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Alpha$_1$-adrenergic receptor blockade in the VTA modulates fear memories and stress responses

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Abstract
Activity of the ventral tegmental area (VTA) and its terminals has been implicated in the Pavlovian associative learning of both stressful and rewarding stimuli. However, the role of the VTA noradrenergic signaling in fear responses remains unclear. We aimed to examine how alpha$_1$-adrenergic receptor (α_1-AR) signaling in the VTA affects conditioned fear. The role of α_1-AR was assessed using the micro-infusions into the VTA of the selective antagonists (0.1–1 μg/0.5 μl prazosin and 1 μg/0.5 μl terazosin) in acquisition and expression of fear memory. In addition, we performed control experiments with α_1-AR blockade in the mammillary bodies (MB) – a brain region with α_1-AR expression adjacent to the VTA. Intra-VTA but not intra-MB α_1-AR blockade prevented formation and retrieval of fear memories. Importantly, local administration of α_1-AR antagonists did not influence footshock sensitivity, locomotion or anxiety-like behaviors. Similarly, α_1-AR blockade in the VTA had no effects on negative affect measured as number of 22 kHz ultrasonic vocalizations during fear conditioning training. We propose that noradrenergic signaling in the VTA via α_1-AR regulates formation and retrieval of fear memories but not other behavioral responses to stressful environmental stimuli. It enhances the encoding of environmental stimuli by the VTA to form and retrieve conditioned fear memories and to predict future behavioral outcomes. Our results provide novel insight into the role of the VTA α_1-AR signaling in the regulation of stress responsiveness and fear memory.

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1. Introduction

Impaired fear regulation and fear conditioning are the core symptoms of stress-related disorders, and they might lead to the persistent flashbacks, nightmares, and intrusions of fear memories in posttraumatic stress disorder (PTSD) (Arnsten et al., 2015). Fear conditioning provides a useful model of psychopathology observed in the aftermath of stressful experience (Mahan and Ressler, 2012; Maren and Holmes, 2016). However, the neurobiological underpinnings of the formation and consolidation of fear memories are still not fully elucidated.

Dopamine (DA) signaling within the mesocorticolimbic brain structures is known to be involved in behavioral responses to both stressful and rewarding stimuli (Bromberg-Martin et al., 2010). Firing of some DA and GABAergic neurons which are typically located in the caudal ventral tegmental area (cVTA), briefly increases in response to footshock, conditioned aversive stimuli, restraint stress or social defeat stress (Anstrom et al., 2009; Anstrom and Woodward, 2005; Brischoux et al., 2009; Guarraci and Kapp, 1999), indicating that stress potently modulates VTA activity as well as DA signaling. In addition, exposure to stressful stimuli increases VTA GABAergic and decreases DAergic neuronal activity during the encoding of negative prediction error (Cohen et al., 2012). However, the underlying mechanisms that regulate VTA activity, its impact on behavioral stress responses and the formation of fear memories remains elusive.

The noradrenaline (NA) system encompassing NA neurons in the locus coeruleus (LC) and the area A1 and A2 in the medulla oblongata (for detailed neuroanatomy of the NA system see: Robertson et al. (2013) has been demonstrated to control the activity of VTA DA and non-DA neurons (Geisler and Zahm, 2005; Masana et al., 2011; Mejías-Aponte et al., 2009). Importantly, salient stimuli upregulate NA system activity similarly to the DA system, inducing burst firing of the LC NA neurons and subsequent phasic NA release at terminals (Bouret and Richmond, 2009; Park et al., 2012) suggesting a role of NAergic signaling in learning and memory. Accordingly, NA signaling has a facilitating role in acquisition and/or reconsolidation of emotional memories (Bernardi et al., 2009; Cahill et al., 1994; Dębiec et al., 2011; Ferry et al., 1999; Furini et al., 2010; Gelinas and Nguyen, 2005; McGaugh and Roozendaal, 2002; Milton et al., 2008; Sara et al., 1999; Schutsky et al., 2011). This is consistent with clinical studies suggesting elevated NA responsiveness in PTSD (Geracioti et al., 2001; Southwick et al., 1993) and the crucial role of NA signaling within the mesocorticolimbic brain structures in encoding fear memory (Wingenfeld et al., 2015); for review see: (Holmes and Quirk, 2010; Mueller and Cahill, 2010). Consequently, α_1-AR blocker prazosin proved to be effective in attenuating some symptoms of PTSD (Birnbaum et al., 1999; Germain et al., 2012; Koola et al., 2014; Raskind et al., 2013, 2007, 2003; Taylor et al., 2008, 2006); for review see: (Arnsten et al., 2015).

Despite the established NA system projections to the VTA and the receptor mechanisms, behavioral consequences of NA signaling in the VTA are poorly understood. Recently, it has been shown that VTA NA signaling is involved in cocaine-related behaviors (Goertz et al., 2015). In contrast, there are no reports to date of the role of VTA NA signaling in stress-related behaviors. Our study aimed to examine the role of the NA signaling via α_1-AR in the VTA in the acquisition and retrieval of conditioned fear memories. Exploring the receptor mechanisms of individual stress responsiveness is crucial for understanding the processes that determine susceptibility or resilience to diseases related to a traumatic stress or challenging situation.

2. Experimental procedures

2.1. Subjects

Male Sprague Dawley rats (280–350 g) were acquired from Charles River (Sulzfeld, Germany) and an Institute of Pharmacology PAS (Krakow, Poland) breeding facility. Animals were housed five per cage in a temperature and humidity controlled room (20–22 °C, 40–50% humidity), on a 12 h light/dark cycle (lights on at 7 a.m.), with *ad libitum* access to food and water. Before any surgical procedures, rats were allowed to acclimate to the facility for one week. After surgery, all animals were housed singly. All behavioral tests were performed during the light phase of the cycle. All experimental procedures were conducted according to the EU Guide for the Care and Use of Laboratory Animals and were approved by the Committee on the Ethics of Animal Experiments at the Institute of Pharmacology, Polish Academy of Sciences (Krakow, Poland) as well as the Committee on the Ethics of Animal Experiments at the Jagiellonian University.

2.2. Drugs

Prazosin hydrochloride (Praz; 0.1–1 μg, Sigma-Aldrich, Germany) – a selective α_1-AR antagonist – was dissolved in PBS and sonicated before microinjections. Terazosin hydrochloride (Teraz; 1 μg, Sigma-Aldrich, Germany) – another selective α_1-AR antagonist with better solubility – was dissolved in PBS. All drugs were infused into the VTA in a volume of 0.5 μL (Praz: 0.24–2.38 nmol/side; Teraz: 2.36 nmol/side) at a rate of 0.5 μL/min, using a Hamilton 25 gauge syringe. After infusion, the internal cannula was left in place for one additional minute to allow adequate absorption of the drug. The doses for all experiments were calculated based on previous work from our laboratory and others' demonstrating the ability of prazosin administration to modulate behavior (Azami et al., 2010; Ecke et al., 2012; Goertz et al., 2015).

2.3. Surgery

All rats were habituated to handling by the experimenters for at least five consecutive days prior to surgery. Rats were anesthetized with ketamine HCl (100 mg/kg, i.m., Biowet-Puławy, Poland) and xylazine (10 mg/kg, i.m., Biowet-Puławy, Poland) and placed in a stereotaxic frame (Stoelting Europe, Ireland) for intracranial cannula implantation. All coordinates were obtained from the rat brain atlas (Paxinos and Watson, 2007) with anteroposterior (AP), mediolateral (ML) and dorsoventral (DV) positions referenced from Bregma. Bilateral guide cannula (Plastics One, Roanoke, VA, USA) were placed dorsal to the VTA (AP −5.2 mm, ML ± 0.5 mm, DV −7 mm). In addition, for control experiments, additional group of animals had bilateral guide cannulas placed in the mammillary body (MB) region (mammillary nuclei and medial supramammillary (AP − 4.5 mm, ML ± 0.5 mm, DV − 8.2 mm). Next, four anchor screws (Agnthos, Sweden) were mounted in the skull and dental cement (Duracryl, SpofaDental, Czech Republic) was used to ensure stability of the cannula. Guide cannula patency was ensured by inserting a matching dummy infusion cannula and a dust cap. After the surgery, animals were given an anti-inflammatory

and analgesic drug (Tolfedine 4%, i.p., Vetoquinol Biowet, Poland) and glucose (5 ml) to prevent dehydration. For the first three days after the operation, animals were treated with antibiotics added to the drinking water (Sul-Tridin 24%, Biowet-Pulawy, Poland). Rats were given at least a week to recover after the intra-VTA cannula implantation.

2.4. Footshock sensitivity

In order to assess animals' individual pain threshold, rats were placed in the conditioning chamber (Med Associates, St. Albans, USA) equipped with a metal grid floor. The electric footshock current was gradually increased from 0 mA, until the animal showed the first signs of pain – notice, flinch (retraction of at least one paw), vocalizing and jumping. At this moment, the current was immediately switched off (Szklarczyk et al., 2012; Vazdarjanova and McGaugh, 1998).

2.5. Fear conditioning

Fear conditioning consisted of two phases: training (conditional stimulus-unconditional stimulus pairings) and retrieval of the conditioned fear memory (Madarasz et al., 2016; Zelikowsky et al., 2013, 2012). The experimental design is shown in Figure 1A–C.

2.5.1. Training

On day 1, each rat was placed in the conditioning chamber with a metal grid floor, two opaque Plexiglas side walls, two metal side walls, a 24-V house light located on the opaque Plexiglas ceiling, and a white stimulus lamp illuminated by a 24-V bulb and a tone generator, both located on a metal side wall. At 180 s after being placed in the conditioning chamber, all rats received four conditioning trials, each consisting of a 30-s tone (60 dB) and light presentation (conditional stimulus; CS) co-terminated with a 2-s 0.9-mA electric footshock (unconditional stimulus; US). The inter-trial interval (ITI) between each pair of conditioning trials lasted 60 s. Time spent freezing (immobility, except for respiratory movements) was automatically measured during baseline (0–180 s) CS-US pairings and ITIs. The freezing data were recorded, stored, and analyzed as a percent of total time spent in the chamber using ANY-maze software (Stoetling Europe, Ireland). After 4 conditioning trials and an additional 60 s in the chamber, rats were returned to their home cages. The conditioning chamber was then cleaned with 70% ethanol.

2.5.2. Fear memory retrieval

Rats were re-exposed to the conditioning chamber or CS-paired with US 24 and 48 h after training. Two different contexts were used: the conditioning chamber with a metal grid floor (the same chamber from day 1, for contextual fear memory) and a novel Plexiglas chamber with a white stimulus lamp illuminated by a 24-V bulb and a tone generator (for cued fear memory).

In the contextual fear memory test, rats were re-exposed to the shock chamber and time spent freezing was automatically measured for 8 min. In the cued fear memory test, rats were exposed to the new context for 9 min (180 s of acclimatization followed by four 30-s presentations of the conditioned stimuli CS + with a 60-s ITI), during which freezing was scored during acclimatization (baseline), the four 30-s CS+ presentations and the ITIs. After each test, all rats were returned to their home cages. The conditioning chamber was cleaned after each test with 70% ethanol, whereas the Plexiglass chamber was cleaned with distilled water. Both tests were performed on the same subjects. The order of test was counterbalanced so 50% of rats started with contextual fear memory retrieval on day 2 followed by cued fear memory retrieval on day 3. Second half of animals on day 2 received cued fear memory retrieval, followed by contextual fear memory testing 24 h later.

2.5.3. Experimental scheme

To study the effects of α_1-AR blockade in the VTA on conditioned fear memory, rats were given intra-VTA drug micro-infusion immediately before being placed in the conditioning chamber on day 1. Next, freezing was measured during day 1 to evaluate impact of α_1-AR blockade on stress responses to the CS-US pairings as well as during day 2 followed by day 3 to demonstrate retrival of fear memories acquired on day 1 (Curzon et al., 2011; Madarasz et al., 2016).

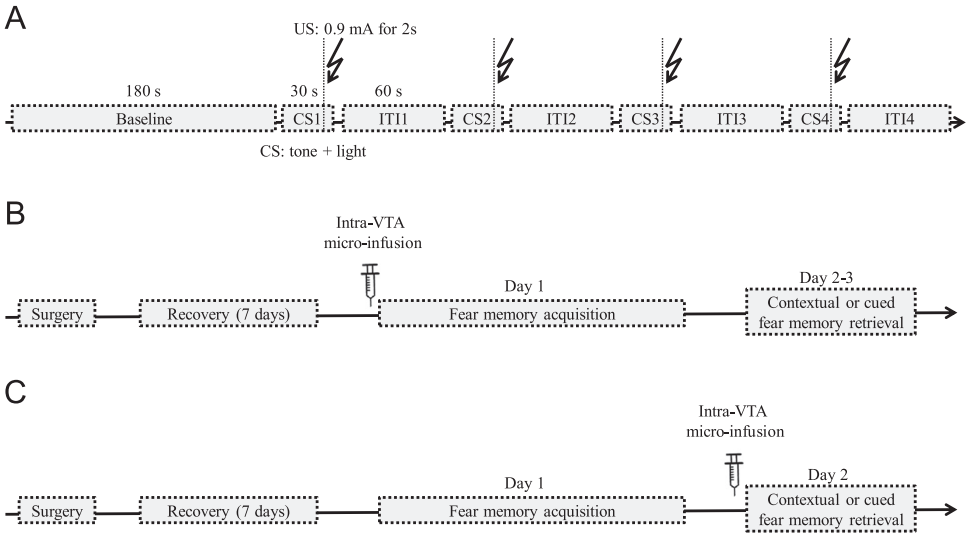

Figure 1 The experimental timeline and schedule of intra-VTA micro-infusions during fear conditioning. (A) Animals after intra-VTA cannula implantation surgeries and recovery underwent the fear conditioning during day 1. All rats received four conditioning trials, each consisting of a conditioned stimulus co-terminated with an electric footshock followed by an inter-trial interval (ITI). (B and C) At 24 h and 48 h later, expression of contextual and cued fear memory was measured in the conditioning chamber (for contextual fear memory) or a novel Plexiglas chamber (for cued fear memory). To evaluate the effects of α_1-AR blockade on acquisition or retrieval of fear memory, drugs were infused into the VTA on day 1 (B) or day 2 (C), respectively, immediately before testing.

For investigation of the intra-VTA α_1-AR blockade effects on fear memory retrieval, separate groups of rats were given intra-VTA drug micro-infusion immediately before being placed in the conditioning chamber (contextual fear memory) or in the novel chamber (cued fear memory) on day 2. Next, freezing was measured during day 2 to evaluate impact of α_1-AR blockade on the expression of conditioned fear.

2.6. Open field test

The open field test was performed in a square apparatus ($80 \times 80 \times 60$ cm) with matte black walls and floor. The center of the apparatus was illuminated at 150 lx. Immediately after the VTA micro-infusion, animals were placed in the center of the open field and left inside for 30 min. Rats' behavior was recorded and analyzed using ANY-maze videotracking software. Distance travelled during the test was used as a measure of locomotor activity, and time spent in the central zone of the apparatus was used a measure of anxiety-like behavior. After each rat, the apparatus was cleaned, using 70% ethanol and dried with a cleaning cloth.

2.7. Light/dark box test

The light/dark box test was performed in a custom-made Plexiglas apparatus consisting of two distinct chambers. The light chamber ($28 \times 21 \times 16$ cm) was made from opaque Plexiglas and was uncovered and lit with white light at 900 lx. The dark chamber ($16 \times 21 \times 16$ cm) was matte black, with a cover in the same color, providing a safe enclosure with very limited lightning. A small opening in the dark chamber (13×6.5 cm) enabled the animals to freely explore the apparatus. Rats were placed in the light chamber, facing away from the transition site, and left to explore the box for 10 min. Behavioral activity was recorded using a camera mounted above the apparatus, and was analyzed using ANY-maze video-tracking software. Rats were considered to be in any given zone, when their head and 3/4 of the body were in that area. Other behaviors, such as latency to the first entrance to the dark chamber, number of full-body transitions between chambers, line crossings (number of stretches from the dark chamber into the light chamber with at least part of the body but not all four feet present in the light chamber), rearings (animal stands on hind paws) were also calculated. Frequency of rearings in the light chamber was standardized by the duration of time each subject spent in that chamber (number of rearings/duration of time in light $\times$ 100). After each rat, the apparatus was cleaned using damp paper towels.

2.8. Ultrasonic vocalizations

Rats' ultrasonic vocalizations were recorded during fear conditioning acquisition phase. Rat calls were recorded using an UltraSoundGate Condenser Microphone CM16 (Avisoft Bioacoustics, Germany), which was positioned 30 cm above the fear-conditioning chamber. The microphone was connected to an ultrasound-recording device (UltraSoundGate 116 Hb, Avisoft, Berlin, Germany) and to Avisoft RECORDER software set for sampling at 200,000 Hz and 16 bit recording. For acoustic analysis, the recordings were transferred to Raven Pro 1.4 interactive sound analysis software (Cornell Lab of Ornithology, Bioacoustics Research Program, USA) and fast Fourier transform was conducted (512 FFT-length, Hamming windows and 75% overlap). A lower cut-off-frequency of 20 kHz was used to reduce background noise. Each call was selected manually on the computer screen by the trained investigator.

2.9. Locomotor activity

Locomotor activity was assessed in the same apparatus that was used in the open field test. The apparatus was illuminated with a light intensity of 20 lx, providing just enough illumination for the videotracking software to work correctly. Immediately after the VTA micro-infusion, animals were placed in the center of the apparatus and left inside for 30 min. Each rat behavior was recorded and analyzed using ANY-maze video-tracking software. Distance travelled during the test was used as a measure of locomotor activity. The apparatus was cleaned using 70% ethanol and dried with a cleaning cloth between each rats.

2.10. Histological verification of cannula placement

Animals were anesthetized with pentobarbital (150 mg/kg i.p., Biowet-Pulawy, Poland), after which 0.5 μL Chicago Sky-Blue dye (Sigma-Aldrich, Germany) was bilaterally microinjected into the VTA or the MB. Immediately after dye micro-infusion, animals were decapitated; their brains were removed and placed in 4% paraformaldehyde for 72 h. Brains were sliced (200 μm) with the vibratome (model VT1000S, Leica Biosystems, Germany) and the diffusion of the dye was analyzed to verify the accuracy of cannula placement. All data from subjects with misplaced cannula were removed from the analysis (Table S1, map of the cannula misplacements in Figure S1A). Representative cannula placements are shown in Figures 2 and S1B. Due to a sufficient number of cannula misplacement subjects in fear conditioning experiments, effects of α_1-AR prazosin administration anterior (less than 4.8 mm from Bregma; $n=3$) or dorsal (less than 7 mm from Bregma; $n=3$) to the VTA were also evaluated. Moreover, control experiment with injections in MB area was conducted. Such misplacement control allowed exclusion of possible behavioral effects of α_1-AR signaling outside the VTA, but in proximity of other α_1-AR expressing brain regions such as the posterior hypothalamus (anterior to the VTA) and the red nucleus (dorsal to the VTA), or MB region (ventral and anterior to the VTA).

2.11. Statistics

Data were analyzed for normal distribution with the Kolmogorov-Smirnov test (Statistica 7, Stat-Soft, Poland). Behavioral effects of intra-VTA drug administration were analyzed using Student's t test or one-way ANOVA with Newman Keuls post hoc comparison (Statistica 7, Stat-Soft, Poland; GraphPad Prism Software, San Diego, CA). In addition, behavioral effects of intra-VTA drug administration over CS presentations, ITIs or time were analyzed using two-way repeated-measures ANOVA with Newman Keuls post hoc comparison. All factors and levels of ANOVA according to the particular drug micro-infusion and behavioral test are listed in Table S3. Statistical significance was set at $P<0.05$. All data values are presented as the means $\pm$ SEM.

3. Results

3.1. α_1-AR blockade in the VTA with terazosin decreases freezing response during CS-US pairings

Prazosin micro-infusions on day 1 (experimental timeline on Figure 3A) had no effects on the total time spent freezing during baseline (Figure 3B; $F_{(3,34)} = 0.50$, n.s.), as well as during CS-US pairings (Figure 3C; $F_{(3,34)} = 1.47$, n.s.) or ITIs (Figure 3D; $F_{(3,34)} = 0.71$, n.s.). Intra-VTA prazosin administration did not affect immediate learning during acquisition of fear memory, as prazosin micro-infusions did not change the increase in fear responses to the CS after

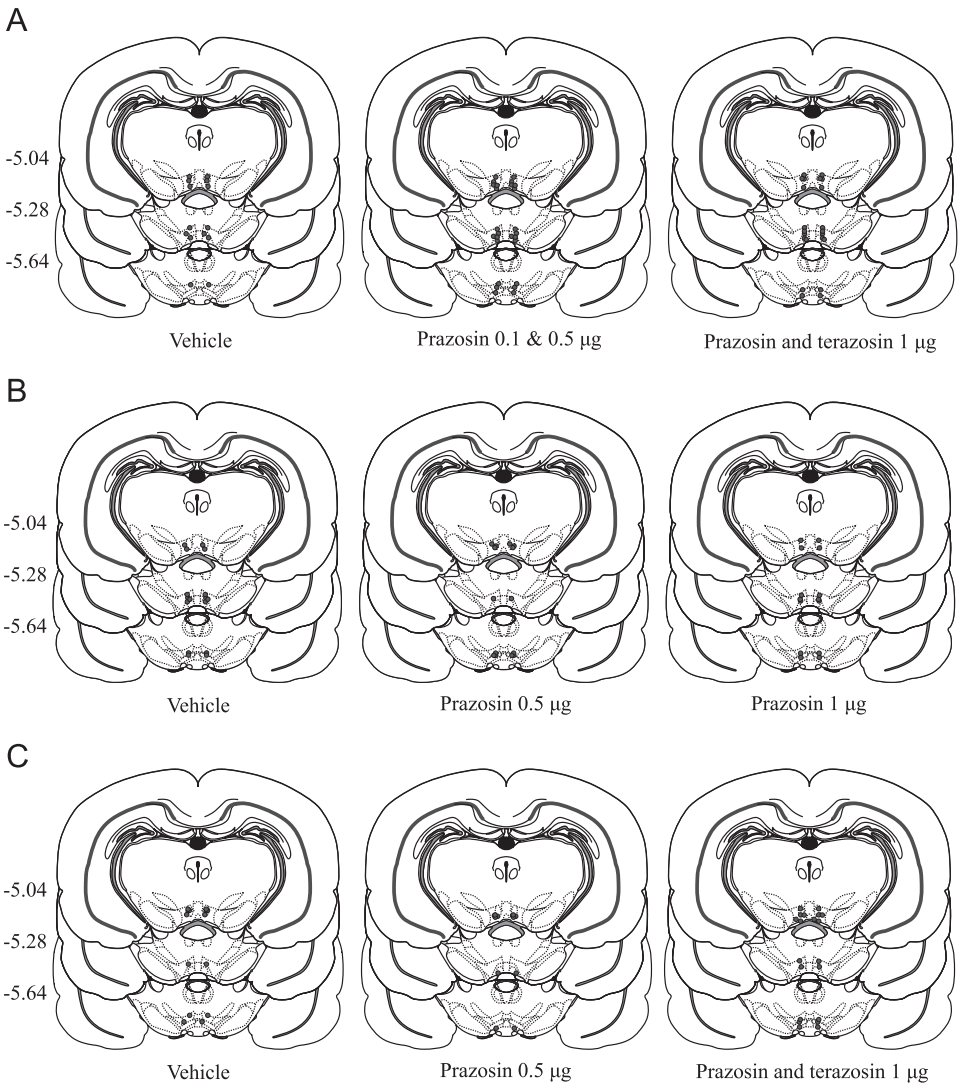

Figure 2 Representative VTA micro-infusion cannula placements in (A) the fear conditioning, (B) the light/dark box and (C) the open field tests. Drawings adapted from Paxinos and Watson (2007).

subsequent footshocks (Figure 3E; CS presentation: $F_{(3,102)} = 79.83$, $p<0.0001$, post hoc test $p<0.01$ for CS1 vs. CS4; dose $F_{(3,102)} = 0.32$, n.s.; CS presentation × dose $F_{(9,102)} = 2.21$, $p<0.05$, post hoc test n.s.) or consecutive ITIs (Figure 3F; CS presentation: $F_{(3,102)} = 12$, $p<0.0001$, post hoc test $p<0.01$ for ITI1 vs. ITI4; dose $F_{(3,102)} = 0.71$, n.s.; CS presentation × dose $F_{(9,102)} = 1.21$, n.s.).

Interestingly, intra-VTA α_1-AR blockade with another selective antagonist terazosin attenuated total time spent freezing during fear memory acquisition during baseline (Figure 3B; $t = 2.48$, df = 24, $p<0.05$), CS-US presentations (Figure 3C; $t = 2.19$, df = 24, $p<0.05$) and ITIs (Figure 3D; t = 3.64, df = 24, $p<0.01$), indicating possible functions of the VTA α_1-AR signaling in fear responses. Terazosin treatment decreased freezing in response to all subsequent CS-US pairings (Figure 3E; dose $F_{(1,24)} = 4.83$, $p<0.05$, post hoc test $p<0.05$; CS presentation × dose $F_{(3,72)} = 0.36$, n.s.) and all 4 ITIs (Figure 3F; dose $F_{(1,24)} = 13.24$, $p<0.01$, post hoc test $p<0.05$; ITI exposure × dose $F_{(3,72)} = 1.48$, n.s.) but did not change immediate learning as there was similar increase in freezing after subsequent CS-US and ITI exposure (CS presentation $F_{(3,72)=}65.3$, $p<0.001$, post hoc test $p<0.05$ for CS1 vs. CS4 and ITI exposure $F_{(3,72)} = 11.02$, $p<0.001$, post hoc test $p<0.05$ for ITI1 vs. ITI4).

3.2. α_1-AR blockade in the VTA prior to CS-US pairings (day 1) affected expression of fear memory (day 2)

Intra-VTA micro-infusion of prazosin on day 1 (experimental timeline on Figure 4A;) dose-dependently attenuated the acquisition of conditioned fear induced by context (Figure 4B; $F_{(3,34)} = 2.9$, $p<0.05$, post hoc test $p<0.05$) during fear memory retrieval on day 2 and 3. In addition, prazosin treatment, while having no effects on freezing during baseline in the novel environment (Figure 4C; $F_{(3,34)} = 2.9$, n.s.), decreased total time spent freezing in response to CS (Figure 4D; $F_{(3,34)} = 3.08$, $p<0.05$, post hoc

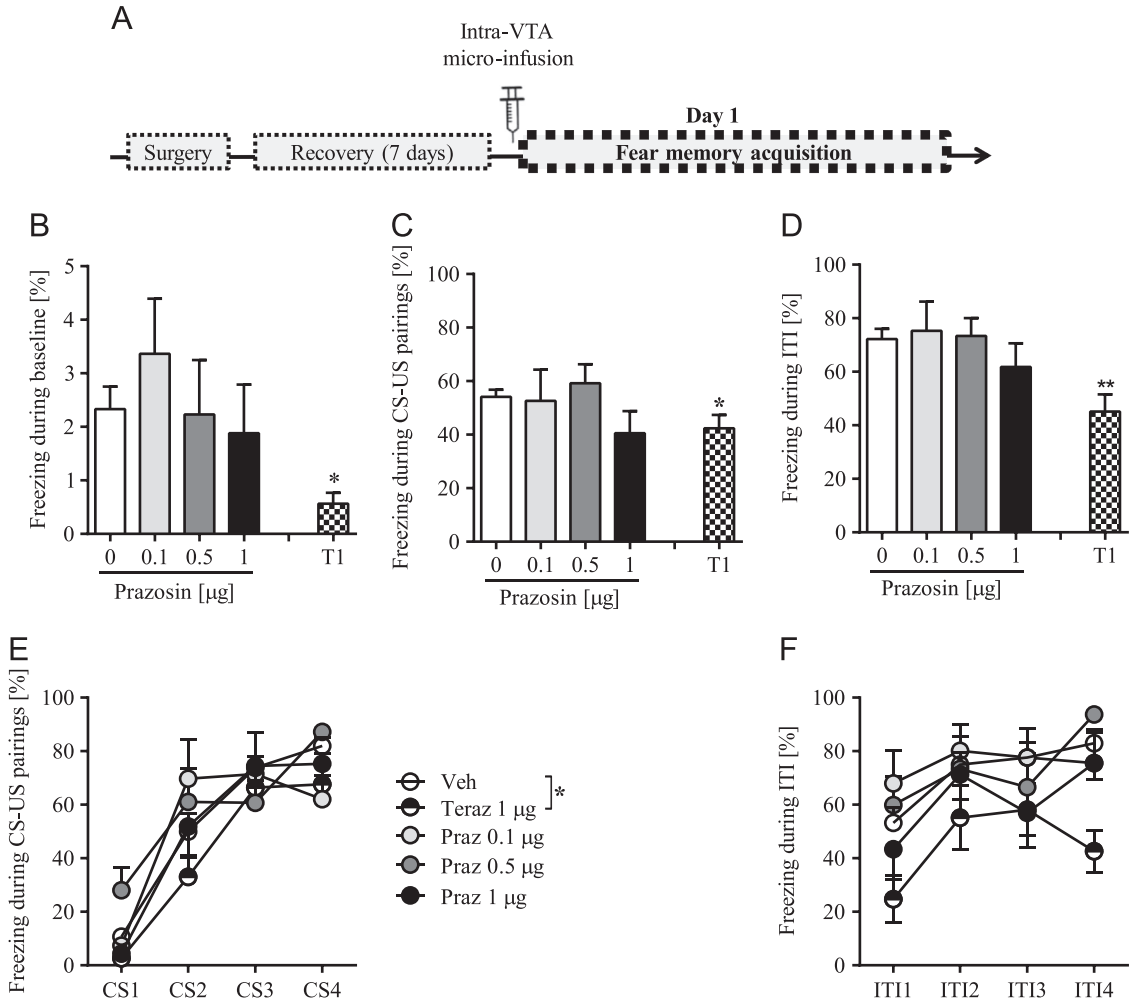

Figure 3 Effects of α_1-AR blockade in the VTA during the acquisition of fear memory. (A) The experimental timeline and schedule of intra-VTA micro-infusions during fear conditioning. (B-F) Intra-VTA micro-infusion of terazosin (T1; 1 µg/side) but not prazosin (Praz; 0.1-1 µg/side) decreased total time spent freezing during (B) baseline, (C) CS-US exposure and (D) ITIs on day 1. Terazosin effects were present (E) throughout CS-US conditioning and (F) during ITI. Data are presented as the mean+SEM of the percentage of time spent freezing. *$p<0.05$, ***$p<0.001$ vs. vehicle group (white column or white circles).

test $p<0.05$). Intra-VTA administration of prazosin did not change freezing during ITI (Figure 4E; $F_{(3,34)} = 0.18$, n.s.).

More-detailed analysis revealed that the effects of α_1-AR antagonist on fear memories were present beginning at the third and fourth CS presentation (Figure 4F; CS presentation × dose: $F_{(9,102)} = 2.54$, $p<0.05$, post hoc $p<0.001$ for Praz 1 vs. Veh during CS3 and CS4), preventing the increase in fear responses after subsequent CS presentation and the recall of fear memory. In addition, prazosin treatment had no effects on freezing during subsequent ITIs (Figure 4G; ITI exposure × dose: $F_{(9,102)} = 0.55$, n.s.; ITI exposure: $F_{(3,102)} = 0.01$, n.s.; dose: $F_{(3,34)} = 0.18$, n.s.), supporting a selective role for the VTA α_1-AR signaling in the acquisition of fear memories.

Similarly to the effects of prazosin, intra-VTA micro-infusion of terazosin potently decreased freezing induced by context (Figure 4B; $t = 3.88$, df = 24, $p<0.001$), had no effects during baseline in novel environment (Figure 4C; $t = 0.44$, df = 24, n.s.) and attenuated total time spent freezing in response to CS previously associated with footshock (Figure 4D; $t = 5.26$, df = 24, $p<0.001$). In addition, terazosin treatment decreased freezing in response to third and fourth CS presentation (Figure 4F; CS presentation × dose: $F_{(3,72)} = 0.35$, $p<0.001$, post hoc $p<0.001$ for Teraz 1 vs. Veh during CS3 and CS4). Furthermore, in contrast to prazosin treatment, terazosin micro-infusion attenuated freezing during all four ITIs (Figure 4G; dose: $F_{(1,72)} = 10.14$, $p<0.05$, post hoc test $p<0.01$; ITI exposure × dose: $F_{(3,72)} = 0.53$, n.s.; ITI exposure: $F_{(3,72)} = 0.26$, n.s.).

Analysis of α_1-AR blockade prior to fear memory acquisition (intra-VTA administration on day 1) in the VTA cannula misplacement subjects (localizations of misplacement cannulae are shown on Figure S1A) demonstrated no effects of prazosin micro-infusion, when administered anterior (contextual fear memory: $t = 0.19$, df=4, n.s.; cue fear memory: t = 0.86, df = 4, n.s.; data not shown) or dorsal (contextual fear memory: $t = 0.92$, df = 4, n.s.; cue fear memory: $t = 0.02$, df = 4, n.s.; data not shown) to the VTA. It diminishes the possibility that the observed behavioral effects of intra-VTA α_1-AR blockade were due to the spread of prazosin outside the VTA and into other α_1-AR expressing brain regions. However, the number of cases ($n = 3$ per each group: anterior and dorsal cannula misplacements) is not

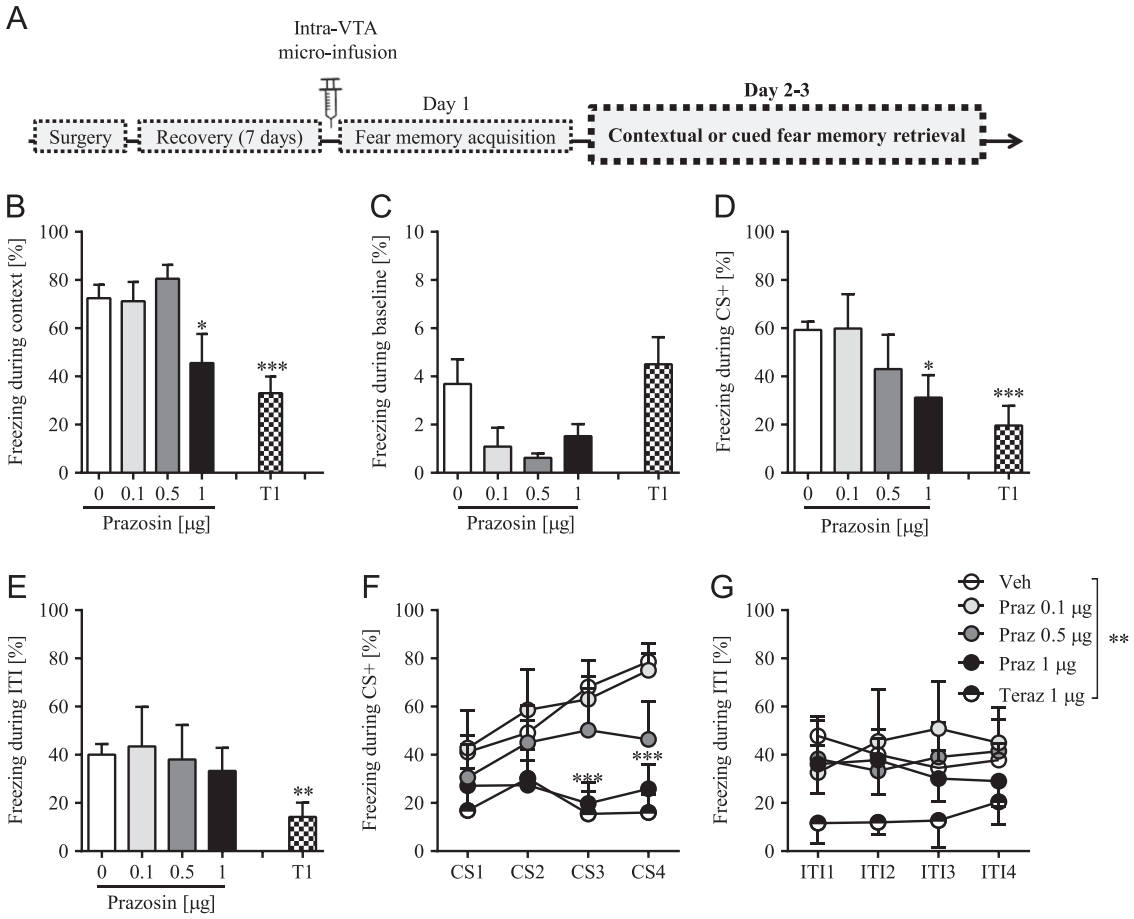

Figure 4 Decreased fear memory expression (on day 2) after α_1-AR blockade in the VTA prior to CS-US pairings (on day 1). (A) The experimental timeline and schedule of intra-VTA micro-infusions during fear conditioning. (B) Intra-VTA micro-infusion of both prazosin (Praz; 0.1–1 µg/side) or terazosin (T1; 1 µg/side) on day 1 potently decreased total time spent freezing in response to conditioned context during fear memory retrieval. (C) Both prazosin and terazosin on day 1 had no effects on freezing during baseline in the novel environment, however (D) attenuated total time spent freezing in response to CSs and (E) during ITIs during fear memory retrieval. (F) The effect α_1-AR blockade on day 1 was present in response to CS3 and CS4 during memory retrieval ($p<0.001$). (G) In contrast, only terazosin but not prazosin treatment on day 1 decreased freezing during subsequent ITIs during memory retrieval. Data are presented as the mean+SEM of the percentage of time spent freezing. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. vehicle group (white column or white circles).

sufficient to draw strong conclusions. Thus, we conducted additional experiment in order to examine the effects of intra-MB α_1-AR blockade on the acquisition of conditioned fear (localizations of cannula are shown on Figure S1B). There were no effects on both, fear memory formation (Figure S2A-C; n.s., detailed statistics are presented in Figure Legend) and expression (Figure S2D-G, n.s., detailed statistics are presented in Figure Legends) after α_1-AR antagonist administration into the MB region during the acquisition phase. These results indicate that the effect of α_1-AR blockade on fear memory acquisition was restricted to the VTA but not other α_1-AR-expressing brain region such as the MB.

The potential effects of intra-VTA α_1-AR blockade on fear memory encoding or fear responses were not due to hyperlocomotion or modulation of rats' footshock sensitivity, as both prazosin and terazosin treatment showed no effect on locomotor activity measured as total distance travelled during 30 min in the activity box (Figure 5A; prazosin dose $F_{(2,25)} = 1.05$, n.s.; terazosin $t = 2.82$, df = 18, n.s.) or on stress reactions to applied electrical current (Figure 5B; stress reaction × prazosin dose $F_{(3,48)} = 0.83$, n.s., stress reaction $F_{(3,48)} = 65.84$, $p<0.001$, prazosin dose $F_{(1,16)} = 0.22$, n.s; stress reaction × terazosin dose $F_{(3,48)} = 0.32$, n.s., stress reaction $F_{(3,48)} = 53.31$, $p<0.001$, terazosin dose $F_{(1,16)} = 0.40$, n.s.). In addition, stress reactivity measured as time of 22-kHz USVs during baseline (Figure 5C; $F_{(2,21)} = 1.18$, n.s.), CS-us pairings (Figure 5D; $F_{(2,21)} = 0.36$, n.s.) or ITIs (Figure 5E; $F_{(2,21)} = 0.49$, n.s.) also remained unchanged after intra-VTA α_1-AR blockade during fear memory acquisition.

3.3. α_1-AR blockade in the VTA prior to memory retrieval (day 2) reduces expression of fear memory 24 h after CS-US pairings

Intra-VTA prazosin micro-infusion (in dose which disrupted acquisition of fear memory), immediately prior to fear memory retrieval (placements of the VTA cannula on Figure 2A;

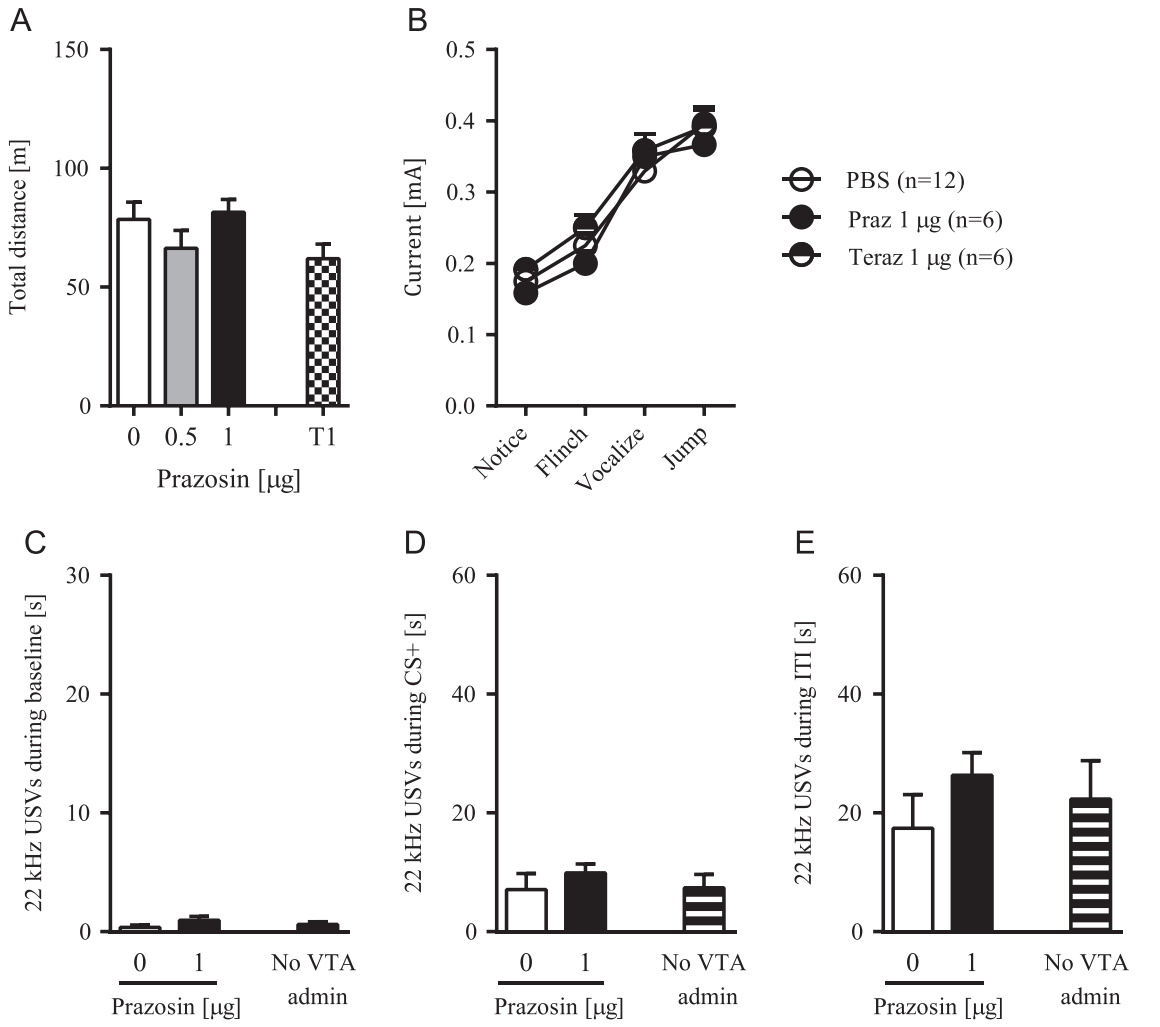

Figure 5 α_1-AR blockade in the VTA does not alter locomotion and footshock sensitivity. Intra-VTA micro-infusion of prazosin (0.5-1 µg/side) or terazosin (1 µg/side) has no effect on (A) locomotor activity measured as total distance travelled and (B) did not influence sensitivity to footshock. (C) Prazosin administration (1 µg/side), similarly to vehicle (0) and no intra-VTA administration, had no effect on the total number of 22-kHz USVs during baseline, (D) CS-US pairings and (E) ITIs on day 1. (F) Placements of the VTA micro-infusion cannula. Data are presented as the mean+SEM.

experimental timeline on Figure 6A), blocked the expression of context-induced (Figure 6B; t=4.63, df=17, $p<0.001$) fear responses. In addition, prazosin treatment, while having no effects on freezing in the novel environment (Figure 6C; t = 0.80, df = 13, n.s.), attenuated CS-evoked fear responses during CS presentation (Figure 6D; t = 3.26, df = 13, $p<0.01$) as well as during ITI (Figure 6E; t = 2.33, df = 13, $p<0.05$). More-detailed analysis of the effects of prazosin on each CS presentation or ITI exposure supported this observation (Figure 6F; dose $F_{(1,13)} = 10.61$, $p<0.01$, post hoc test $p<0.001$; CS presentation × dose $F_{(3.39)} = 2.03$, n.s., CS presentation $F_{(3,39)} = 1.83$, n.s. and Figure 6G; dose $F_{(1,13)} = 5.42$, $p<0.05$, post hoc test $p<0.01$; ITI exposure × dose $F_{(3.39)} = 1.72$, n.s., ITI exposure $F_{(3,39)} = 0.38$, n.s.). These results indicate that the effects of prazosin were selective for CS memory retrieval rather than general anxiety. In addition, there were no preexisting differences between control and terazosin-treated animals as subject demonstrated similar freezing during acquisition phase of fear conditioning (Figure S3A-E; n.s.; detailed statistics in the Figure Legend). Our results indicate that intra-VTA α_1-AR signaling appears to be necessary for both acquisition and retrieval of fear memory.

3.4. α_1-AR blockade in the VTA has no effects on anxiety-like behavior and locomotion

There were no effects of intra-VTA administration of prazosin on anxiety-like behaviors such as total time spent in the light zone (Figure 7A; $F_{(2,\ 24)} = 0.01$, n.s.), latency to first exit from the light zone (Figure 7B; $F_{(2,24)} = 2.25$, n.s.) or other behaviors such as number of rearings (Figure 7C; $F_{(2,24)} = 0.06$, n.s.), number of head line crossings (Figure 7D; $F_{(2,24)} = 0.80$, n.s.), and number of transitions (Figure 7E; $F_{(2,24)} = 0.06$, n.s.). Additionally, prazosin did not change behaviors measured in the open field: time spent

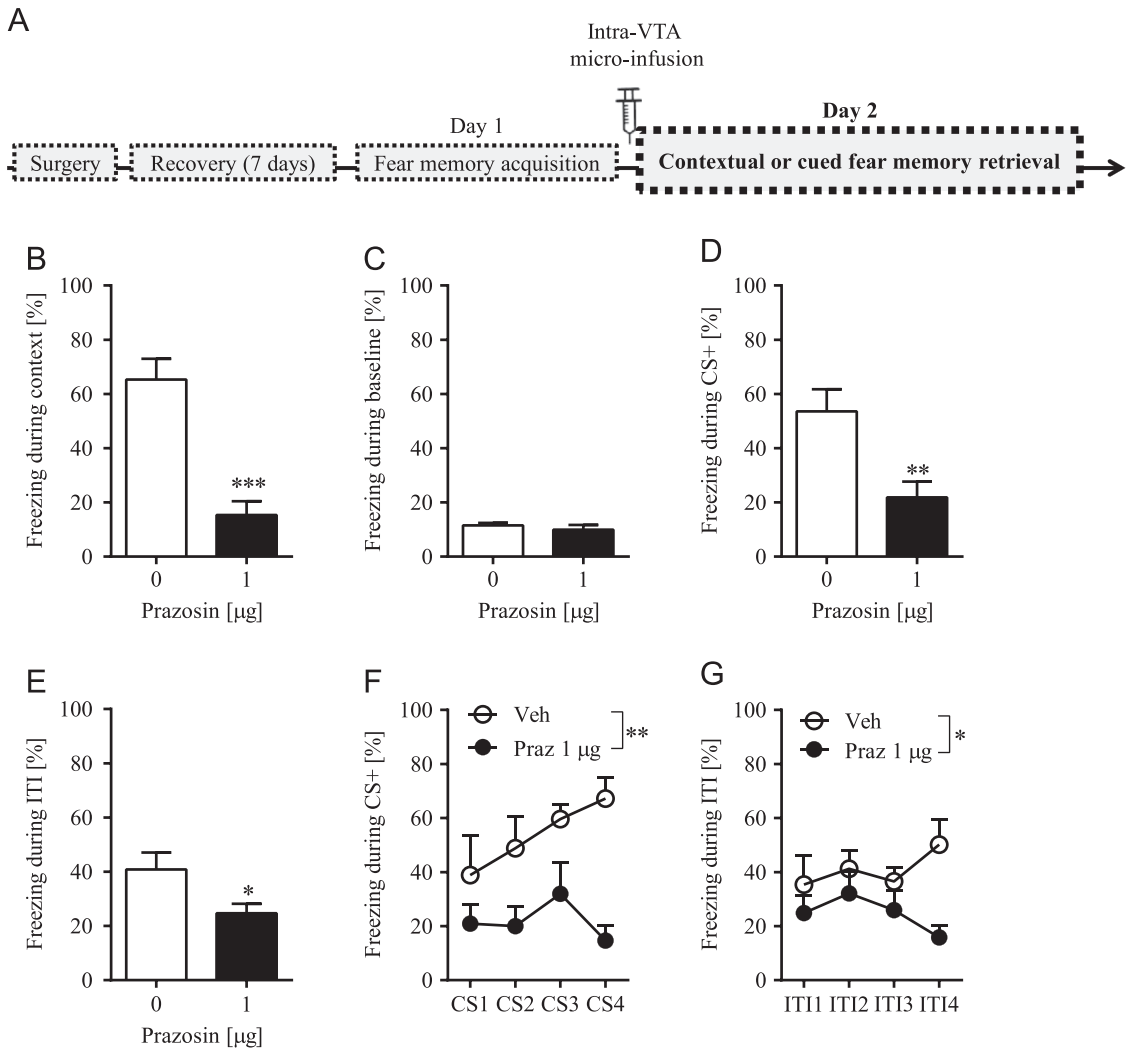

Figure 6 α_1-AR blockade in the VTA immedietly prior fear memory retrieval decreases the expression of conditioned fear memory. (A) The experimental timeline and schedule of intra-VTA micro-infusions during fear conditioning. (B) Intra-VTA micro-infusion of prazosin (1 μg/side) reduced freezing in response to conditioned context, (C) had no effects during baseline in novel environment ($t=0.78$, df = 32, n.s.), (D) attenuated total time spent freezing in response to CSs and (E) during ITIs on day 2. (F) Prazosin (Praz 1 μg/side) treatment decreased freezing to subsequent CS presentations as well as (G) during subsequent ITIs (time × treatment interaction $F_{(3,39)}=1.72$, n.s., time $F_{(3,39)}=0.38$, n.s., treatment $F_{(1,13)}=5.42$, $p<0.05$, post hoc test $p<0.01$). Data are presented as the mean+SEM of the percentage of time spent freezing. ***$p<0.001$, **$p<0.01$ vs. the vehicle group (white column or white circles).

in the center zone (Figure 7F; $F_{(2,24)} = 3.24$, $p = 0.057$) and total distance travelled (Figure 7G; $F_{(2,24)} = 1.32$, n.s.).

4. Discussion

4.1. Functional role of the VTA α_1-AR signaling

Here, our data show that intra-VTA α_1-AR blockade attenuated both acquisition and retrieval of cued as well as contextual fear memories. These results indicate that the VTA α_1-AR signaling is involved in conditioned fear memories, irrespective of their nature (i.e., contextual vs. cued) and are the first demonstrations of a crucial role of the VTA NAergic signaling in fear memory acquisition and retrieval.

We find that intra-VTA α_1-AR blockade with prazosin did not change freezing during fear memory acquisition phase but impaired expression of conditioned fear. In contrast, intra-VTA α_1-AR blockade with terazosin – another selective α_1-AR antagonist, attenuated fear responses to CS-footshock pairings during fear memory acquisition. Such terazosin effects on freezing during CS-US conditioning, including decreased fear response to novel environment (freezing during baseline on day 1) might suggest that intra-VTA α_1-AR blockade regulates locomotor activity or other behaviors. However, intra-VTA α_1-AR blockade had no effect on locomotion as both, prazosin and terazosin treatment, did not change distance traveled during the locomotor activity test. Furthermore, intra-VTA α_1-AR blockade had no effects on footshock sensitivity, anxiety-like behaviors or footshock-induced dysphoric affective state (evidenced by 22-kHz USV).

These results indicate that VTA α_1-AR signaling effectively regulates the encoding of stress memories. Such postulated

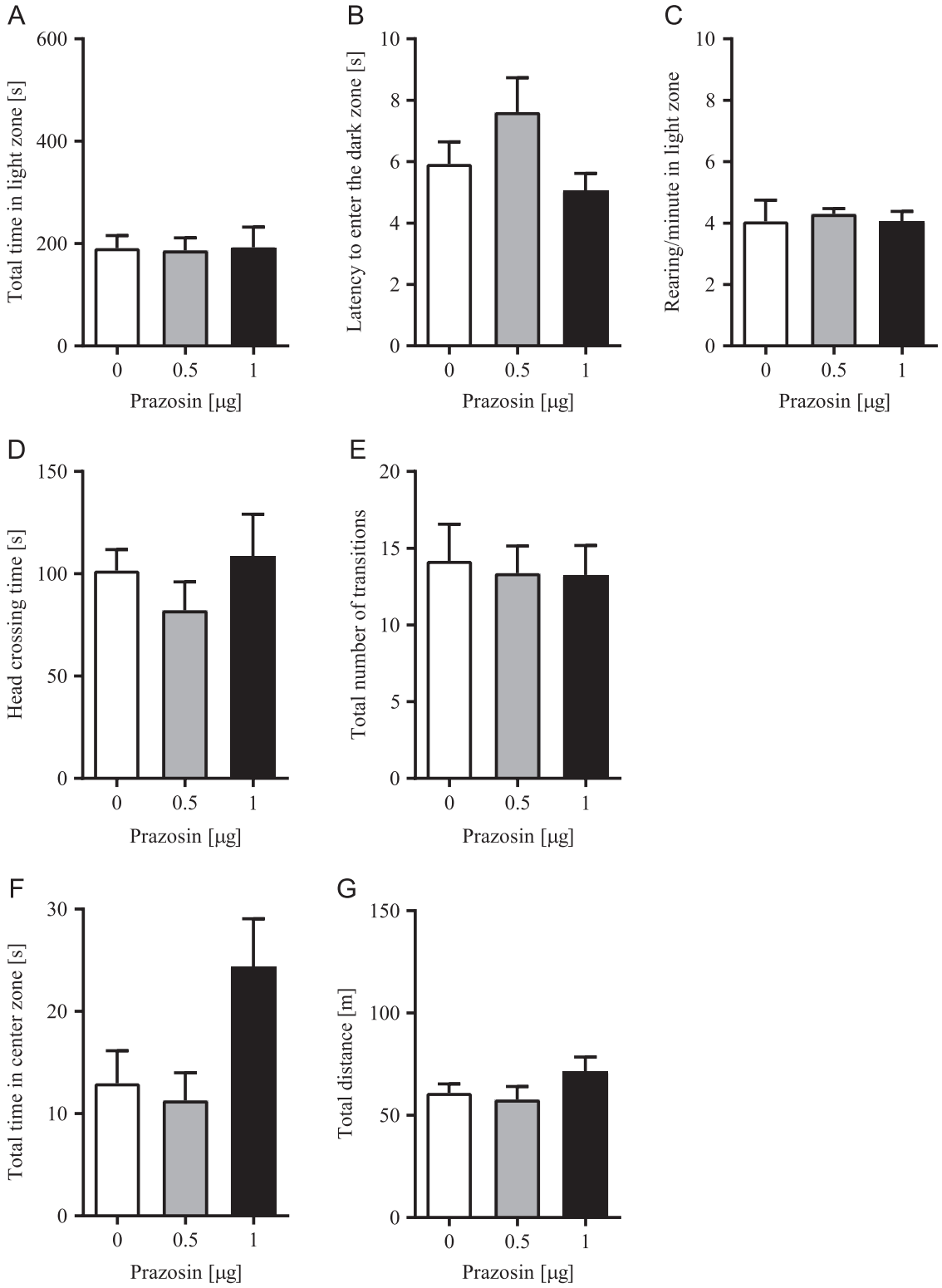

Figure 7 α_1-AR blockade in the VTA has no effect on anxiety-like behaviors. Intra-VTA micro-infusion of prazosin (0.5–1 μg/side) did not change behavior measured in the light/dark box (A–E) and during anxiogenic open field test (F and G). Data are presented as the mean + SEM.

functional role of VTA α_1-AR signaling is not surprising as NA system activity encodes selective attention, vigilance and stress responses (Aston-Jones and Cohen, 2005; Carter et al., 2010; Sara, 2009). The major noradrenergic inputs to the VTA include parts of the ventrolateral medulla (area A1) and the nucleus of the solitary tract (area A2) (Mejías-Aponte et al., 2009). The A1 and A2 cell groups were implied previously as modulating memory for emotionally arousing learning

conditions and stress responses (Clayton and Williams, 2000; Kerfoot and Williams, 2011; Rinaman, 2011; Williams et al., 2000). Moreover, A1 and A2 noradrenergic neurons were propose to serve as sensory relays to the prefrontal cortex regulating arousal and attention states (Robertson et al., 2013). Our data suggest the importance of noradrenergic signaling in the VTA in conditioned fear responses. We propose that in stressful situation, noradrenaline inputs (specifically via α_1AR) regulate the VTA activity and participate in fear reactions to aversive conditioned stimuli.

Mechanistically, the NA signaling in the VTA modulates neuronal activity via α_1-AR and α_2-AR, whereas β-adrenergic receptor protein expression has not been reported to date. The NA signaling via α_2-ARs, which are located presynaptically on the NAergic terminals, serves as a negative feedback mechanism and decreases NA release (Philipp et al., 2002). In contrast, majority of α_1-ARs are found on unmyelinated axons; however, α_1-ARs are also found on both glutamatergic and GABA-ergic axon terminals as well as on neuronal dendrites and on glial cells in the VTA (Mitrano et al., 2012; Rommelfanger et al., 2009). Interestingly, intra-VTA prazosin potently attenuates phasic DA release at terminals evoked by electrical VTA stimulation or systemic cocaine administration (Goertz et al., 2015). Thus, it can be proposed that salient stressful stimuli upregulate NAergic activity in the VTA and via α_1-ARs regulate downstream DA signaling necessary for the encoding of the emotionally disturbing event.

In particular, activity of the VTA DA terminals in the amygdala (AMG) might be important for the possible interpretations of the behavioral effects of our intra-VTA α_1-AR blockade. The DA projections from the VTA to the AMG are activated during presentations of aversive CS (de Souza Caetano et al., 2013; Nader and LeDoux, 1999; Stephens, 2005) and DA signaling is necessary for acquisition of cue-dependent fear memory (Fadok et al., 2009). In addition, intra-AMG administration of a DA D_1 receptor (D_1R) antagonist or intra-VTA administration of a DA D_2 receptor (D_2R) agonist (both leading to decreased D_1R signaling in the AMG) attenuates retrieval of conditioned fear memory (de Souza Caetano et al., 2013; Nader and LeDoux, 1999). The AMG DA signaling is proposed to enhance the signal-to-noise ratio (via enhancing strong inputs into the postsynaptic elements), which leads to increased AMG neuronal excitability enabling association between the CS and US (Stephens, 2005). Together with our results, it can be suggested that stressful stimuli upregulate the NAergic neuronal activity which then drives activity of the VTA DA neurons and subsequent DA release at terminals to enable fear memory acquisition and retrieval.

4.2. Relevance of α_1-AR signaling in stress-related disorders

Currently available pharmacotherapies for stress-related disorders include antidepressants and anxiolytic drugs (Parsons and Ressler, 2013), which are frequently poorly tolerated or ineffective. Interestingly, recent clinical studies demonstrated increasing evidence for the therapeutic effects of prazosin treatment in improving selected symptoms of PTSD as well as anxiety disorders (Birnbaum et al., 1999; Germain et al., 2012; Koola et al., 2014; Raskind et al., 2013, 2007, 2003; Taylor et al., 2008, 2006); for review see Arnsten et al. (2015). Furthermore, prazosin treatment has been suggested to enhance the effectiveness of exposure therapy in PTSD patients due to decreasing dysphoric hyperarousal and re-experiencing symptoms that often occur early in the course of exposure therapy and prior to therapeutic reductions (Arnsten et al., 2015). These positive effects of prazosin were hypothesized to involve its ability to weaken amygdala activation, thus facilitating the therapeutic response. Despite these promising results, the effectiveness of prazosin in treating stress-related disorders remains debated (Abul-Husn et al., 2015; George et al., 2016; Petrakis et al., 2016; Writer et al., 2014). The results of our study provide evidence for a crucial role of the α_1-AR signaling in the VTA – a novel brain locus of potential prazosin therapeutic effects in anxiety and stress-related disorders.

4.3. Conclusions

Our results identified a novel functional role for VTA α_1-AR signaling in the formation and expression of conditioned fear memory. Demonstration of the role of VTA α_1-AR signaling may help to understand the mechanism underlying α_1-AR antagonist pharmacotherapy for selective symptoms in stress-related disorders such as PTSD, anxiety and panic disorder. We propose that the VTA α_1-AR signaling helps to encode the environmental stimuli accompanying stressful encounters, improving the formation and retrieval of conditioned fear memories and prediction of future behavioral outcomes. Our data provide new insight into the noradrenergic mechanisms that underlie the encoding of fear memories.

Role of founding source

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Authors contributions

WBS designed and performed experiments, analyzed results, performed histological verifications as well as wrote the manuscript. KS performed parts of behavioral experiments and histological verifications as well as wrote parts of the Introduction section of the manuscript. WBS, KS, KP and GD have performed animals' surgeries. AK performed parts of behavioral experiments. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2017.05.008.

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