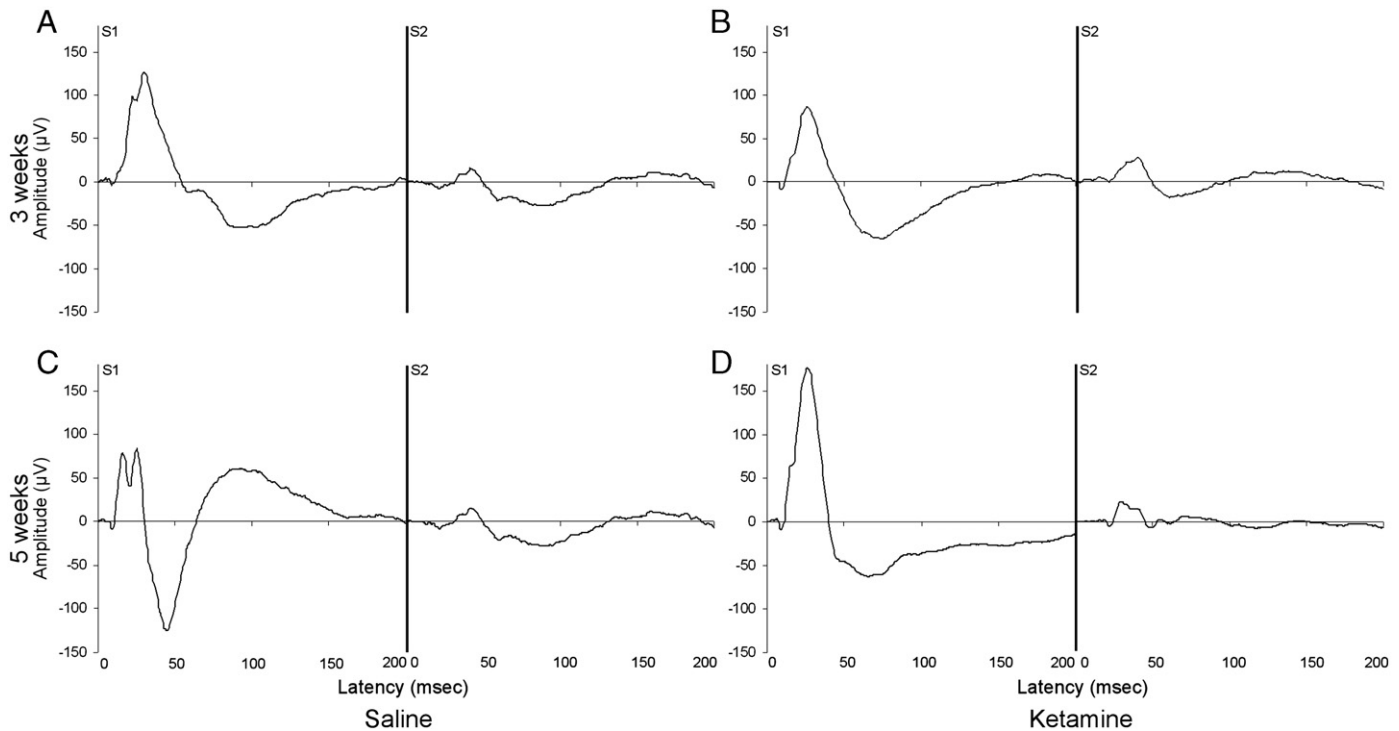


Fig. 3. Grand average AEP readings for each treatment, test session and stimulus condition in the gating task are shown. Grand averages of (A) 0.09% saline and (B) 5.0 mg/kg ketamine-treated mice, 3 weeks after the cessation of the 4-week, daily administration schedule. (C) Saline- and (D) ketamine-treated grand averages, 5 weeks after the cessation of treatment. S1 represents the response to the first of two white-noise bursts, while S2 represents the response to the second.

Novelty

A *t* test on the epochs generated from baseline corrected novel and standard tone difference waveforms did not produce any significant results. The rmANOVAs on each epoch did not identify an interaction between ketamine condition and stimulus type (novel vs. standard) within the MMN epochs {(50–75 ms) [$F(1, 13) = 1.74, p = 0.210$] and (75–100 ms) [$F(1, 13) = 0.40, p = 0.540$]}.

Amplitude, gating and latency

The overall pattern of auditory event-related potentials for the P20, N40 and P80 is shown in Fig. 3.

P20

Though a rmANOVA on baseline corrected data for P20 amplitude did not yield any significant interactions, as expected there was a significant main effect of stimulus order (S1 vs. S2), confirming that S2 was gated relative to S1 [$F(1, 12) = 54.37, p < 0.001$]. Our analyses also revealed a trend toward increased P20 amplitude 5, as opposed to 3, weeks after the cessation of treatment [$F(1, 12) = 4.37, p = 0.059$]. An evaluation of P20 latency also demonstrated that chronic ketamine administration resulted in a significant increase in S1 latency [$F(1, 12) = 5.80, p = 0.033$] (Fig. 4).

N40

A rmANOVA on baseline corrected data for N40 amplitude revealed only a significant interaction between test week and stimulus [$F(1, 12) = 6.66, p = 0.024$], as S1 increased between weeks 3 and 5 [$MS = 2760.5, p < 0.001, df = 12.00$] (Fig. 5). In addition, analyses demonstrated a main effect of stimulus, as S2 was again significantly gated relative to S1 [$F(1, 12) = 25.45, p < 0.001$], and a trend toward increased N40 amplitude at week 5 [$F(1, 12) = 4.24, p = 0.062$].

P80

A rmANOVA on baseline corrected data for P80 amplitude revealed a significant interaction between treatment and stimulus, indicating differing effects of saline and ketamine on auditory sensory gating [$F(1, 12) = 5.02, p = 0.045$]. Post hoc analyses revealed that ketamine-treated mice exhibited a significant decrease in S1 amplitude as compared to saline-treated subjects [$MS = 956.69, p = 0.016, df = 23.34$] (Fig. 5). The P80 component also displayed a trend toward decreased amplitude when treated with ketamine, though this result did not quite reach significance [$F(1, 12) = 4.21, p = 0.063$]. A latency evaluation showed that there was also a significant decrease between weeks 3 and 5 [$F(1, 12) = 12.38, p = 0.004$].

Discussion

The current study examines both immediate and long-lasting effects of chronic ketamine administration on contextual fear

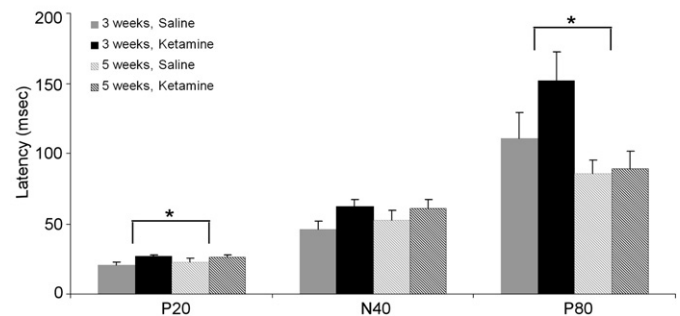


Fig. 4. S1 latencies of the P20, N40 and P80 components of the auditory ERP following chronic saline (gray) or 5.0 mg/kg ketamine (black) treatment. Asterisk indicates either a significant ketamine-induced increase in P20 latency across both test weeks, or a decrease in P80 latency between weeks 3 and 5. Overall, ketamine increased the S1 latency of all components at both time points. Data are presented as mean \pm SEM.

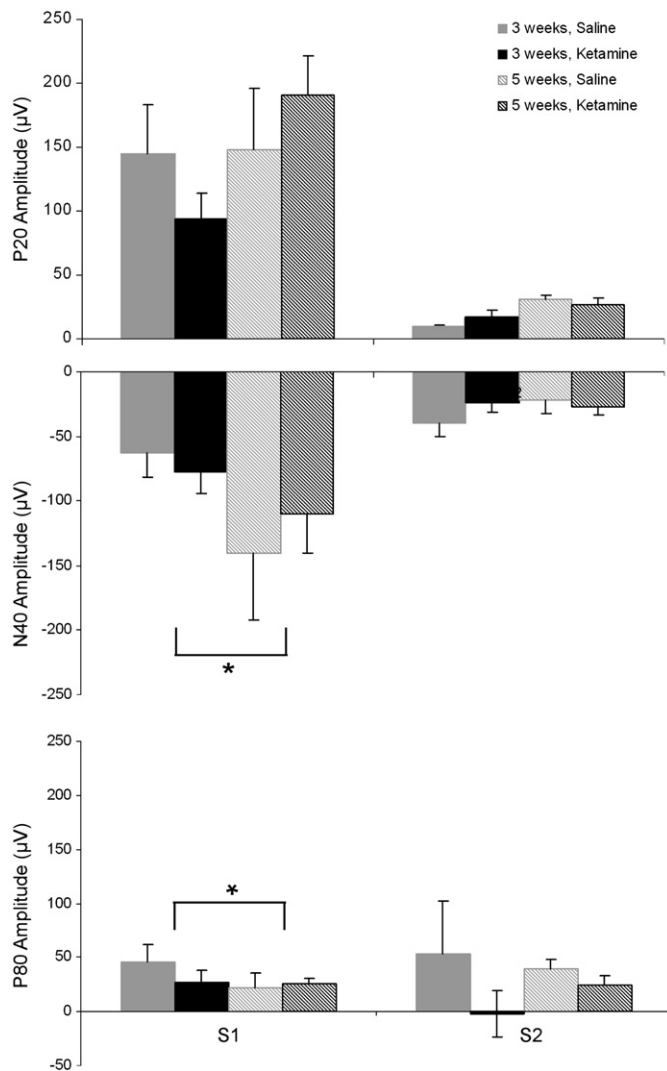


Fig. 5. Gating data for the P20, N40 and P80 AEP components three and five weeks after cessation of chronic saline (gray) or 5.0 mg/kg ketamine (black) treatment. Asterisk indicates either a significant interaction between N40 gating and test week, or P80 gating and treatment condition. Post hoc analyses revealed that both of these results were mediated by effects on S1 amplitude, which increased between weeks 3 and 5, and was decreased by ketamine. Data are presented as mean \pm SEM.

conditioning, detection of pitch deviants and auditory gating. Murine studies have repeatedly shown that acute ketamine treatment results in impaired contextual fear conditioning (Pietersen et al., 2006, 2007; Calzavara et al., in press). In those studies, the authors suggest that this deficit is caused by the blockage of NMDA receptors (NMDARs), which are requisite for such Pavlovian conditioning. Additionally, ketamine causes widespread apoptotic neurodegeneration after both single and chronic administrations (Scallet et al., 2004; Wang et al., 2005; Young et al., 2005; Majewski-Tiedeken et al., 2008). Such neurodegeneration has been identified in the thalamus, amygdala, parietal cortex and hippocampus, among other regions. Because all four of these regions are involved in the fear conditioning pathway, it is possible that ketamine-induced cell death, and not only the blockage of NMDARs, in any of these regions can result in fear conditioning impairments (Bissiere et al., 2007; Matus-Amat et al., 2007; Zanoveli et al., 2007; Keene and Bucci, 2008). Because experiments in this study were conducted 48 h after injections and the half-life of ketamine in mice is approximately 13 min, it is unlikely that the drug was still bound to NMDARs during data collection (Maxwell et al., 2006). Though the pharmacokinetics of ketamine's metabolites, norketamine and dehydroketamine, have not been

studied in mice, data from other small animals suggest that they are largely cleared within 24 h (Pedraz et al., 1985; Pypendop and Ilkiw, 2005). Therefore, results in the CFC paradigm support our hypothesis that the detrimental effects of ketamine were caused not by acute NMDAR blockade, but more lasting, potentially apoptotic, mechanisms.

Despite ketamine's link to impaired fear conditioning, one caveat of the current study is our inability to identify which phase of the learning process was affected by drug. Studies show that the thalamus, hippocampus and sensory cortex are all involved in conditioned response (CR) acquisition, hippocampus and amygdala are important for memory consolidation, and the amygdala is the primary site of conditioned stimulus (CS)-unconditioned stimulus (US) convergence (LeDoux, 1995; Davis and Shi, 2000; Maren, 2001). The hippocampus and amygdala also play a role in the retrieval, or expression, of contextual fear memories, which is one of the physiological functions ultimately being assessed in our two experimental test sessions. Because ketamine-treated mice exhibited normal fear conditioning after two weeks of injections, it appears that they were able to acquire fear memories and retrieve them at that time point. However, because there was only one training session, the task at the second time point was more difficult and isolated to memory retrieval. The selective impairment during the second test session also suggests that ketamine may alter system consolidation, which is the gradual reorganization of brain connections that support memory. This process is generally slower and longer-lasting than synaptic consolidation, which is complete within hours of training and involves changes in more localized circuits. One of the key characteristics of system consolidation is that memories shift from hippocampus- to prefrontal cortex-dependence. The effect of ketamine on CFC two weeks after training may therefore be due to a decreased ability to strengthen the cortical connections involved in this process (Frankland and Bontempi, 2005).

Though CFC testing was only performed at the two discussed time points, data collected in the gating and novelty paradigms also suggest that repeated ketamine administration has lasting effects on brain functioning. Alternatively, our hypothesis regarding MMN was not supported. The lack of a MMN effect in the current study suggests that the disruption of MMN following acute ketamine is due to receptor blockade, rather than the lasting changes secondary to previous exposure. Despite the lack of effect of chronic ketamine on novelty recognition, the drug did produce the predicted results in terms of several different AEP components. For instance, similar to a previously published study from our laboratory, ketamine caused a significant increase in P20 latency, and generally increased the latencies of the N40 and P80 components, as well (Maxwell et al., 2006). ERP component latency is generally thought to be a measure of processing speed, which is the rate at which a person responds to a given stimulus. As discussed in Amann et al., (2008), an increase in processing speed would subsequently decrease the likelihood that information held with one's working memory could be accessed before it is lost or diminished (Fry and Hale, 2000; Amann et al., 2008). The reported effects of chronic ketamine on ERP component latency are therefore consistent with its effects on CFC, and lend further support to the proposed link between the temporal characteristics of electrophysiological recordings and behavioral outcomes.

Similar to its effects on latency, we also found that chronic ketamine had negative consequences in terms of P80 amplitude and gating, as gating was decreased over both test weeks. The mouse P80 displays morphology, relative latency and pharmacological response properties similar to those of the human P200 (Siegel et al., 2003; Maxwell et al., 2004b). Clinical electrophysiology studies consistently show that the P200 exhibits decreased amplitude and inhibition in patients with schizophrenia (Roth et al., 1981; Ford et al., 1999). Such changes are usually manifest as a reduction in the ability to gate repeated stimuli, as mediated by a decrease in S1 amplitude. Therefore, the effects of

chronic ketamine treatment on P80 amplitude and gating support both the NMDAR blockade and chronic ketamine models of abnormal P80/P200 amplitudes after auditory stimuli, as seen in schizophrenia. The P200 has multiple generators and due to their analogous nature, it is believed that the mouse P80 does, as well (Wunderlich and Cone-Wesson, 2001; Sheehan et al., 2005). However, because the recording technique utilized in this study measures whole-brain activity, it is impossible to determine which regions were affected by ketamine and responsible for the gating deficit observed. Because immunohistochemical analyses were not performed, it is also not possible to confirm that the long-lasting effect of ketamine on P80 amplitude, and P20 latency, was due to mechanisms other than NMDAR blockade.

The current study builds upon previous reports assessing the effects of chronic ketamine treatment on the mouse AEP. Until now, such studies have only evaluated these effects immediately and one week after a 14-day daily administration regimen. Therefore, this study demonstrates that continued ketamine administration has even longer-lasting effects on AEP components than previously recognized. Such lasting effects also add support to the belief that repeated recreational ketamine use could have long-term effects on brain function and information processing.

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