Journal of Neuroscience Methods 177 (2009) 355–360

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Neck electromyography is an effective measure of fear behavior

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ARTICLE INFO

Article history:
Received 30 August 2008
Received in revised form 15 October 2008
Accepted 15 October 2008

Keywords:
Fear
Freezing
Electromyogram
Electroencephalogram
Neck
Sleep

ABSTRACT

Conventional methodology for examining conditioned fear has involved visual-based technologies such as cameras and direct observation. These methods are excellent screening tools of fear behavior (i.e. freezing). However, they do not provide a physiological output, which could be useful to examine neurophysiological correlates of fear. We found that dorsal neck electromyography can be used as a method to score fear behavior and is as effective as visual-based scoring. Since electromyogram is a physiological measure it can be used in either the light or dark to score freezing behavior, without use of any camera or visual observation. We also show that electromyogram-based scoring methodologies, in conjunction with electroencephalogram, are useful to discriminate fear from sleep.

1. Introduction

Genetic mouse models are a fundamental tool to examine the molecular basis of behavior (Fanselow and Poulos, 2005; Ko et al., 2005; Mogil et al., 2000; Steenland et al., 2008; Tang et al., 1999; Wei et al., 2002; Zhao et al., 2005). Conditioned fear is one of the most widely used methodologies to examine emotional memory in rodents (LeDoux, 2000). Fear behavior can be identified as an absence of movement (Blanchard and Blanchard, 1972; Bolles, 1970; Fanselow, 1984; LeDoux et al., 1986), and is known as freezing. However, a similar definition holds for the behavioral description of sleep, which is also seen as a state of immobility (Siegel, 2005). Thus, it is essential that; freezing, which consists of a defensive behavior (Kim and Jung, 2006; LeDoux, 2000; McNaughton and Corr, 2004), be dissociated from sleeping, which ultimately disengages an animal from the external environment. Confusion over identifying these two behaviors could become and issue when examining fear memory in genetic mice which also demonstrate alterations in sleep (Espinosa et al., 2004; Steenland et al., 2008).

The most common method to examine fear behavior is through direct visual observation or through use of motion sensitive cameras (Anagnostaras et al., 2000; Blanchard and Blanchard, 1972; Bolles, 1970; Espinosa et al., 2004; Fanselow, 1984; Kopec et al., 2007; LeDoux et al., 1986; Marchand et al., 2003; Takahashi, 2004). Direct visual observation can produce inaccuracies in measurement due to varying reaction times and attention. Camera systems do not provide a physiological correlate of behavior, but infrared cameras are available for recordings in the dark (Takahashi, 2004). Thus, it would be useful for fear measurement methods to provide both a physiological correlate and for freezing behavior to be recorded regardless of the time of day.

Trace-fear conditioning in rodents, involves the association of a neutral conditioned stimulus (CS; generally a tone) with an aversive unconditioned stimulus (US; shock) over a time interval (referred to as the trace interval). The acquisition and retention of trace-fear memory requires the amygdala, hippocampus and prefrontal cortex (Bangasser et al., 2006; Buchel et al., 1999; Burman and Gewirtz, 2007; Carter et al., 2006; Chowdhury et al., 2005; Crestani et al., 2002; Han et al., 2003; Knight et al., 2004, 2006; McEchron et al., 1998, 2000; McEchron and Disterhoft, 1999; Misane et al., 2005; Runyan et al., 2004). Methodologies which might permit precise timing of a freezing behavior, such as an electromyography (EMG), may be helpful for examining the neural correlates which dictate fear. Additionally, some evidence suggests that contextual information may influence trace-fear conditioning (McEchron et al., 1998; Quinn et al., 2002). Accordingly, conducting trace-fear conditioning in the dark would eliminate confounding information of the environmental context. The current study validated a simple, inexpensive, physiological method to examine trace-fear conditioning in the dark or light, utilizing EMG of the neck.

Abbreviations: CS, conditioned stimulus; EEG, electroencephalogram; EMG, electromyogram; NREM, non-rapid eye movement; US, unconditioned stimulus.

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doi:10.1016/j.jneumeth.2008.10.020

2. Materials and methods

2.1. Animals

Surgery was performed on 6 C57BL6 mice for in vivo electrophysiology studies (8–14 weeks old). Mice were maintained on a 12:12-h light–dark cycle (lights on at 8:00 a.m.) and had access to food and water ad libitum. Procedures conformed to the recommendations of the Canadian Council on Animal Care and the University of Toronto Animal Care Committee approved the protocols.

2.2. Surgical preparation

Mice were anesthetized with 1–2% isoflurane which was mixed with oxygen (30% balanced with nitrogen) and delivered to the mice via nose cone throughout the surgery. All electrodes were pre-attached to a miniature connector. The abdomen and scalp of mice were shaved and then cleaned with iodine (Triadine) and alcohol. The skull of the mouse was fixed into a stereotaxic adapter (502063, WPI, Sarasota, Fl, USA) mounted on a stereotaxic frame (Kopf Model 962, Tujunga, CA, USA). Three small holes (1.19 mm diameter) were drilled into the skull for differential frontal–parietal recordings. Electrodes, consisting of a wire attached to a jeweler's screw (with contact end ground flat), were fixed into the holes to record EEG (electroencephalogram) at the following coordinates relative to bregma: frontal cortex (AP—2.2, ML—1.0), parietal cortex (AP—2.2, ML—2.5) and ground (AP—3.0, ML—3.0). For dorsal neck muscle recording, left and right nuchal muscles were exposed and Teflon-coated stainless steel electrodes (part# AS632, Cooner Wire) were sutured (4.0 silk) to each muscle to record neck EMG. All wires and connectors were secured to the skull with dental cement and cyanoacrylate glue (Krazy glue). Mice were injected, intraoperatively with buprenorphine (0.05 mg/kg, SC) as an analgesic, and 1.0 ml sterile saline (IP) for hydration. Mice were placed on a warm heating pad until they showed signs of ambulation, and were permitted to recover ~14 days prior to recording.

2.3. Electrophysiological recordings

On the day of the experiment, a light-weight cable was connected to the assembly on the mouse head. The signals were routed through a commutator (Crist Instruments, Hagerstown, MD, USA). The mice were placed in a shock chamber (Med Associates, St. Albans, VT) to either examine fear conditioning or to record EEG. The shock chamber was situated in a sound attenuated cubicle (ENV-017 M, Med Associates, St. Albans, VT, USA). Electrophysiological signals were amplified and filtered (Super-Z headstage amplifiers and BMA-400 amplifiers and filters, CWE Inc., Ardmore, PA, USA) as follows: cortical EEG 1000X at 1-100 Hz and neck EMG 2000X at 100–1000 Hz.

2.4. Trace-fear conditioning

Trace-fear conditioning was performed in an isolated shock chamber. The CS was an 80 dB white noise, delivered for 15 s, and the US was a 0.75-mA-scrambled foot-shock for 0.5 s. The mouse was acclimated for 60 s, and presented with ten CS–trace–US–ITI trials (trace of 30 s, inter-trial interval (ITI) of 210 s). For the camera-based method, data were video recorded using FreezeFrame Video-Based Conditioned Fear System and analyzed by Actimetrics Software (Coulbourn Instruments, Wilmette, IL). In addition, whenever freezing behavior was observed directly, the experimenter delivered a 3-V pulse to the analog to digital converter, via depression of a foot pedal. The pulse was recorded with Spike2 software alongside the EMG record. Freezing was defined as the absence of movement (of at least 1 s) with the exception of breathing for visual and camera-based methods. Since neck EMG is well known to correlate with an animal's movements, we tentatively defined EMG-based freezing as an absence of muscle twitch activity (of at least 1 s). All three scoring methodologies were conducted simultaneously to examine whether or not EMG freezing measures would correlate with visual-based freezing measures.

2.5. Analyses and statistics

For all comparisons, differences were considered significant if the null hypothesis was rejected at $P < 0.05$ using a two-tailed test. Repeated-measures ANOVA (RM-ANOVA) was performed and followed by post hoc comparisons with the Bonferroni-corrected P to infer statistical significance for EMG magnitude comparisons. Freezing behavior was analyzed using Pearson-product moment correlation to examine the validity of using EMG as a measure for conditioned freezing behavior. Analyses were performed using Sigma-Stat (SPSS Inc., Chicago, IL, USA). Data were plotted with SigmaPlot (Systat Software, San Jose, CA, USA).

For the sleep study the mice were scored to be in non-rapid eye movement (NREM) sleep if the EMG was of low muscle tone and the EEG was characterized by high voltage slow waves (1–4 Hz range). Wakefulness was characterized by low voltage, high frequency EEG, and muscle tone associated with movement. EEG was analyzed in 5 s epochs for periods of wakefulness and NREM sleep using Spike2 software. Scripts for EEG analysis (Sudsa-version 2.2) were obtained from CED. Fast-Fourier transform was used to convert EEG waveforms into total power (μV^2), which was binned every 5 s for the following frequency bands: $\delta 1$ (0.5–2 Hz), $\delta 2$ (2–4 Hz), θ (4–7.5 Hz), $\beta 1$ (7.5–13 Hz), $\beta 2$ (13–20 Hz) and α (20–30 Hz). All neck EMG recordings were smoothed (25 ms time constant) and rectified (Spike2 software, 1401 interface, CED Ltd., Cambridge, UK) to calculate the area under the curve. For EMGs during active wakefulness and NREM sleep, the area under curve was calculated every 5 s. For EMG-based freezing behavior the magnitude of the EMG was determined for the length of the freezing bout and normalized to a 5-s period for comparison to other behavioral states.

3. Results

Fig. 1A shows the placement of the neck-EMG recording electrodes to examine freezing behavior and sleep. Fig. 1B shows an example of a baseline EMG recording and how the signal was processed for quantification.

Animals were conditioned in the trace-fear paradigm while their fear was simultaneously recorded via visual observation, freeze frame camera and with neck EMG electrodes. Fig. 2A shows simultaneous visual scoring with camera and neck recordings during trace-fear conditioning. The animal was found to develop visually observable freezing behavior during the conditioning. The automated camera system also detected this freezing behavior. Interestingly, the neck muscle EMG was found to shift from twitch-like muscle activity during visually observed movements to a non-twitch activity, with a stable level of muscle tone. All methods of collecting freezing behavior data appeared to correlate with one another. The percentage of freezing during the tone and during the trace interval was quantified for 10 trials of conditioning. Fig. 2B–D shows correlations plotted for the percentages of freezing for all three data collection methods. Fig. 2B shows that there was a strong statistically significant positive correlation between the visual scoring and the camera scoring ($r^2 = 0.82$, $p < 0.001$). Fig. 2C shows that there was a strong statistically significant positive cor-

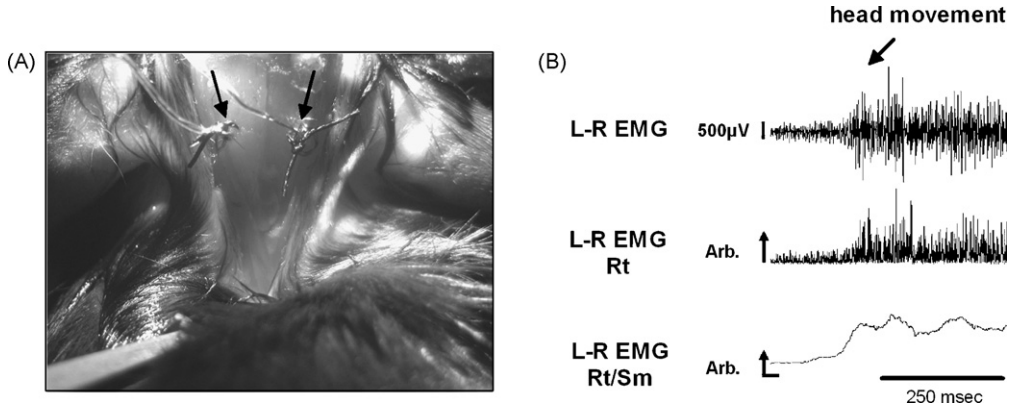

Fig. 1. Surgical preparation. (A) Left and right neck muscles (arrows). The muscle overlying the neck muscles (nuchals) has been gently pulled back for better visualization. Two loops of exposed wire were made at the end of a Teflon coated stainless steel wire. The loops were then sewn to left and right muscles. (B) Representative neck EMG traces. The upper trace is and EMG recorded between left (L) and right (R) neck muscles. The second trace is the same signal which has been rectified (Rt). The third signal is the same signal rectified and smoothed (Rt/Sm). Upward arrows denote an increase in movement. Arb., is arbitrary units.

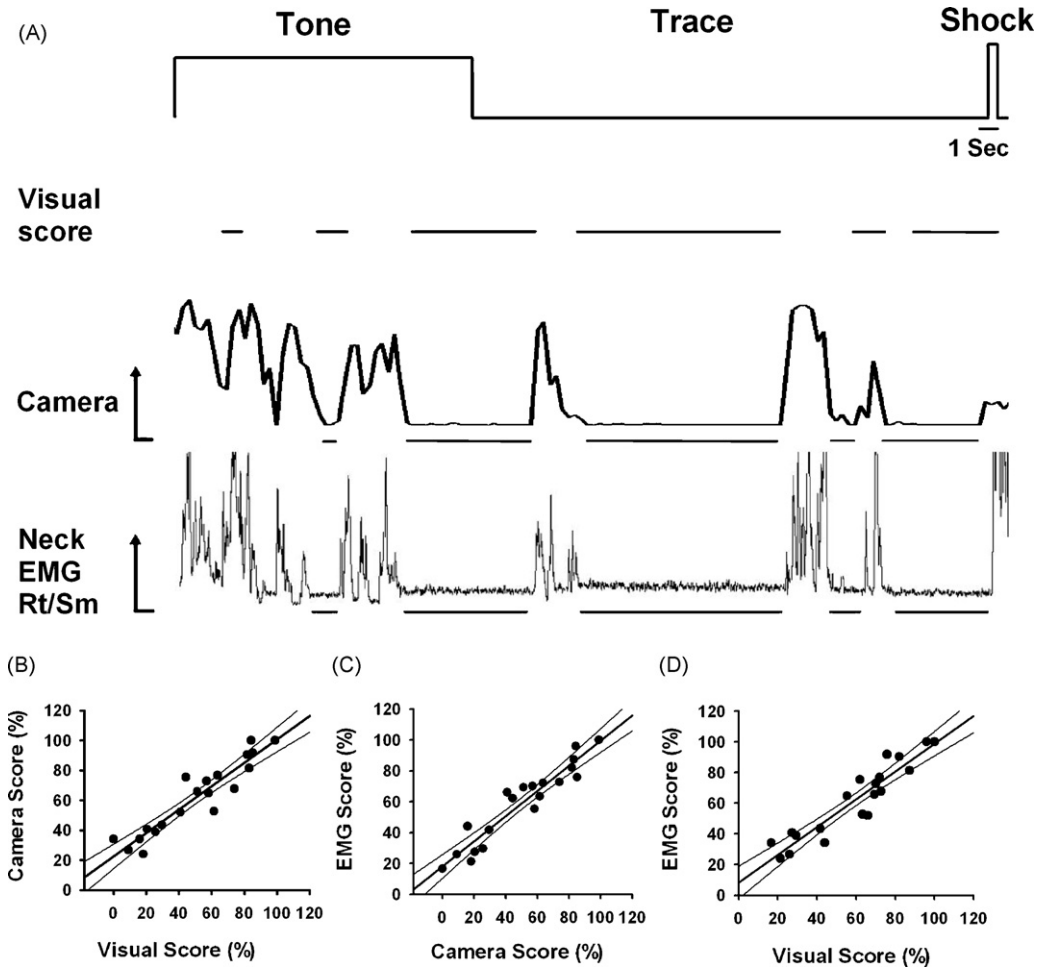

Fig. 2. Neck EMG recordings can provide measurements of fear behavior. (A) Simultaneous recordings from a visual observer, freeze-frame camera, and neck EMG during the trace-fear conditioning paradigm (during 10th training trial). The conditioning protocol is depicted at the top, showing that a tone was played followed by an interval (trace interval) then by a shock. It was readily observed that the rectified and smoothed EMG signal paralleled the camera-based movement detection, with periods of quiescent EMG activity (no twitches) correlating with periods of inactivity. Under each measurement, the freezing behavior duration is indicated, similar to the visual-based scoring. Visual-based scoring paralleled both the EMG and the camera-based scoring methods, however, it was obvious that the latencies of the detection were longer (based on observer reaction times). (B–D) Scatter plots and regression lines (+/−95% confidence intervals) for the percentage freezing during tone and trace intervals for each trial of the 10 trace-fear training trials. Comparisons are made for each of the fear conditioning scoring methods. All scoring methods were highly correlated with one another. Abbreviations as for Fig. 1.

relation between the camera scoring and the neck EMG scoring ($r^2 = 0.88$, $p < 0.0001$). Fig. 2D shows that there was a strong statistically significant positive correlation between the EMG scoring and the visual scoring ($r^2 = 0.83$, $p < 0.0001$). Based on these findings we conclude that EMG scoring is as accurate as both the visual method and the camera method. Thus, the EMG-based method would be sufficient to yield measures of fear behavior during any point in a 24-h day, whether lights are on or off.

Fear behavior is not always easy to discriminate from sleep when visually observing rodents. Importantly, fear memory is often studied when rodents would naturally be sleeping (i.e. lights on phase). We therefore, examined whether the automated camera system and simple EMG recording could mistake sleep for fear memory in an animal which was not fear conditioned. Fig. 3A shows an example of the automated camera system detecting fear behavior in an untrained animal. Consistently, an examination of the EMG suggests an absence of movement. However, in this experiment, simultaneous EEG was conducted to examine brain wave signatures associated with sleep. Fig. 3B shows that the addition of and EEG recording identifies this state as NREM sleep rather than fear. The EEG is characterized by high voltage slow waves compared to the wakefulness that follows the sleep. Fig. 3C and D shows quantified EMG activity and EEG power confirming the behavioral states of the mouse.

We next examined whether the magnitude of the EMG would be useful for discriminating freezing behavior from both NREM sleep and wakefulness. EMG values were quantified for five chronically instrumented animals during EMG-based freezing behavior, wakefulness and NREM sleep. RM-ANOVA revealed that there was a significant effect of the recorded state on neck muscle EMG

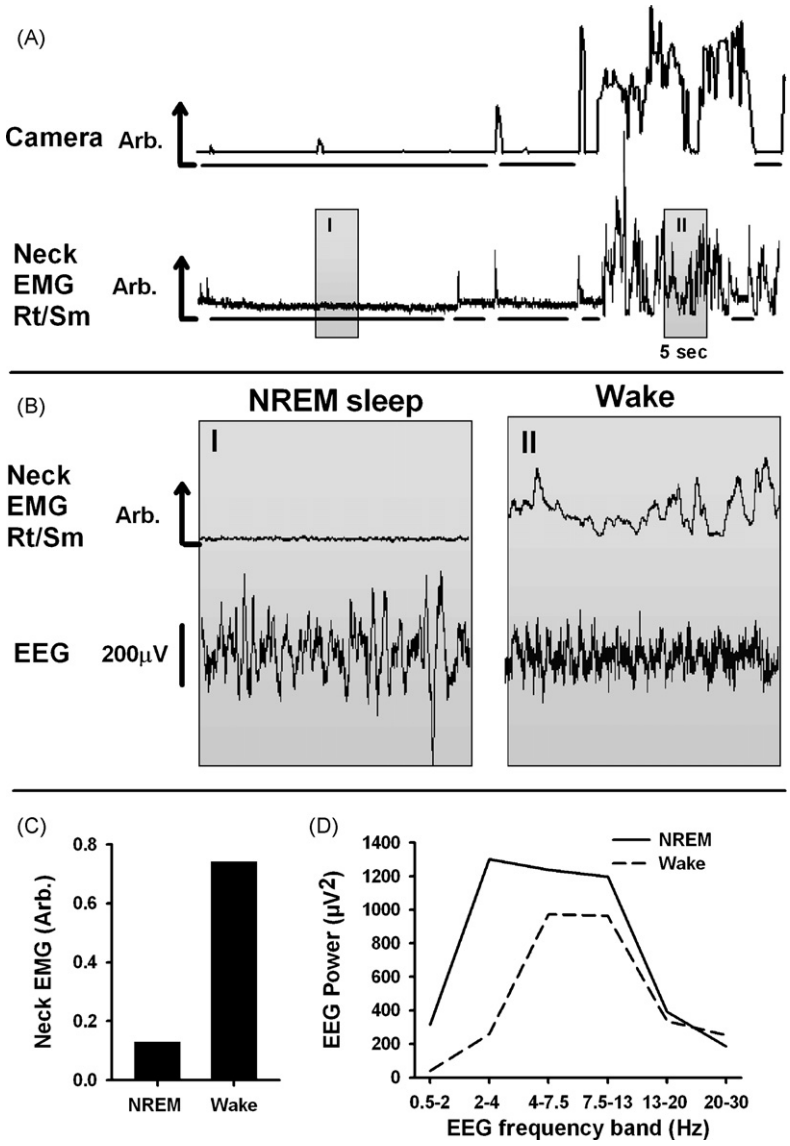

Fig. 3. Conventional visual-based measures and EMG-based measures can falsely score sleep as fear behavior. (A) Shows a simultaneous recording with a freeze-frame camera while EMG activity was recorded during the light phase, when mice normally sleep. There is a long period of behavioral quiescence, as detected by the automated camera system and also viewed with neck muscle EMG recording. The animal then appears to become active toward the end of the measurement period. Freezing behavior is scored below each trace. (B) When the EEG activity was observed during this period (shaded boxes in A and B) it was revealed that the mouse was actually sleeping (NREM) as indicated by high voltage slow waves in the EEG (panel I). The animal can be then seen to arouse from sleep (panel II) as revealed by high frequency low amplitude EEG and the increase in EMG activity. Thus, camera and even EMG methodologies may be recording sleep behavior when they should be recording fear behavior. (C) Reduced neck EMG is observed during NREM sleep in comparison to arousal from sleep. (D) Quantification of EEG frequency bands during NREM sleep, demonstrating an increase in power of high voltage slow frequencies relative to wakefulness. Abbreviations as for Fig. 1.

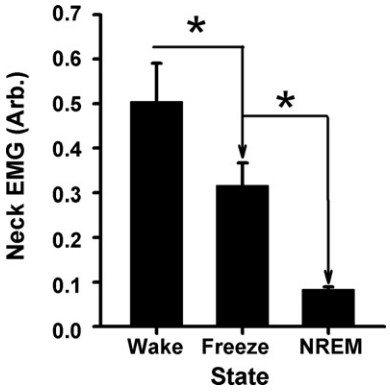

Fig. 4. Quantitative EMG can be used to help discriminate freezing behavior from wakefulness and NREM sleep. (A) Grouped data ($n = 5$) showing that each state could be quantitatively discriminated based on muscle tone measurements. Abbreviations as for Fig. 1, * is $p < 0.05$.

($F_{(2,10)} = 20.17$, $P = 0.001$). Fig. 4B shows that there was a significant decrease in EMG from wakefulness to freezing behavior ($t = 2.90$, $P = 0.032$), and from freezing behavior to NREM sleep ($t = 3.04$, $P = 0.013$). The finding suggests that the magnitude of the EMG is sufficient to discriminate each of these states.

We therefore, suggest that EMG-based freezing behavior is characterized by neck muscle activity that is greater in magnitude than NREM sleep but less than that of wakefulness. In addition, freezing behavior occurs in the absence of neck muscle twitches and must last for at least for 1 s to be scored.

4. Discussion

The current investigation sought to examine an inexpensive alternative approach for fear behavior scoring, to examine mice across the natural light–dark cycle. We have shown that freezing behavior can be scored with use of a simple EMG record. We also show that the way in which freezing is behaviorally defined overlaps with the way that sleep is defined. That is; immobility with the exception of breathing. Thus, EEG recordings in conjunction with EMG recording can be helpful to discriminate fear behavior from sleep.

A major advantage of recording neck EMG for freezing behavior is that it provides precise timing of when the freezing occurs which is crucial for the investigation of the neural correlates of trace-fear memory. Moreover, the EMG activity can be collected using the same analog-digital converter as that for recording EEG or brain cells.

The major drawbacks to the EMG method described here are that it requires surgery and there is currently no automated method to score the EMG-based freezing activity. Thus, rapid screening would be problematic. However, this does not preclude using a camera system and automated scoring method to do initial screening. The examination of the neurophysiology could then be followed up with the EMG-based methodology to score freezing.

It has been suggested that resting or sleeping, as scored via visual observation, only occurs after extinction training of at least 20 min (Marchand et al., 2003). Preliminary observations from our laboratory also suggest that this is the case. Accordingly, studying animals that have altered sleep habits might cause these animals to fall asleep during memory testing or extinction which would be detected as freezing behavior. We have shown that visual-based measures of freezing and EMG measures can mistake sleep for freezing behavior. However, we also show that the magnitude of the EMG can help clarify if an animal is sleeping or in a state of fear. Thus, it is recommended that EMG and EEG be used to verify the true behavioral state of an animal during memory testing of fear conditioning paradigms.

Acknowledgements

M.Z. was supported by the Canadian Institutes of Health Research (84256). H.W.S. was supported by the CGS-NSERC Scholarship.

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