Neurobiological correlates of state-dependent context fear

Mariah A.A. Meyer, Kevin A. Corcoran, Helen J. Chen, Sonia Gallego, Guanguan Li, Veda V. Tiruveedhula, James M. Cook and Jelena Radulovic

1Department of Psychiatry and Behavioral Sciences, Northwestern University, Chicago, Illinois 60611, USA; 2Department of Chemistry and Biochemistry, University of Wisconsin–Milwaukee, Milwaukee, Wisconsin 53211, USA

Retrieval of fear memories can be state-dependent, meaning that they are best retrieved if the brain states at encoding and retrieval are similar. Such states can be induced by activating extrasynaptic γ-aminobutyric acid type A receptors (GABA$_{A}$R) with the broad α-subunit activator gaboxadol. However, the circuit mechanisms and specific subunits underlying gaboxadol’s effects are not well understood. Here we show that gaboxadol induces profound changes of local and network oscillatory activity, indicative of discoordinated hippocampal–cortical activity, that were accompanied by robust and long-lasting state-dependent conditioned fear. Episodic memories typically are hippocampus-dependent for a limited period after learning, but become cortex-dependent with the passage of time. In contrast, state-dependent memories continued to rely on hippocampal GABAergic mechanisms for memory retrieval. Pharmacological approaches with α-subunit-specific agonists targeting the hippocampus implicated the prototypic extrasynaptic subunits (α4) as the mediator of state-dependent conditioned fear. Together, our findings suggest that continued dependence on hippocampal rather than cortical mechanisms could be an important feature of state-dependent memories that contributes to their conditional retrieval.

Important events are typically encoded into memories that are readily accessible for retrieval. However, some memories are formed so that they are inaccessible to retrieval unless the brain is in a similar affective, stress- or drug-induced state as during encoding (Janet 1889; Braun 1984; Spiegel et al. 2011). These memories, known as state-dependent memories, have been studied extensively using various psychoactive drugs (Barnhart and Abbott 1967; Bustamante et al. 1970; Bruins Slot and Colpaert 1999; Romieu et al. 2006; Sanday et al. 2013), with the majority of examples coming from activators of GABAR (Overton 1964; Patel et al. 1979). These receptors also mediate state-dependent learning induced by non-GABAergic drugs, such as morphine (Zarrindast et al. 2006), that act through inhibitory neurons. GABAR type B agonists, such as baclofen, are ineffective (Nakagawa et al. 1993), supporting the view that state-dependent learning is primarily a GABA$_{A}$R-mediated phenomenon. In line with these findings, the GABA$_{A}$R agonist gadoxadol (GBX), which specifically activates extrasynaptic GABA$_{A}$R (Chandra et al. 2006), is capable of eliciting state-dependent contextual fear conditioning (Jovasevic et al. 2015).

Initially, memory retrieval processes are thought to depend on hippocampal–cortical interactions, but with the passage of time, the retrieval process becomes increasingly independent of the hippocampus and predominantly regulated by the cortex (Squire and Alvarez 1995). Interestingly, GBX potently activates hippocampal neurons while inhibiting cortical neurons, as indicated by up-regulation of immediate-early gene activity (Jovasevic et al. 2015). This finding suggests that one of the features of GBX-induced brain states supporting state-dependent fear conditioning is a change in the coordination of hippocampal–cortical activity. This is not surprising in view of GBX effects on local and circuit network activity in various brain regions (Jessen et al. 2014), however, the specific effects within hippocampal–cortical networks are not well defined.

GABA$_{A}$R are heterooligomeric pentamers composed of subunits α$_{1-6}$, β$_{1-4}$, γ$_{1-3}$, δ, ε, θ, π, and π$_{1-3}$ arranged around a central chloride-conducting pore (Whiting 2003; Olsen and Sieghart 2008, 2009); though most are composed of two α, two β, and one γ, δ, or ε subunit (Sieghart and Sperr 2002). Although the presence of the δ subunit correlates with extrasynaptic localization of GABA$_{A}$R (Belelli et al. 2009) and confers the sensitivity of GABA$_{A}$R to GBX (Meera et al. 2011), GBX binds nonspecifically to all α subunits of GABA$_{A}$R (Ebert et al. 1997). Though α$_{4,6}$ subunits seem to preferentially respond to GBX (Brown et al. 2002; Meera et al. 2011), the contribution of individual α/δ subunit complexes to state-dependent fear conditioning is not known.

Here we investigated the mechanisms by which GBX induces state-dependent learning and memory. As reflected in changes of local field potentials (LFPs) and behavioral pharmacological effects we studied GBX effects on hippocampal–cortical interactions. We also used GABA$_{A}$R subunit-specific agonists to study the ability of individual α subunits to mediate state-dependent learning.

Results
Induction of state-dependent learning by GBX
To induce state-dependent contextual fear, mice were injected intrahippocampally (i.h.) with vehicle (Veh) or GBX before contextual fear conditioning (Veh- and GBX-conditioned groups,
Effects of GBX on hippocampal–cortical LFPs

We next measured LFPs in the dorsal hippocampus (DH), retrosplenial cortex (RSC), and anterior cingulate cortex (ACC) to test how GBX affects activity in a hippocampal–cortical network that is necessary for contextual memory retrieval (Frankland et al. 2006; Corcoran et al. 2016). On each test day, there were three recording sessions: in the home cage prior to and 30 min after an intraperitoneal (i.p.) injection, and during post-injection testing for fear to the conditioning context. On the first test day, mice were injected with Veh; on the second day they were injected with GBX. Similar to i.h. infusions of GBX, i.p. injections caused a reduction in freezing to the conditioning context (t(16) = 4.29; P = 0.005; data not shown). Recordings were analyzed for power in DH and peak coherence between DH-RSC and DH-ACC in the δ, θ, and γ frequency bands.

Injection of Veh caused no changes to δ, θ, or γ power in DH, or to DH-RSC or DH-ACC θ peak coherence (Fig. 2A,C,D). GBX increased DH δ power (F(6, 12) = 8.52; P = 0.005), whereas γ power (F(6, 12) = 9.58; P = 0.0033 Fig. 2B,C) and RSC-DH θ peak coherence (F(6, 12) = 5.99; P = 0.016) decreased.

In the conditioning chamber on the Veh test day, DH δ power decreased (F(6, 12) = 5.59; P = 0.019) and θ power increased (F(6, 12) = 5.41; P = 0.021) compared with the post-injection home cage recording (Fig. 2C). θ Peak coherence also increased in the DH-RSC (F(6, 12) = 29.19; P < 0.001) and DH-ACC (F(6, 12) = 21.29; P < 0.001) site-pairs (Fig. 2D). In the conditioning chamber during the GBX retrieval test, δ power remained elevated above the pre-injection baseline, and there were no changes in θ or γ power relative to the post-injection recording in the home cage. Retrieval test-related increases in DH-RSC and DH-ACC θ peak coherence were also blocked by GBX (Fig. 2D), suggesting discoordinated hippocampal–cortical activity during the memory test.

Role of GABA_\text{A-R} α subunits in state-dependent contextual fear conditioning

To test whether GBX-induced state-dependent learning depends on the activity of specific GABA_\text{A-R} α subunits, we compared the abilities of α_1, α_2 preferential agonists to induce state-dependent contextual freezing. Mice were injected i.h. with Veh, the α_1, α_2 agonist GBX, the α_1,3 preferential agonist zolpidem (Pritchett and Seeburg 1990; Langer et al. 1992; Crestani et al. 2000), or the α_5 preferential agonists SH-053-R-CH3-2’F (Savić et al. 2008) or MP-III-022 (Stamenić et al. 2016) before contextual fear conditioning. Memory retrieval was assessed over three subsequent days, alternating between off- and on-conditioning drug tests. Only mice that were conditioned on GBX differed in their freezing between testing (n = 7 mice per group; main effect of conditioning drug F(1,12) = 11.7, P = 0.0051 and main effect of test F(2,24) = 4.2, P = 0.0278 Fig. 4A); all other groups froze similarly across test days (zolpidem, SH-053-R-CH3-2’F; n = 8–9 mice per group; no main effects; all F < 2.7, all P > 0.081 Fig. 4B,C; MP-III-022; n = 7–8 mice per group; main effect of conditioning drug F(1,12) = 9.491, P = 0.0095 Fig. 4D).

Thus, state-dependent contextual fear memory is not encoded ubiquitously by GABA_\text{A-R} α-subunit activation.

GABA_\text{A-R} agonists can sometimes substitute for one another in recovering state-dependent memories (Nakagawa and Iwasaki 1995). We therefore tested
whether the agonists described above can substitute for GBX in recovering retrieval of state-dependent context memories. Mice were injected i.h. with Veh or GBX before contextual fear conditioning and then tested for memory retrieval on consecutive days after i.h. Veh, GBX, zolpidem, SH-053-R-CH3-2′F, and MP-III-022 (Fig S; n = 9–10 mice per group; main effect of conditioning drug $F_{1,16} = 5.5$, $P = 0.0328$ and main effect of test drug $F_{1,64} = 3.16$, $P = 0.0196$). The freezing behavior of Veh-conditioned animals was significantly reduced by GBX (compared with freezing during the Veh test), and conversely, the freezing behavior of GBX-conditioned mice was significantly elevated on GBX when compared with Veh. Freezing during retrieval testing on zolpidem, SH-053-R-CH3-2′F, and MP-III-022 was indistinguishable from freezing during the Veh test, demonstrating that other GABA AR agonists could not substitute for GBX to induce retrieval of state-dependent contextual fear, such that retrieval is also GABA AR α-subunit activation-specific.

Retrieval of memory for contextual fear conditioning is associated with decreased δ and increased θ power, along with increased θ peak coherence between DH and both RSC and ACC (Corcoran et al. 2016). We found that pretest injection of GBX blocked all of these retrieval-related changes. Consistent with previous findings (Vyazovskiy et al. 2005), GBX also increased δ power, which is inversely correlated with arousal and wakefulness (Bódizs et al. 2001; Dang-Vu et al. 2008), and is typically considered important for offline consolidation of memory during sleep (Binder et al. 2014; Westerberg et al. 2015), rather than for online memory retrieval. Thus, the changes in power and coherence seen after GBX administration may be a neural representation of a state shift supporting the processing of memories with limited access.

It has been shown that the hippocampus has a time-limited role in retrieval of contextual fear memory (Kim and Fanselow 1992; Frankland et al. 2006). This time-dependence serves as a fundamental characteristic of systems consolidation theories, in which the circuits that support memory recall shift (Squire and Kandel 2000; Dudai 2004). One of the central tenets of the systems consolidation hypothesis is that the hippocampus functions as a temporary storage site for information, whereas permanent storage depends on a broadly distributed cortical network (Lashley 1950; McClelland et al. 1995; Squire and Alvarez 1995). Even with the hippocampal independence of remote memories being less certain than it once was (Goshen et al. 2011; Wiltgen and Tanaka 2013), because GBX caused discoordination of LFP coherence across a hippocampal-cortical network, we tested whether GBX-induced state-dependent contextual fear would shift cortically over time as predicted by systems consolidation theories. In activating hippocampal extrasynaptic GABA AR at varying times, we observed that GBX-conditioned animals retrieved contextual fear memories, whether tested 48 h or 29 d post-conditioning.

Thus, activation of the subset of hippocampal GBX-responsive GABA AR was sufficient to retrieve state-dependent contextual fear memory. Our findings suggest that contextual fear memories encoded in a state-dependent manner remain trapped within the region that encodes them, the hippocampus, and do not become cortically dependent with the passage of time. This finding is in line with previous work showing that state-dependent memories are subcortical in nature and that suppression of cortical activity...
does not impair retrieval of state-dependent memory (Girden and Culler 1937; Jovasevic et al. 2015).

Most rodent studies on fear conditioning and state-dependent memory use male subjects (Overton 1991; Lebron-Milad et al. 2012). It has recently been suggested, however, that freezing behavior, the passive response traditionally used to quantify Pavlovian fear conditioning, may differ between sexes (Gruene et al. 2015). We tested the ability of GBX to elicit state-dependent contextual fear in male and female mice. Both sexes displayed significantly higher freezing when tested on the drug that was present at conditioning, an effect that was found at both recent and remote time points. Thus, at least in this paradigm, state-dependent fear conditioning is sex-independent.

Of note, 8-9-wk-old male or female C57BL/6N mice were obtained from a commercial supplier (Harlan), individually housed on a 12-h light–dark cycle (lights on at 7 a.m.), and allowed ad libitum access to food and water. All animal procedures were approved by Northwestern University’s Animal Care and Use Committee in compliance with U.S. National Institutes of Health standards.

Cannulations and electrode surgeries
Mice were anesthetized with 1.2% tribromoethanol (vol/vol). Using a stereotaxic apparatus, bilateral 26-gauge guided cannulas (Plastic One) were implanted, as described previously (Corcoran 1997). Animals were allowed 10 days for postsurgical recovery before the commencement of fear conditioning.

Materials and Methods
Animals

Figure 3. Intrahippocampal GBX promotes recent and remote retrieval of state-dependent contextual fear memory. Freezing of Veh-conditioned mice was similar between Veh and GBX tests. GBX-conditioned mice froze significantly more when tested on GBX, both at recent and remote memory tests. Similar findings were obtained with male (A) and female (B) mice. (** P < 0.01, (*** P < 0.001 versus Veh test, within respective recent or remote time frame. Error bars represent standard error of the mean.

Figure 4. GABAAR α1,3,5-subunit preferential agonists do not induce state-dependent contextual fear conditioning. (A) Freezing behavior of GBX-conditioned mice was dependent upon testing conditions. (B) The GABAAR α1-subunit preferential agonist zolpidem did not affect freezing behavior. (C) GABAAR α3-subunit preferential agonist SH-053-R-CH3-2F conditioned and (D) MP-III-022 were also ineffective. (*) P < 0.05 versus Off-Drug test. Error bars represent standard error of the mean.
et al. 2011), targeting the dorsal hippocampus (DH) (1.7 mm posterior, ±1.0 mm lateral, and 2.0 mm ventral to bregma, according to the mouse brain atlas (Paxinos and Franklin 2004)). For electrode surgery, mice were implanted with insulated silver wires (100 µm diameter) aimed at RSC (1.8 mm posterior, 0.4 mm lateral, 0.75 mm ventral to bregma), DH (1.5 mm posterior, 1.0 mm lateral, 1.75 mm ventral), and ACC (1.3 mm anterior, 0.4 mm lateral, 1.75 mm ventral). All electrodes were placed in the left hemisphere. A gold screw lowered into the skull near the right parietal/occipital bone suture served as a reference and ground electrode. Two stainless steel jeweler’s screws were inserted in the skull to anchor the headcap. All wires were soldered to a 6-pin connector to which the recording devices were later attached, and the assembly was fixed to the skull with acrylic. Mice were allowed at least 72 h to recover from surgery prior to behavioral procedures.

Pharmacological treatments

Drugs were injected i.h. at a volume of 0.25 µL per side at a rate of 0.5 µL min⁻¹ or i.p. at a volume of 200 µL 20-30 min prior to fear conditioning or retrieval test. α1-δ- and/or α2-δ-subunit-containing GABAAR were activated by gaboxadol hydrochloride (0.5 µg per DH, dissolved in artificial cerebrospinal fluid; Sigma-Aldrich) also known as 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP); α1-δ-subunit-containing GABAAR were activated by zolpidem (25 µg per DH, 100% dimethyl sulfoxide (DMSO); Sigma-Aldrich); and α2-δ-subunit-containing GABAAR were activated by SH-053-R-CH3-2F (20 µg per DH, dissolved in 90% DMSO; synthesized at University of Wisconsin–Milwaukee) or MP-III-022 (12.5 µg per DH; dissolved in 60% DMSO; synthesized at University of Wisconsin–Milwaukee).

LFP recordings

On subsequent days following contextual fear conditioning, mice were tested for memory retrieval after i.p. injection of Veh (0.9% saline; 0.2 mL and GBX (4 mg/kg in 0.2 mL 0.9% saline). LFP recordings began as soon as mice were connected to wireless four-channel NeuroLogger recording devices (TE Systems), and continued until the end of each test session (up to 55 min total). Continuous recordings were made with a sampling rate of 500 Hz. Preamplification, analog-to-digital conversion (unity gain buffer, AC input range ±750 µV, 1000x gain, ADC resolution 8bits), and data storage all occurred on the NeuroLogger. After each session, the NeuroLogger was removed and data were downloaded to a computer for later analysis.

For each of the test days, recordings were converted to a Matlab-compatible format for analysis of the 3 min mice were in their home cages just prior to drug injection, a 3 min period in the home cage beginning 30 min post-injection and ending immediately before mice were placed in the conditioning chambers, and the 3-min test sessions. Spectral analyses were performed using open-source Chronux (http://Chronux.org) algorithms as described previously (Kay and Freeman 1998; Rojas-Libano et al. 2014). Power and coherence spectra were computed for the δ (1–4 Hz), θ (4–12 Hz), and γ (30–80 Hz) frequency bands across each 3 min recording session using 35 half-overlapping 10-sec windows with four tapers (resulting in a frequency resolution of 1.4 Hz). Coherence was then transformed to z-coherence using the inverse hyperbolic tangent transform (Kay and Freeman 1998). There was no filtering. The peak frequency within each band was taken as the center frequency, and coherence at this peak was used as the dependent measure. Coherence, by definition, is normalized by power, allowing for direct comparison across subjects. Peak coherence and average power were calculated for each mouse in each session and used for statistical analysis.

Fear conditioning

Contextual fear conditioning was performed in an automated system (TSE Systems) as previously described (Corcoran et al. 2011). Briefly, mice were exposed for 3 min to context, followed by a footshock (3 sec, 0.8 mA, constant current). In state-dependent contextual fear subunit specificity experiments, mice were exposed for 3 min to context, followed by a footshock (2 sec, 0.7 mA, constant current). Twenty-four hours later, mice were tested for memory retrieval. Testing consisted of 3 min in the conditioning context, during which freezing was measured every 10 sec. Freezing was expressed as a percentage of the total number of observations during which the mice were motionless.

Analysis

Changes in power and peak coherence across recording sessions were determined separately for Veh and GBX using repeated-measures ANOVA. Following each significant F ratio, Tukey’s HSD tests were used to determine the significance of two comparisons: (1) preinjection vs. post-injection in the home cage, and (2) post-injection in the home cage versus post-injection during the retrieval test. Changes in freezing were determined using two-way repeated-measures ANOVA. Following each significant F ratio, Tukey’s HSD tests were used to determine the significance of comparisons between two time points and Bonferroni tests were used to determine the significance of comparisons between three or more time points. Verification of electrode or cannula placements was made from coronal sections through DH, RSC, or ACC. Statistical analyses were performed using GraphPad Prism or StatView software.

Acknowledgments

This work was supported by NIMH grant MH078064 to J.R., the NIH grants MH096463 and NS076517 to J.M.C., the NIH Neurobiobiology of Information Storage training grant MH067564 to M.A.A.M., and the NUCATS grant UL1TR001422 and a Davee award to K.A.C. Additionally, we thank Anita Guedea for assistance with the behavioral experiments and the personnel at the University of Wisconsin-Milwaukee’s Shimadzu Laboratory for Advanced and Applied Analytical Chemistry and the Milwaukee Institute for Drug Discovery who assisted with the synthesis of MP-III-022 and SH-053-R-CH3-2F.

References

Barnhart SS, Abbott DW. 1967. Dissociation of learning and meprobamate. Psychol Rep 20:520–522.

Belelli D, Harrison NL, Maguire J, Macdonald RL, Walker MC, Cope DV. 2009. Extrasynaptic GABAA receptors: form, pharmacology, and function. J Neurosci 29:12757–12763.

Binder S, Ravolh J, Bor J, Marshall L. 2014. Transcranial slow oscillation stimulation during NREM sleep enhances acquisition of the radial maze task and modulates cortical network activity in rats. Front Behav Neurosci 7:220.

Blanchard RJ, Blanchard DC. 1969. Crouching as an index of fear. J Comp Physiol Psychol 67:370–375.

Bödazu R, Kántor S, Szabo G, Szács A, Éross L, Halász P. 2001. Rhythmic hippocampal slow oscillation characterizes REM sleep in humans. Hippocampus 11:747–753.

www.learnmem.org
Spiegel D, Loewenstein RJ, Lewis-Fernández R, Sar V, Simeon D, Vermetten E, Cardena E, Brown RJ, Dell PF. 2011. Dissociative disorders in DSM-5. *Depress Anxiety** 28: 824–852.

Squire LR, Alvarez P. 1995. Retrograde amnesia and memory consolidation: a neurobiological perspective. *Curr Opin Neurobiol* 5: 169–177.

Squire LR, Kandel ER. 2000. *Memory: from mind to molecules*. Macmillan, New York.

Stamenić TT, Poe MM, Rehman S, Santraš A, Drivoč B, Scholze P, Ernst M, Cook JM, Savič MM. 2016. Ester to amide substitution improves selectivity, efficacy and kinetic behavior of a benzodiazepine positive modulator of GABAA receptors containing the α5 subunit. *Eur J Pharmacol* 791: 433–443.

Sun C, Sieghart W, Kapur J. 2004. Distribution of α1, α4, γ2, and δ subunits of GABAA receptors in hippocampal granule cells. *Brain Res* 1029: 207–216.

Vyazovskiy VV, Kopp C, Bösch G, Tobler I. 2005. The GABAA receptor agonist THIP alters the EEG in waking and sleep of mice. *Neuropharm* 48: 617–626.

Weingartner H, Adefris W, Eich JE, Murphy DL. 1976. Encoding-imagery specificity in alcohol state-dependent learning. *J Exp Psychol Hum Learn Mem* 2: 83–87.

Westerberg CE, Florczak SM, Weintrab S, Mesulam M, Marshall L, Zee PC, Paller KA. 2015. Memory improvements via slow-oscillatory stimulation during sleep in older adults *Neurobiol Aging* 36: 2577–2586.

Whiting PJ. 2003. GABA-A receptor subtypes in the brain: a paradigm for CNS drug discovery? *Drug Discov Today* 8: 445–450.

Wiltgen BJ, Tanaka KZ. 2013. Systems consolidation and the content of memory. *Neurobiol Learn Mem* 106: 365–371.

Zarrindast MR, Noorbakhshnia M, Motamedi F, Haeri-Rohani A, Rezayof A. 2006. Effect of the GABAergic system on memory formation and state-dependent learning induced by morphine in rats. *Pharmacology* 76: 93–100.

Received March 10, 2017; accepted in revised form June 7, 2017.