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The basolateral amygdala can mediate the effects of fear memory on sleep independently of fear behavior and the peripheral stress response

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ABSTRACT

Fear conditioning associated with inescapable shock training (ST) and fearful context re-exposure (CR) alone can produce significant behavioral fear, a stress response and alterations in subsequent REM sleep. These alterations may vary among animals and are mediated by the basolateral nucleus of the amygdala (BLA). Here, we used the $GABA_A$ agonist, muscimol (Mus), to inactivate BLA prior to CR and examined the effects on sleep, freezing and stress-induced hyperthermia (SIH). Wistar rats (n = 28) were implanted with electrodes for recording sleep, data loggers for recording core body temperature, and with cannulae aimed bilaterally into BLA. After recovery, the animals were habituated to the injection procedure and baseline sleep was recorded. On experimental day 1, rats received ST (20 footshocks, 0.8 mA, 0.5 s duration, 60 s interstimulus interval). On experimental day 7, the rats received microinjections (0.5 μl) into BLA of either Mus (1.0 μM; n = 13) or vehicle (Veh; n = 15) prior to CR (CR1). On experimental day 21, the animals experienced a second CR (CR2) without Mus. For analysis, the rats were separated into 4 groups: (Veh-vulnerable (Veh-Vul; n = 8), Veh-resilient (Veh-Res; n = 7), Mus-vulnerable (Mus-Vul; n = 7), and Mus-resilient (Mus-Res; n = 6)) based on whether or not REM was decreased, compared to baseline, during the first 4 h following ST. Pre-CR1 inactivation of BLA did not alter freezing or SIH, but did block the reduction in REM in the Mus-Vul group compared to the Veh-Vul group. These data indicate that BLA is an important region for mediating the effects of fearful memories on sleep.

1. Introduction

The conditioned fear paradigm is a powerful classical conditioning procedure in which an association is formed between an explicit neutral stimulus (generally a light or auditory stimulus) or situational context and an aversive stimulus (usually footshock) (Davis, 1992a, 1992b). After training, the previously neutral explicit stimulus or context assumes fear-inducing qualities similar to the aversive stimulus and produces similar behavioral and physiologic outcomes (Misslin, 2003; Nijsen et al., 1998; Stiedl, Tovote, Ogren, & Meyer, 2004). Changes in sleep also can be fear-conditioned; i.e., evoking fearful memories produce changes in sleep in the period after fear was evoked that are similar to those that occur after the initial fearful stressor. However, the relationship of fear conditioning to sleep is complex. The best evidence of this complexity is that fear conditioning and the stress response are not predictive of subsequent alterations in sleep. For example, extensive training using inescapable shock (IS) as the aversive stimulus can significantly reduce rapid eye movement (REM) sleep and training with escapable shock (ES) can produce significant increases in REM sleep (Sanford, Yang, Wellman, Liu, & Tang, 2010; Yang, Wellman, Ambrozewicz, & Sanford, 2011) whereas indices of fear (freezing) and stress (stress-induced hyperthermia (SIH)) are similar for both conditions (Yang et al., 2011). Given increasing evidence that REM is important for the processing of emotional (Walker & van der Helm, 2009) and traumatic memories (Mellman, Bustamante, Fins, Pigeon, & Nolan, 2002; Mellman, Pigeon, Nowell, & Nolan, 2007), understanding the neural processes by which fear and stress can produce directionally different alterations in sleep is likely key to understanding sleep disturbances in disorders such as posttraumatic stress disorder (PTSD), which is viewed as arising from abnormal functioning of the brain's fear system (Shvil, Rusch, Sullivan, & Neria, 2013).

The amygdala is central in current concepts of fear conditioning (e.g., (Myers & Davis, 2007)), it is hyperactive in PTSD (Bremner

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et al., 2005), and it has an established role in regulating fear- and stress-induced alterations in sleep, especially REM sleep (Liu, Yang, Wellman, Tang, & Sanford, 2009; Liu et al., 2011; Wellman, Yang, Ambrozewicz, Machida, & Sanford, 2013). The central nucleus of the amygdala (CNA) (Inagaki, Kawai, Matsuzaki, Shiosaka, & Tohyama, 1983; Peyron, Petit, Rampon, Jouvet, & Luppi, 1998; Price, Russchen, & Amaral, 1987; Semba & Fibiger, 1992), along with the lateral division of the bed nucleus of the stria terminalis (BNST) (Amaral, Price, Pitkanen, & Carmichael, 1992; Davis & Whalen, 2001), projects to brainstem REM sleep regulatory regions. The basolateral nucleus of the amygdala (BLA) has output to both CNA and BNST (Amaral et al., 1992; Davis & Whalen, 2001) and likely regulates the influence of fearful experiences and memories on REM sleep via these descending pathways. Several studies have reported that damage to, or inactivation of, BLA prior to or after fear conditioning (e.g., (Cousens & Otto, 1998; Koo, Han, & Kim, 2004; Maren, 1998; Maren, Aharonov, & Fanselow, 1996; Sacchetti, Lorenzini, Baldi, Tassoni, & Bucherelli, 1999)) or prior to context re-exposure (CR) (Helmstetter & Bellgowan, 1994; Muller, Corodimas, Fridel, & LeDoux, 1997) attenuates freezing in the fearful context. These studies have been taken to support a role for BLA in the acquisition and consolidation of fear conditioning. However, functional inactivation of BLA using the $GABA_A$ agonist muscimol (Mus) after single trial fear conditioning did not prevent learning, thereby suggesting that BLA is important for fear acquisition, but not fear memory consolidation (Wilensky, Schafe, & LeDoux, 2000). Thus, there is still some question as to the putative role of BLA in the acquisition and consolidation of fear memory.

BLA appears to be critical for the formation of fear memories that can impact sleep. Microinjections of the corticotropin releasing factor antagonist, antalarmin (ANT) into BLA of rats prior to shock training (ST) blocked both IS-induced reductions in REM sleep and the formation of memories that alter sleep without blocking fear memory as indicated by contextual freezing (Wellman et al., 2013). By comparison, global inactivation of BLA with microinjections of Mus, prior to ST blocked the post-training reduction in REM sleep seen in vehicle treated rats (Wellman, Fitzpatrick, Machida, & Sanford, 2014). Furthermore, in Mus treated rats, REM sleep after re-exposures to the fearful context was at baseline levels and freezing was significantly attenuated. Together, these data indicated that BLA is an important regulator of stress- and fear-induced alterations in sleep and that it is critical for the acquisition of fear memories that can impact sleep.

We recently found that outbred Wistar rats can show different REM responses to IS that are independent of freezing and SIH. Some show pronounced decreases in post-ST REM whereas others do not show reductions compared to baseline levels, thereby suggesting individual differences in the sleep response to stress (Wellman et al., 2016). Differences in REM also were observed with post-ST inactivation of BLA. However, post-ST inactivation of BLA blocked the conditioned reduction in REM without blocking freezing or reducing SIH (Wellman et al., 2016). These data suggest that activity in BLA during memory consolidation is important for determining the subsequent effects of fearful memories on REM, but not for forming memories of fearful events. The role that BLA may play in the recall of fearful memories that impact REM is not known.

In this study, we trained rats with ST and inactivated BLA with microinjections of Mus prior to CR and examined the relationship between fear behavior and sleep on two exposures to the fearful context alone. Our goal was to assess whether pre-recall inactivation of BLA could alter fear memory as assessed by fear behavior and fear-induced alterations in sleep. We also recorded core body temperature in order to assess SIH as an index of the stress response.

2. Materials and methods

2.1. Subjects

The subjects were 28 ninety-day-old Wistar rats obtained from Harlan Laboratories (Frederick, MD). Upon arrival, the rats were individually housed in polycarbonate cages and given *ad lib* access to food and water. The rooms were kept on a 12:12 light:dark cycle with lights on from 07:00 to 19:00 h. Light intensity during the light period was 100–110 lux and less than 1 lux during the dark period. Ambient room temperature was maintained at 24.5 ± 0.5 °C.

2.2. Surgery

Beginning one week following arrival, the rats were anesthetized with isoflurane (5% induction; 2% maintenance) and implanted with skull screw electrodes for recording their electroencephalogram (EEG) and stainless steel wire electrodes sutured to the dorsal neck musculature for recording their electromyogram (EMG). Leads from the recording electrodes were routed to a 9-pin miniature plug that mated to one attached to a recording cable. Bilateral guide cannulae (26 ga.) for microinjections into BLA were implanted with their tips aimed 1.0 mm above BLA (A 2.6, ML ± 4.8, DV 8.0 (Kruger, Saporta, & Swanson, 1995)). The recording plug and cannulae were affixed to the skull with dental acrylic and stainless steel anchor screws. During the same surgery, temperature recorders (SubCue Standard Dataloggers, Canadian Analytical Technologies Inc. Calgary, Alberta, Canada) were implanted intraperitoneally. Ibuprofen (15 mg/kg) was made available in their water supply for relief of post-operative pain. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals* and were approved by Eastern Virginia Medical School's Animal Care and Use Committee (Protocol # 13-003).

2.3. Drugs

Mus (muscimol hydrobromide, 5-aminomethyl-3-hydroxyisoxazole) was obtained from Sigma–Aldrich, St. Louis, MO, USA. It was prepared in pyrogen-free distilled water as a vehicle (Veh; 1.0 μM)) and was sonicated for 20 min to ensure that the drug was dissolved completely. A fresh solution was prepared for each experimental day.

2.4. Procedures

All experimental manipulations were conducted during the fourth h of the light period such that sleep recording would begin at the start of the fifth hour. This resulted in 8 h of light period recording on each experimental day.

Home cages were changed at least 3 days prior to injection day. The same room was used for animal housing and sleep recording. The microinjections and behavioral testing were conducted in a separate room from that used for recording.

2.4.1. Sleep recording

For recording sleep, each animal in its home cage, was placed on a rack outfitted for electrophysiological recording and a lightweight, shielded cable was connected to the miniature plug on the rat's head. The cable was attached to a commutator that permitted free movement of the rat within its cage. EEG and EMG signals were processed by a Grass, Model 12 polygraph equipped with model 12A5 amplifiers and routed to an A/D board (Model USB-2533, Measurement Computing) housed in a personal computer.