Behavioural Brain Research 201 (2009) 8–13

Contents lists available at ScienceDirect

Behavioural Brain Research

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

Research report

Female fear: Influence of estrus cycle on behavioral response and neuronal activation☆

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ARTICLE INFO

Article history:
Received 8 August 2008
Received in revised form 9 January 2009
Accepted 15 January 2009
Available online 23 January 2009

Keywords:
Innate fear
Trimethylthiazoline (TMT)
Estrus cycle
Estrogen

ABSTRACT

Our observation that male rat's innate fear response differed with hormonal status, as well as the higher prevalence of fear and anxiety disorders in human females led to the current investigation of the impact of phases of the estrus cycle on innate fear responding. Female rats in different phases of the cycle were exposed to an innate fear-inducing stimulus (2,5-dihydro-2,4,5-trimethylthiazoline, TMT odor) and monitored for changes in behavior and brain activation. Behavioral data showed freezing responses to TMT were significantly enhanced during estrus as compared to other phases of the cycle. This data was supported by significant increases in pixel intensity in cortical and sub-cortical regions in estrus compared to proestrus and diestrus.

Imaging results demonstrated significant increases in brain activation in the somatosensory and insular cortices when comparing estrus to diestrus. There were significant increases in neural activity in the bed nucleus of the stria terminals (BNST) and septum in estrus as compared to proestrus. Additionally, the hippocampus, hypothalamus, olfactory system, and cingulate cortex show significant increases in the estrus phase when compared to both diestrus and proestrus. Taken together, these results suggest that the female's hormonal status may be correlated with alterations in both neuronal and behavioral indices of fear.

1. Introduction

Anxiety disorders affect more than 40 million individuals in the United States each year and have a yearly economic cost of $50 billion [26]. Studies suggest that females have a higher prevalence of anxiety disorders and phobias than their male counterparts [28]. In fact, the national comorbidity survey reports that women have a phobia prevalence rate of 15.7%, compared to a rate of 6.7% for men [30].

Phobias and other anxiety disorders have been linked to alterations in both conditioned and innate fear responses [33,46]. Although the literature is replete with studies on conditioned fear much less is known about innate fear and the possible impact of gender. Reports examining sex differences in stress responsivity demonstrate higher levels and enhanced release of the stress hormones adrenocorticotrophin (ACTH) and corticosterone in female rodents responding to stressors [6,12]. In addition to general stressors, female rodents have been shown to exhibit more defensive behaviors than males when exposed to predator induced stressors [3,4,29,31], suggesting a link to hormonal status. The possible role of alterations in gonadal hormones like estrogen and progesterone on female fear behavior appears complex and multidimensional. For example, while some rodent studies show that estrogen administration increases in anxiety-like behaviors [40], others report anxiolytic-like effects [56] and still others show no significant effect [38]. Much work needs to be done to elucidate the possible mechanisms and brain regions involved in these possible conflicting reports. Functional imaging provides an innovative way to view the neural correlates of a stimulus. When using fearful face recognition as an fMRI stimulus, women show a different pattern of activation in the left amygdala and other regions such as the prefrontal cortex. In fact, the female fear recognition response was characterized by increased habituation in the amygdala followed by increased activity in the hippocampus in human studies [8]. Independent of the current lack of consensus in this field, the important impact of hormones on psychiatric disorders in women have been supported by reports showing that the occurrence of mood disorders is greatest during times when gonadal hormones are in flux [45,57,58].

Recently several models of predation stress have been utilized to interrogate the neural mechanisms underlying innate fear. Behaviorally, 2,4,5-trimethylthiazoline (TMT), a molecule produced by

☆ This publication was made possible by Grant Number R01 MH067096-01 from the National Institute of Mental Health. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIMH.

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doi:10.1016/j.bbr.2009.01.019

the gland secretions of the red fox [55] has been shown to elicit fearful behaviors in rodents, including freezing [16], defensive burying [25], and avoidance [7]. With this in mind, the current study was designed to assess the influence of the estrus cycle in modulating innate fear response. We have examined both behaviorally and with functional MRI the TMT-induced innate fear response of female rats (at different phases of the cycle). We hypothesize that changes in phases of the cycle will correlate to heightened behavioral and neuronal responses to predator stress.

2. Materials and methods

2.1. Animals

Sexually mature female Sprague–Dawley rats (250–300 g) were obtained from Harlan Sprague–Dawley Laboratories (Indianapolis, IN). Animals were housed in Plexiglas cages (two to a cage) and maintained in ambient temperature (22–24 °C) on a 12-h light:12-h dark schedule (lights on at 09:00 h). Food and water were provided *ad lib*. All animals were acquired and cared for in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals (#80–23, Revised 1996). These studies were approved by the IACUC Committee of the University of Massachusetts Medical School.

2.2. Acclimation procedure

For imaging experiments, animals were acclimated to the restraint device for 3 days according to a previously published procedure [10]. Briefly, animals were lightly anesthetized with 2% isoflurane secured in a dual coil rodent restrainer developed for fMRI (Insight Neuroimaging Systems LLC, Worcester, MA). A plastic semicircular headpiece with blunted ear supports that fit into the ear canals was positioned over the ears. Lidocaine paste (2%) was added to points of mechanical restraint, e.g., bridge of the nose and ear canals to minimize any pain or discomfort during the study. The head was placed into the cylindrical head holder with the animals' incisors secured over a bite bar and the ears were positioned inside the head holder. The body of the animal was placed in a custom-fitted cylindrical body tube. The body restrainer isolates all of the body movement from the head restrainer and minimizes motion artifact [32], while allowing for unrestricted respiration. The head holder and body tube were subsequently placed in a black opaque tube "mock scanner" with a tape recording of scanner noises. Scanner noises were identical to the precise imaging protocol to which rats would later be exposed during the experimental imaging protocol.

2.3. Estrus cycle assessment

Estrus cycle was determined by virginal smear examination [9]. Female in diestrus, proestrus and estrus phases were used in this experiment.

The vagina presents cyclic changes according to hormonal fluctuations, so estrus cycle can be determined by virginal smear examination [9]. Epithelial cells exfoliated from the vaginal wall can be collected onto a swab, smeared onto a slide, and examined under the microscope; the presence of cornified cells is indicative of estrus. In this study, all rats had vaginal smears to determine estrus cycle stage. This involved sampling the cells of the vaginal canal with sterile saline using plastic pipette. The recovered solution containing cells were placed on microscope slides. Subsequent cell cytology was examined under low or medium-power with a light microscope. Cell descriptions, as described by Sharp and LaRegina [49], were used to classify rats as being in diestrus, proestrus, or estrus.

Usually in diestrus phase, there is an abrupt decrease in superficial epithelial cell numbers and a marked increase in basal and parabasal epithelial cell numbers, many neutrophils are present at first with a marked decrease in 1–2 days, red cells may or may not be present. Simply, there were a variety of cell types along with leukocytes in diestrus. In proestrus, epithelial cells are all non-cornified basal, parabasal, and intermediate cells gradually decreasing in number, superficial epithelial cells appear by 2nd or 3rd day and increase in number over time. Erythrocytes are numerous and gradually decrease in number. Leukocytes disappear by the last day or two of proestrus and large and small intermediate cells along with red cells predominate.

Specifically, in proestrus the majority of cells were large, round and nucleated. In estrus phase, epithelial cells are mostly large flat angular cornified epithelial cells that become wrinkled and irregular as estrus progresses. Red cells may be observed microscopically throughout estrus, leukocytes appear on the last day or two of estrus. Large number of leukocytes indicate the end of estrus. In estrus the majority of cells were cornified.

2.4. Behavioral assessment

Rats were tested to assess their behavioral response to each scent and control prior to imaging studies. Briefly, a separate group of female rats ($n = 8$ per group) were removed from their home cage between 9:00 and 11:00 am EST (to minimize the impact of circadian rhythms) and allowed to habituate to the environment (plastic container) for two 10 min sessions. The following day, the rats were placed in the same environment (Plexiglass cage, 40 cm in length, 20 cm in breadth, 20 cm in height) and 100 μl of either water (no odor), lemon scent, and TMT odor (Phero Tech Inc., British Columbia, Canada) were presented in this order. Between each scent the animal was allowed to return to its home cage for a resting period of 10 min. This quantity of TMT has been used in past behavioral and imaging experiments to examine innate fear responses in our laboratory [10].

The animal remained in each condition for 5 min. Each test session was scored as well as videotaped for future analysis. The videotapes were then blindly scored for fear behaviors, particularly freezing behavior. The freezing response consisted of cessation of all movement except those necessary for breathing [23].

2.5. Magnetic resonance imaging

All images were acquired using a 4.7T/40 cm horizontal magnet equipped with a Bruker BioSpec console and a 20 Gauss/cm magnetic field gradient insert (inner diameter, 12 cm) capable of a 120 μs rise time (Bruker, Billerica, MA, USA). High resolution multislice anatomical images for each subject were obtained using a fast spin-echo pulse sequence (RARE, or rapid acquisition relaxation enhanced) with relaxation time (TR) = 2.0 s, effective excitation time (TE = 12 ms), matrix = 256 × 256, field of view (FOV) = 3.0 cm × 3.0 cm, eighteen 1.0-mm slices. Subtraction of these data sets confirmed there was no significant movement of the animal over the imaging session. Subsequent functional imaging was performed at a resolution of $64^2 \times 18$ slices with the same FOV and slice thickness with RARE sequence of TR = 2.5 s, TE = 7 ms, 16 echo train length, average = 2, number of repetitions = 30, total acquisition time = 10 min. The first 10 repetitions were used for acclimation and control. Following baseline data acquisition, the stimulus (TMT, British Colombia, Canada) was introduced and acquisition continued for an additional 20 repetitions.

2.6. Data processing

Following the functional sequences repeated anatomy images were collected and compared to the initial anatomy image sets. Motion artifact was assessed by: (1) subtraction of anatomical data across the imaging session, (2) qualitative analysis of time series movies looking for voxel displacement, and (3) analysis of raw data time series for course spikes. The time series movies correlated with course spike activity. The multiple data sets collected from these imaging sessions showed very little motion artifact using these criteria. On the rare occasion there would be a course spike usually caused by movement of the mouth such as in swallowing. The data for these images were excluded.

Each subject was registered or aligned to a fully segmented rat brain atlas that delineates more than 1200 distinct anatomical sub-volumes within the brain based on 2D atlas textbooks [42,52]. These detailed regions are collected into some major regions of the brain, e.g. amygdaloid complex, cerebrum, hippocampus, bed nucleus of the stria terminals (BNST), etc. The anatomy volumes were aligned to the atlas volume using interactive manual registration. The affine registration involved translation, rotation, and scaling in all three dimensions, independently.

The matrices that transformed the subject's anatomy volume to the atlas space were used to embed each slice within the atlas. All transformed pixel locations of the anatomy images were tagged with the segmented atlas major and minor regions creating a fully segmented representation of each subject. The inverse transformation matrix $[\mathbf{T}_i]^{-1}$ for each subject (i) was also calculated. An interactive GUI facilitated these alignments [59].

Unpaired t-test statistics were performed on each individual subject within their original coordinate system. The control period was repetitions 2–9. The stimulation

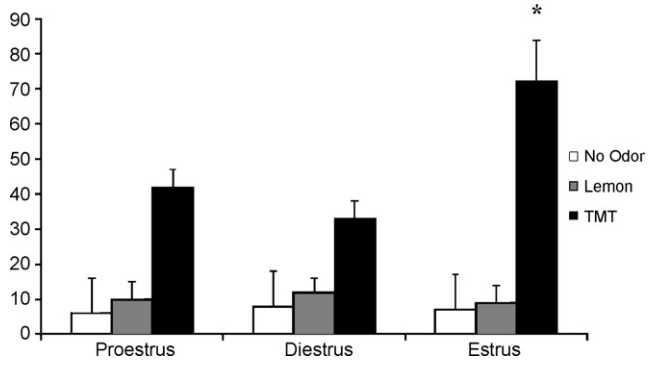

Fig. 1. Behavioral response to TMT and lemon scent across the estrus cycle. Error bars represent S.E.M. Time spent freezing, defined as an animal remaining motionless for at least 3 s was recorded. The estrus rat froze significantly longer than both the proestrus and diestrus phases ($p = 0.03$). There were no significant differences between estrus phases in response to lemon scent ($p > 0.05$). Additionally there were no significant differences between phases in freezing in response when no scent stimulus was presented ($p > 0.05$).