



# Antagonism of corticotropin releasing factor in the basolateral amygdala of resilient and vulnerable rats: Effects on fear-conditioned sleep, temperature and freezing<sup>☆</sup>

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## ABSTRACT

The basolateral nucleus of the amygdala (BLA) plays a significant role in mediating individual differences in the effects of fear memory on sleep. Here, we assessed the effects of antagonizing corticotropin releasing factor receptor 1 (CRFR1) after shock training (ST) on fear-conditioned behaviors and sleep. Outbred Wistar rats were surgically implanted with electrodes for recording EEG and EMG and with bilateral guide cannulae directed at BLA. Data loggers were placed intraperitoneally to record core body temperature. The CRFR1 antagonist, antalarmin (ANT; 4.82 mM) was microinjected into BLA after shock training (ST: 20 footshocks, 0.8 mA, 0.5 s duration, 60 s interstimulus interval), and the effects on sleep, freezing and the stress response (stress-induced hyperthermia, SIH) were examined after ST and fearful context re-exposure alone at 7 days (CTX1) and 21 days (CTX2) post-ST. EEG and EMG recordings were scored for non-rapid eye movement sleep (NREM), rapid eye movement sleep (REM) and wakefulness. The rats were separated into 4 groups: Vehicle-vulnerable (Veh-Vul; n = 10), Veh-resilient (Veh-Res; n = 11), ANT-vulnerable (ANT-Vul; n = 8) and ANT-resilient (ANT-Res; n = 8) based on whether, compared to baseline, the rats showed a decrease or no change/increase in REM during the first 4 h following ST. Post-ST ANT microinjected into BLA attenuated the fear-conditioned reduction in REM in ANT-Vul rats on CTX1, but did not significantly alter REM in ANT-Res rats. However, compared to Veh treated rats, REM was reduced in ANT treated rats on CTX2. There were no group differences in freezing or SIH across conditions. Therefore, CRFR1 in BLA plays a role in mediating individual differences in sleep responses to stress and in the extinction of fear conditioned changes in sleep.

## 1. Introduction

Experimental fear conditioning is an important model for examining how stressful events, through the formation of fear memories, can produce lasting effects on behavior and psychological health. In this paradigm, an association is formed between an explicit neutral cue (generally a light or auditory stimulus) or situational context and an aversive stimulus (usually footshock) (Davis, 1992). Subsequently, presentation of the cue or context elicits behavioral and physiologic outcomes similar to those induced by the original stressor (Nijsen et al., 1998; Stiedl et al., 2004).

Changes in sleep also can be fear-conditioned. However, fear-conditioned changes in sleep can vary depending on stressor characteristics and with individual differences. For example, rapid eye movement sleep (REM) can be reduced after training with inescapable shock (IS)

and increased after training with escapable shock (ES) (Sanford et al., 2010; Yang et al., 2011). Some outbred Wistar rats show reductions in REM during the first 4 h after training with IS whereas others show normal amounts or even increased REM shortly after training with IS (Wellman et al., 2017; Wellman et al., 2016). Evoking contextual fear memories produce virtually identical directional changes in REM to those observed in response to the initial stressor. These differences in REM in putatively vulnerable (Vul, reduced REM) and resilient (Res, normal/increased REM) rats occur even though indices of fear (behavioral freezing) and stress (stress-induced hyperthermia (SIH)) are virtually identical across groups (Sanford et al., 2010; Wellman et al., 2017; Wellman et al., 2016; Yang et al., 2011). The importance of differences in REM after stress is not fully understood; however, reduced and fragmented REM have been linked to the onset of post-traumatic stress disorder (PTSD) (Mellman et al., 2002; Mellman et al.,

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2007), which is viewed as arising from abnormal functioning in the brain's fear system (Shvil et al., 2013). There also is increasing evidence that REM is important for the processing of emotional (Walker and van der Helm, 2009) and traumatic memories (Mellman et al., 2002; Mellman et al., 2007). Thus, REM may play a significant role in processing stressful emotion, and it may be a useful marker of differences in the stress response that are not captured by standard behavioral and physiological measures of fear memory and stress (Wellman et al., 2016). The differences in REM in the Vul and Res rats may also reflect differences in the time course of the restoration of sleep homeostasis after experiencing stress (Tang et al., 2005).

The basolateral nucleus of the amygdala (BLA) has an established role in the acquisition and consolidation of fear conditioning (e.g., (Cousens and Otto, 1998; Koo et al., 2004; Maren, 1998; Muller et al., 1997)). It also regulates fear- and stress-induced alterations in sleep, especially REM (Wellman et al., 2013), and it plays a critical role determining how fear memories impact sleep. For example, inactivation of BLA with microinjections of the GABA<sub>A</sub> receptor agonist, muscimol, prior to training with IS blocked post-training reductions in REM and attenuated contextual freezing and fear-conditioned reductions in REM (Wellman et al., 2014). However, post-training inactivation of BLA with muscimol did not alter REM after shock training: Vul animals showed decreases and Res animals did not. Post-training inactivation of BLA did block the subsequent fear-conditioned reduction in REM in Vul rats whereas REM in the Res rats was not altered. Pre-context inactivation of BLA also attenuated the reduction in REM in the Vul rats, but did not significantly alter REM in the Res rats (Wellman et al., 2017). The changes in REM were independent of freezing and SIH in both Vul and Res animals regardless of whether BLA was inactivated after shock training or prior to context re-exposure. These data suggest that individual differences in BLA functioning may mediate resilience and vulnerability to stress as manifested by alterations in REM.

Corticotropin releasing factor (CRF) plays a significant role in mediating central nervous system responses to stressors (Heinrichs et al., 1995; Koob, 1999) and has roles in anxiety and conditioned fear (Liang et al., 1992; Swerdlow et al., 1989). CRF also plays a role in fear-conditioned alterations in sleep and microinjections of the CRF receptor 1 (CRFR1) antagonist, antalarmin (ANT) locally in the central nucleus of the amygdala (CNA) prior to fearful context re-exposure (Liu et al., 2011) can block fear-induced reductions in REM in rats without blocking fear-induced freezing. In BLA, microinjections of ANT prior to IS can block both IS- and fear-induced reductions in REM (Wellman et al., 2013). These alterations in REM can occur without blocking fear-induced freezing (Liu et al., 2011; Wellman et al., 2013). It is not known whether post-training antagonism of CRFR1 in BLA plays a role in the consolidation of fear memories and their effects on REM. It also is not known whether CRFR1 in BLA plays a role in mediating individual differences in the effects of fear memory on REM. To assess these possibilities, we separated Wistar rats into Vul and Res groups based on REM amounts observed in the first 4 h after training with IS and examined stress-induced and fear-conditioned changes in sleep as previously described (Wellman et al., 2017; Wellman et al., 2016). In a subset of animals, we microinjected ANT into BLA immediately after ST to determine whether CRFR1 in BLA has a role in mediating individual differences in ST-induced and fear-conditioned changes in sleep. Additionally, we recorded core body temperature to assess SIH as an index of the stress response and we examined behavioral freezing as an index of fear memory.

## 2. Subjects and methods

### 2.1. Subjects

The subjects were 37 nine-week-old, male 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 lx and < 1 lx during the dark period. Ambient room temperature was maintained at  $24.5 \pm 0.5^\circ\text{C}$ .

### 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  $\pm$  4.8, DV 8.0 (Kruger et al., 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 in a subset of rats. 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.

### 2.3. Drugs

ANT (antalarmin hydrochloride, *N*-Butyl-*N*-ethyl-2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-*d*]pyrimidin-4-amine hydrochloride) was obtained from Sigma-Aldrich, St. Louis, MO, USA. It was prepared in pyrogen-free distilled water (Vehicle (Veh); ANT: 4.82 mM) and was sonicated for at least 20 min to ensure that the drug was dissolved completely. A fresh solution was prepared for each experimental day. This dosage was chosen because we previously found that it did not alter spontaneous sleep when microinjected into BLA, but when microinjected prior to fear acquisition, it prevented both footshock and fearful context induced reductions in REM (Wellman et al., 2013).

### 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 h. This resulted in 8 h of light period recording on each experimental day.

Home cages were changed at least 3 days prior to each treatment 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. The signals were digitized at 256 Hz and collected in 10 s epochs using the SleepWave™ (Biosoft Studio) data collection program.

The rats were allowed a minimum post-surgery recovery period of 14 days prior to beginning the experiment. Once recovered, the animals were randomly assigned to one of two groups: ANT after ST (ANT;  $n = 16$ ) or Veh after ST (Veh;  $n = 21$ ) for studies of its effects on ST and

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