Published in final edited form as:

J Neurosci Methods. 2013 July 15; 217(0): . doi:10.1016/j.jneumeth.2013.03.023.

Behavioral Effects of Acclimatization To Restraint Protocol Used for Awake Animal Imaging

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Abstract

Functional MRI of awake rats involves acclimatization to restraint to minimize motion. We designed a study to examine the effects of an acclimatization protocol (5 days of restraint, 60 minutes per day) on the emission of 22-kHz ultrasonic vocalizations and performance on a forced swim test (FST). Our results show that USV calls are reduced significantly by day 3, 4 and 5 of acclimatization. Although rats show less climbing activity (and more immobility) in FST on day 5 compared to the 1st day of restraint acclimatization, the difference is gone once animals are given a 2 week hiatus. Overall, we show that animals adapt to the restraint over the five day period, however, restraint may introduce confounding behavioral outcomes that may hinder the interpretation of results derived from awake rat imaging. The present data warrant further testing of the effects of MRI restraint on behavior.

Keywords

fMRI; Rats; Restraint; Acclimatization; Ultrasonic Vocalizations; Forced Swim Test

1. Introduction

There are a rising number of studies employing functional MRI in rodents to study brain function and anatomy. Issues arise with the use of anesthetics and paralyzing agents that make the method less attractive for those in neuroscience fields. On the other hand, imaging animals while awake and restrained raises concerns regarding distress and the possibility of an ensuing depressive state. Anesthetized preparations are used extensively for fMRI studies in rats. The methods are not suitable for many longitudinal applications under ideal experimental conditions where the same populations of animals are imaged at multiple time points. Many studies utilize surgical methods to compensate for the loss of vital homeostatic mechanisms. Additional paralyzing agents are often required to further reduce movement to levels sufficient for stable imaging. These invasive methodologies are not favorable for long-term studies in rats. There are also significant lines of evidence suggesting that agents typically used for anesthetizing animals can significantly suppress neuronal activity and modify specific patterns of neuronal activity and metabolism (Wang et al., 2011; Williams et

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al., 2010; Masamoto et al., 2009). In addition to the neuronal actions, anesthesia can influence global cerebrovascular reactivity and therefore anesthetics may impact the magnitude of the blood oxygen level dependent (BOLD) signal through disparate effects on cerebral blood flow (CBF), cerebral blood volume (CBV) and other hemodynamic parameters (Sicard et al., 2003). Our laboratory has utilized methods to image awake rats as an alternative to imaging under anesthesia. Before this is done, however, animals must be acclimated to the restraint conditions and MR pulse sequence noise (Figure 1)(King et al., 2005). The procedures for acclimation are carried out for 5 days prior to collection of imaging data. Acclimation is done to simulate the actual imaging experimental setup during data collection. Movements during scanning (for example, slow shifts in head position, sustained or transient leg motion, chewing, vocalizations) and physiological motion (pulsations due to cardiac and respiratory cycles) can significantly reduce the quality of anatomical and functional MR scans. Although some physiological variables adjust to the daily intermittent restraint conditions involved in acclimatization and there are improvements in image quality (as measured by increased signal to noise and contrast), no behavioral demonstration of the efficacy of our acclimation protocol exists. In addition the question still remains whether reductions in motion observed after restraint conditioning are symptomatic of a depression-like state induced by the protocol. We begin to address this point in the present study. Several aversive stimuli have been shown to elicit 22-kHz vocalizations in rats (as reviewed by Panksepp, 2002). Therefore, we use 22-kHz ultrasonic calls as an index of negative affect to behaviorally assay acclimatization. The forced swim test is used to assay depressive-like symptoms at the onset and completion of the restraint conditioning protocol.

2. Methods

2.1 Subjects

Male Long Evans rats weighing 300–400 grams were purchased from Charles River Laboratories (Wilmington, MA). Animals were pair housed under a 12 hour light:dark cycle (lights off at 1900 hrs). Water and Purina rat chow were provided *ad libitum*. Rats were acquired and cared for in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals (8th Edition, 2011) and adhere to the National Institutes of Health and the American Association for Laboratory Animal Science guidelines. The Institutional Animal Care and Use Committee at Northeastern University approved the protocols used for this study. Animals were subjected to an acclimatization procedure similar to one previously reported to minimize motion artifact during fMRI scanning (King et al., 2005). We recorded USV calls in a groups of rats ($n = 11$). Another group of rats ($n = 9$) were subjected to a FST on days 1 and 5 of acclimatization and this was compared to 9 animals that were tested but not acclimated and 10 animals acclimated to restraint but tested two weeks after the final acclimatization session ($n = 8$).

2.2 Acclimation to fMRI restraint procedures

Rats were acclimated for 5 days to MRI restraint procedures (King et al., 2005). On each day, they were anesthetized with 2–4% isoflurane gas anesthesia and placed into a replica of the restraint unit used for functional magnetic resonance imaging (fMRI) studies (insightMRI, Shrewsbury, MA) (Figure 1). A topical anesthetic of 4% lidocaine cream was applied to the skin around the ear canals and over the bridge of the nose before the animal was placed inside the restraint unit. This minimized discomfort on pressure points around the head. Most of the restraint was around the head and above the shoulders; therefore, animals were in a hunched posture with limbs free and unrestrained. The head holder has a bite bar over which the incisors were placed and held by lowering and locking-in a plastic nose bar. Plastic ear bar guides were locked in place by lateral screws, thereby preventing

lateral movement. A pair of shoulder bars was lowered behind the animal's neck, thereby further restricting vertical movement. Once the setup was complete, the rat was placed inside a sound attenuation box and USV calls and respiratory rates were recorded. Following the recording session animals were removed from the sound attenuation box and placed in a secondary chamber and exposed to a recording of an fMRI imaging to recapitulate the imaging environment. The acclimatization procedure was repeated over 5 days, during which the time spent under restraint was 60 minutes per day.

2.3 Ultrasonic vocalization recording

Rats were placed inside a sound attenuation chamber (Med-Associates, St. Albans, VT). In order to minimize the confounding influence of the isoflurane anesthesia used to place the animals in the restraint, scoring of the data did not begin until 5 minutes after it was evident from the spectrograms that the animals were awake. Ultrasonic vocalizations were recorded using a condenser microphone that is sensitive to frequencies from 10–250kHz (model CM16 Avisoft Bioacoustics, Germany). Sound detection and storage was achieved by interfacing with an UltraSoundGate USB digital-to-analog converter and amplifier (USG 116, Avisoft). A laptop PC running Avisoft RECORDER software was used to store acoustic data as wav files for analysis of spectral features. USV's were continuously monitored using a frequency window between 10–70kHz and spectrograms were generated online using a Fast Fourier Transform size of 256, a sampling rate of 140kHz, 16-bit data format. Acoustic datasets were imported into SASLab Pro (Avisoft) and spectrograms were generated for the analysis of vocalization around the 22-kHz range. Filtering was used to reduce background noise and a trained observer manually quantified the total number of specific spectrogram waveforms that were observed over time. 22-kHz calls were classified using several criteria: Calls with a bandwidth of 1–6kHz and frequency of 18–32kHz were considered 22-kHz calls (Brudzynski et al., 1993). SASLab Pro (Avisoft), enabled us to accurately determine call duration, frequency, and bandwidth. All noises shorter than 10ms, with bandwidths shorter than 1Hz, or bandwidths greater than 6hz, were excluded from analysis. 22-kHz call frequency ranged from 18 to 31.5kHz. Mean call frequency was 23.87 ± 3.79 kHz. Consistent with short 22-kHz calls, observed call duration ranged from 10.8 to 351ms.

2.4 Forced Swim Test

For FST, a 5-gallon cylindrical pail (dimensions: 14.5" tall and 11.75" width) with clear white walls was filled with water to half its capacity. Care was taken to maintain room temperature water. Water was changed after each test in order to keep it free of feces during the collection of recordings using a digital video camera placed directly over the cylinder facing down. 15-minutes recordings were used for the analysis. All behavior was video recorded, imported to a laptop PC as avi files using Windows Movie Maker and analyzed using ODLog software (Macropod software). Rats were subjected to the restraint protocol each day for 5 days, with an additional FST test on the 1st and last (5th) days. FST was done immediately after their restraint, after a 20-minute break. Control rats were tested on the 1st and 5th day without any manipulations in between. During the gap between the 1st and 5th days of testing animals remained in their home cage in the animal colony. All measurements were scored as duration in seconds. Climbing was scored as vigorous thrashing movements with forepaws, usually against the sides of the tank. Animals tended to show escape-oriented behaviors (jumping, rearing, sniffing or diving). Immobility was scored as the animals making minimum movements necessary to stay afloat, with noticeable absence of escape-oriented behaviors. Statistical analysis USV's was done using a Non-parametric Mann-Whitney test ($p < 0.05$) comparing each subsequent day to day 1. Wilcoxon matched paired test ($p < 0.05$) was used for the FST data.

3. Results

We observed clearly identifiable but infrequent 22-kHz calls on the reconstructed spectrograms. There was a progressive decrease in vocalizations each day (Figure 2a). The decrease in 22-kHz calls became significant by day 3 ($p < 0.05$) when compared to the volume of calls on day 1 of the restraint protocol. The decrease remained significant at day 4 ($p < 0.005$) and day 5 ($p < 0.005$). Respiratory rates for the restraint acclimated animals remained stable around 80 respirations per minute throughout the 5 day experiment (Figure 2a Inset). FST analysis showed significantly reduced climbing activity on day 5 versus day 1 of restraint acclimatization ($p = 0.04$) (Figure 2b). These animals also showed greater duration of immobility on day 5 ($p = 0.04$) (Figure 2c). Animals tested but not acclimated did not show any differences when comparing both FST days (Figure 2b,c, NR groups). Interestingly, a 3rd group tested 2 weeks after the final 5th day of acclimatization did not show any differences when compared to day 1 in non-restrained and restrained groups (Figure 2b,c, R 2wks group).

4. Discussion

One of the concerns of acclimation for awake fMRI studies is that it may chronically stress animals. However, it seems to involve intermittent stress that can have transient and not chronic effects. King and co-workers reported that various physiological variables (e.g., respiratory rates, blood pressure, corticosterone levels) of Sprague-Dawley rats are reduced following 5–8 days of restraint (King et al., 2005). Most measures in their study are reduced near pre-stress baseline levels on day 4–5. Rats appear to habituate to repeated daily 1–2 hour restraint for 4–9 days (Melia et al., 1994; Dhabhar and McEwen, 1997; Dhabhar and McEwen, 1997b). Rats show normal patterns of food intake and heart rate following habituation to restraint (Haleem, 1996; Stamp and Herbert, 2001). Importantly, habituation to repeated daily sessions of restraint is not necessarily indicative of impaired hypothalamic-pituitary-adrenal (HPA) axis function, since rats acclimated to restraint stress still show increased c-Fos activation and corticosterone levels to a novel stressor (Melia et al., 1994). Our present data provides evidence that 22-kHz ultrasonic calls are reduced by day 4 and 5 of acclimatization in comparison to day 1. At the same time struggle movements diminish in these animals when tested on a forced swim assay. One interpretation is that animals are in a state of behavioral despair and are therefore not struggling. However, without intervention, struggle movements return to baseline levels by two weeks after cessation of restraint conditioning. When viewed collectively, these tests point to signs of physical and behavioral adaptations to the restraint conditions. The results support less struggle movements reported previously in Sprague-Dawley rats (King et al., 2005). Therefore rats seem to be fully capable of adapting to the head restraint conditions.

A central question is whether animals adapt to stress and whether observed adaptation is indicative of learning or an overall impairment of HPA axis and a resultant depression-like state. Parry and McElligott (1993) devised a method for head immobilization in awake rats in order to study central regulation of cardiovascular function (Parry and McElligott, 1993). They reported that side-by-side acclimatization of rats to restraint reduced the stress of individual animals that are being acclimated. We have used a similar acclimatization procedure in our studies in which groups of animals are simultaneously exposed to daily sessions of restraint. Heart rate and blood pressure normalized after initial exposure to restraint (Parry and McElligott, 1993) supporting previous work by King and co-workers (King et al., 2005). Barnum et al. (2007) investigated the hyperthermia effects of chronic restraint stress and compared these to other forms of stress such as the social defeat model and isolated cage confinement. They observed that corticosterone and stress-induced hyperthermic responses to restraint stress adapted after 5–6 days of repeated exposure.

However, this was not observed for the social defeat stress model indicating that the two behavioral stress models have different outcomes. Despite the reported evidence of adaptations to stress, there are important long-term effects that should not be overlooked. Naert et al. (2011) report a 'depressed-like state' in rats chronically exposed to repeated restraint stress. This included behavioral changes while in the elevated plus maze indicative of higher anxiety levels, changes in hedonic state as measured by the sucrose preference test, depressed locomotion and a reduction in body weight by 17% (Naert et al., 2011). However, it is important to keep in mind that these studies use very intensive stress exposure schedules, involving 90–120 minutes or more per day. King et al. (2005) reported a maximum time spent under restraint of 90 minutes. In the above-cited study, the authors employed 3 hr daily sessions for 3 weeks (Naert et al., 2011). In addition to being stressful, an extended stress paradigm imposes a physical challenge to animals that can reduce overall activity (see results in Figure 2b,c). Results in Figure 2 show that there is reduction in time spent struggling on the 5th day of restraint in comparison to the first restraint and FST session. The reduced struggle time, or a curtailed effort to escape, may be considered a sign of a depression-like state. However, an alternative explanation is that the effort to escape on the 1st day of restraint and FST is reduced on day 5 because of physical fatigue. However, this is unlikely due to consistent measures of respiratory rate across days. Also, previous work showed decreased heart rate over the acclimation period (King et al. 2005) suggesting fatigue is not a dominant contributor to the observed changes in behavior. Evidence for this comes from a group in Figure 2 showing data for rats restrained for 5 days and then re-tested 2 weeks later (R 2wks). These animals recover their levels of struggle time to levels observed in the first day of testing. This result, in addition to supporting an adaptive behavioral mechanism, also suggests that experiments should ideally be carried out immediately after day 5 of restraint acclimation procedures. Animals may again increase movement if not imaged before two weeks. Reduced exposure time to intermittent daily sessions may therefore minimize the effects seen with longer chronic restraint sessions.

5. Conclusion

We have shown that animals exposed to our restraint conditioning protocol elicit significantly less 22-kHz vocalizations when compared to day one. Using these calls as a behavioral readout for negative affect suggests that animals habituate to the restraint throughout the course of the protocol. We have also shown that animals exhibit decreased mobility in the forced swim test by the end of the restraint protocol, which could suggest the development of depressive-like symptoms. In summary, we show that animals acclimate to restraint over five days however, restraint may introduce confounding factors into the interpretation of results derived from awake animal imaging.

Acknowledgments

Support was provided by NIH grant DA019946. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute on Drug Abuse. The NIH and NIDA had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Abbreviations

BA	Sodium Butyrate
USV	Ultrasonic vocalizati

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Highlights

- We test the efficacy of a behaviorally uncharacterized awake animal fMRI restraint acclimatization protocol
- We measure 22-kHz vocalizations to assay the affective state of rats throughout the restraint acclimatization protocol
- We use the forced swim test to determine if habituation to fMRI restraint is due to engendered learned helplessness state
- The demonstrated efficacy of this protocol enables increased data interpretation reliability in awake animal fMRI

Figure 1. fMRI restraint setup. 1) The restraint setup is designed to restrict head movement in all directions. Screws (black arrows) fixed to the enclosure around the animal's head prevent yaw. Incisors are placed over a bite bar and a plastic nose bar (white arrow) is lowered and locked on top of the snout to prevent pitch. The animal is then placed in a body tube and plastic bars (striped arrows) are inserted immediately in front of the shoulders isolate the head from caudal body movement.

Figure 2.

Results of ultrasonic vocalization measurement and forced swim test. A) 22-kHz ultrasonic calls emitted during daily restraint acclimatization sessions. Inset shows respiratory rates during 5 days of acclimatization. Data presented as mean $\pm$ standard error. Non-parametric Mann-Whitney test, * $p < 0.05$, ** $p < 0.005$. B) Duration (in seconds) of climbing activity C) Duration (in seconds) of immobility on a forced swim test. Both climbing and immobility tests were performed on the first and last day of acclimatization and 2 weeks after the last acclimatization day. Data presented as mean $\pm$ standard error. Abbreviations: RPM, respirations per minute; d1, day 1; d5, day 5; R, restraint; NR, no restraint; R 2 wks, tested 2 weeks after the last restraint session. Wilcoxon paired t-test * $p = 0.04$ R day 5 vs R day 1 for both climbing activity and immobility.