Published in final edited form as:
Pain. 2011 July ; 152(7): 1632–1640. doi:10.1016/j.pain.2011.03.001.

Modulation of nociceptive and acoustic startle responses to an unpredictable threat in men and women

Catherine S. Hubbard[1,2,6], **Edward Ornitz**[1,3,5], **John X. Gaspar**[1], **Suzanne Smith**[1], **Jennifer Amin**[1], **Jennifer S. Labus**[1,3,5], **Lisa A. Kilpatrick**[1,2], **Jamie L. Rhudy**[7], **Emeran A. Mayer**[1,2,3,4,5], and **Bruce D. Naliboff**[1,3,6]

[1]Center for the Neurobiology of Stress, University of California at Los Angeles (UCLA), Los Angeles, CA

[2]Department of Medicine-Digestive Diseases, University of California at Los Angeles (UCLA), Los Angeles, CA

[3]Department of Psychiatry and Biobehavioral Sciences, University of California at Los Angeles (UCLA), Los Angeles, CA

[4]Department of Physiology, University of California at Los Angeles (UCLA), Los Angeles, CA

[5]Brain Research Institute, University of California at Los Angeles (UCLA), Los Angeles, CA

[6]VA Greater Los Angeles Healthcare System, Los Angeles, CA

[7]Department of Psychology, The University of Tulsa, Tulsa, Oklahoma

Abstract

The present study examined whether a moderately aversive abdominal threat would lead to greater enhancement in affect and pain-related defensive responding as indexed by the acoustic startle (ASR) and nociceptive flexion (NFR) reflex in women compared to men. We also predicted sex differences in threat-related autonomic arousal measured by skin conductance responses (SCR) to acoustic startle and noxious sural nerve stimulation. Unpredictable threat was manipulated by alternating 30 s safe ('no abdominal stimulation will be given') and threat ('abdominal stimulation may occur at anytime') periods. The experiment consisted of two blocks, each containing 4 safe and 4 threat periods in which the ASR or NFR was randomly probed 9-21 s following period onset. Unpredicatable abdominal threat potentiated both ASR and NFR responses compared to periods signaling safety. SCRs to acoustic startle probes and noxious sural nerve stimulation were also significantly elevated during the threat versus safe periods. No sex differences in ASR or startle-evoked SCRs emerged. However, nociceptive responding was moderated by sex; females showed significant increases in NFR magnitudes across both safe and threat periods compared to males. Females also showed greater threat potentiated SCRs to sural nerve stimulation than males. Our findings indicate that both affect and pain-related defense and arousal systems are strongly influenced by threat of an aversive, unpredictable event, a situation associated with anticipatory anxiety. Females compared to males, showed greater nociceptive responding and pain modulation

Corresponding Author: Bruce D. Naliboff, Ph.D., CNS: Center for Neurobiology of Stress, UCLA Division of Digestive Diseases, CHS 47-122, MC 737818, 10833 Le Conte Avenue, Los Angeles, CA 90095-7378, TEL: (310) 312-9276, FAX: (310) 794-2864, naliboff@ucla.edu.

The authors declare no conflicts of interest pertaining to this study.

when exposed to an unpredictable threatening context, whereas affect-driven ASR responses showed no such sex differentiation.

1. Introduction

Affective processes can interact with nociception and pain at a variety of levels including pain generation, pain modulation, and pain response [28]. Several negative affective states including anxiety have been consistently associated with increased clinical pain and hyperalgesia in human experimental studies [1,6,13]. However, the relationship between negative affect and pain is complicated by findings from an extensive animal literature [4,20,26] and a few human studies demonstrating that high levels of negative affect inhibit nociceptive and pain-like responses [3,25,34-35]. Rhudy and Meagher have proposed a model to account for these findings which postulates that high intensity imminent threat activates acute defensive systems resulting in pain inhibition while more unpredictable anxiety provoking threats result in pain facilitation [36].

According to the model outlined by Rhudy and Meager, an environment containing an unpredictable, moderately aversive threat is a potent generator of pain facilitation, yet to date, this has not been well studied in humans [26,36,48]. Although a variety of techniques have been employed in animal studies to examine acute and chronic stressors as they relate to nociception and 'pain'-like responses, human investigations have mostly relied upon studying affect by either examining pre-existing individual differences or by viewing or recalling images with varying levels of emotional content [17,23,45]. More recently, Rhudy and colleagues have shown that a non-invasive measure of nociceptive signaling in humans, known as the nociceptive flexion reflex (NFR), is enhanced when viewing unpleasant emotional pictures, suggesting affect-related pain facilitation [38-39,41,43].

The primary purpose of the present study was therefore to examine the modulation of the NFR by an unpredictable, moderately aversive threat using a well-established threat of shock paradigm [29,47]. We have previously shown increases in self-reported negative affect and the acoustic startle reflex (ASR) during periods in which female subjects were threatened with a moderately aversive shock to the abdomen compared to periods that denoted safety, or absence of shock [29,47]. This procedure is considered an unpredictable threat since periods in which an aversive stimulation may occur are relatively long and the stimulus may occur at any time. The current study aimed to extend these findings by examining the effects of unpredictable threat on NFR responding in addition to ASR reactivity and sympathetic arousal. Specifically, we hypothesized that acute periods of unpredictable threat would lead to potentiated ASR and NFR responses indicative of enhanced affective responding and afferent nociceptive signaling, respectively. We also hypothesized that unpredictable threat would potentiate the sympathetic nervous system (SNS) response (indexed by Skin Conductance, SC), and that the intensity of the SNS response would be positively related to ASR and NFR magnitudes. Moreover, in light of prior clinical and experimental evidence demonstrating significant sex-related differences in pain sensitivity and the greater prevalence of chronic pain and anxiety disorders in women compared to men, as well as increased SNS responses in males relative to females [8,18-19,37], we predicted sex differences in NFR and ASR responses, with women showing greater magnitude changes in their threat-related NFRs and ASRs, and men displaying greater SNS responses.

2. Methods

2.1. Subjects

A sample of 60 healthy male (n = 31) and female (n = 29) adults between the ages of 18 and 42 years (mean = 22.15 years, SD = 3.92) were recruited by advertisement and through flyers posted on the UCLA campus and in the surrounding Los Angeles community. After informed consent was obtained, each subject underwent a medical history and physical screening to exclude for 1) the current use of psychoactive medication; 2) psychotic, major depressive, or substance abuse disorders; and 3) major medical or neurological conditions (e.g., epilepsy, diabetes, heart disease, pain disorders). Each subject was administered the Hospital Anxiety and Depression Scales (HADS) to screen for the presence of anxiety or depression symptoms [51]. If a subject scored above the standard clinical cutoff on HAD anxiety or depression measures (> 11), the Mini International Neuropsychiatric Interview (M.I.N.I.) Plus v. 5 [42] was administered by a trained nurse practitioner. Subjects were excluded from the study if they were found on the M.I.N.I. to meet criteria for a current anxiety or depression disorder. Subject's body mass index (BMI) was also assessed along with relevant demographic information (age, ethnicity and sex). Out of the 60 subjects sampled, one subject was dropped from the study prior to the start of the experiment and 11 subjects were discontinued due to reaching pain tolerance prior to evoking a reliable NFR during threshold assessment. Additionally, data from 3 subjects were excluded due to technical recording problems. Therefore, a total of 45 (males = 25; females = 20) subjects completed the entire study protocol (see Table 1). Of these subjects, 45.5% were White non-Hispanic, 25.0% were Hispanic or Latino, 22.2% were Asian, and 6.7% were African American. The majority of female subjects (75%) were naturally cycling while the remaining females (25%) reported current use of an oral contraceptive or some other type of hormone-based method for contraception (Depo-Provera). All procedures were previously approved by The UCLA Institutional Review Board and conducted in accordance with NIH guidelines for research in human subjects.

2.2. Apparatus

Data acquisition, analysis and stimuli presentation were accomplished using LabVIEW 8.6 (National Instruments, Austin, TX) software and A/D multifunction interface board (National Instruments, PCI-6030E). All psychophysiological raw signals were recorded at a sampling rate of 1000 Hz, filtered and amplified with Quad AC and Dual DC Grass Instruments amplifiers (Grass Technologies, 15A54; West Warwick, RI). Skin conductance was measured using a Grass Instruments SCA1 adaptor. The acoustic startle stimulus was generated by the digital to analog converter on the multifunction board and consisted of a 50 ms burst of white noise at 105 dB SPL (0 ms rise time) presented binaurally through a pair of stereophonic headphones (Sony, MDR-V700DJ). For NFR elicitation, transcutaneous electrical stimulation was delivered to the sural nerve using a Digitimer constant current stimulator (Digitimer, Ltd., DS7A; Hertfordshire, England). Sural nerve stimulation consisted of a train of 5 rectangular wave pulses (1 ms width, 3 ms ISI) set to current intensities determined to reliably evoke an NFR response during NFR threshold assessment [38-39]. A Physio2go electronic muscle stimulator (Physio2go, EV807; Victoria, BC) was used to deliver aversive abdominal shocks. The abdominal stimulation site was chosen in anticipation of future studies with the same methodology to study visceral pain although it should be noted that in a prior study of ASR to threat no differences were found between abdominal or arm site threat trials [29]. Each stimulation to the abdomen consisted of a pulse train (260 μs pulse width) lasting 1 s in duration, with a frequency of 130 Hz, and set to a fixed current intensity level of 20.4 mA across individuals. This level of stimulation was chosen to elicit a mild to moderately unpleasant muscle contraction [29,47]. Both stimulator

devices are approved for human use and have built in protection circuits to prevent giving subjects unsafe levels of stimulation.

2.3. Skin conductance response (SCR)

Skin conductance was measured using two Ag-AgCl (Grass Technologies, F-E9; 11 mm diameter) disk electrodes attached to the volar surface of the index and middle finger of the subject's non-dominant hand and was collected for a 3 s pre-stimulus and 8 s post-stimulus window for each of the acoustic startle probes and sural nerve stimuli. Skin conductance level (SCL) in microsiemens (μS) was derived from the mean values of the pre-stimulus windows. Skin conductance response (SCR) was operationally defined as the change in skin conductance from the trough preceding the response to the apex of a response occurring in the 8 s post-stimulus window. SCRs were expressed in μS and log-transformed to increase normality.

2.4. Acoustic startle reflex (ASR)

Electromyogram (EMG) activity of the orbicularis oculi associated with the ASR was recorded from 6 mm electrodes (Gereonics; Mission Viejo, CA) placed beneath the right eye approximately 10 mm apart edge to edge, and 8 mm below the lower lid margin. In addition, vertical electrooculograms (vEOG) were recorded from a pair of 11 mm electrodes (Grass Technologies) placed above and below the left eye to facilitate identification of spontaneous blinks, saccades, and large gaze shifts during offline scoring of orbicularis oculi EMG responses. The ground electrode (Grass Technologies; 11 mm diameter) was placed in the center of the forehead. The impedance level for all electrodes never exceeded 20 kΩ. Raw orbicularis oculi EMG and vEOG signals were filtered with low and high frequency cut-off values of 30 Hz and 1000 Hz and 0.01 and 30Hz, respectively, with the former signal undergoing full-wave rectification and amplified 10,000× and the latter signal amplified 5000×.

Prior to conducting analyses, orbicularis oculi EMG activity was smoothed offline using a 2 ms moving average. Peak startle amplitude was defined as the highest point within a window from 20 ms to 105 ms following startle onset relative to a 200 ms pre-stimulus baseline period and was expressed in microvolts (μV). ASR magnitude scores were log transformed to increase normality. Trials with excessive EMG activity or vEOG responses during the 200 ms preceding the startle stimulus and during the first 20 ms following the startle stimulus were rejected since these are often indicative of spontaneous blinks or saccades. Trials with no discernable EMG response onset evident during the 20 to 80 ms interval following the onset of the acoustic startle probe were scored as zero. Three subjects had more than half their trials rejected or designated as zeroes and were excluded from all subsequent ASR analyses, leaving a total of 42 out of 45 subjects in which ASR analyses were conducted.

2.5. Nociceptive flexion reflex (NFR)

The procedure for individually determining the current intensity (mA) needed to reliably evoke an NFR was adapted from Rhudy and France's previously published protocol [11,33,38-39]. For NFR elicitation, a bipolar stimulating electrode (Grass Technologies, F-E10S2) was placed behind subject's left ankle, directly over the sural nerve. NFR recording was accomplished via surface EMG activity obtained from a pair of 20 mm diameter electrodes (Grass Technologies) placed over the biceps femoris muscle of the ipsilateral limb, approximately 10 cm above the superior popliteal fossa. Impedance levels for all NFR recording and stimulation electrodes were always below 10 kΩ. Raw EMG data were bandpass filtered at 10-300 Hz, amplified 20,000× and full-wave rectified. Sural nerve stimulation was delivered at pseudorandomized intervals of 15-30 s to minimize habituation.

After each stimulation, subjects were asked to verbally rate their pain using a scale displayed on the computer screen ranging from 0, representing “no sensation”, to 100, representing “maximum tolerable” level of stimulation (Fig. 1).

For all subjects, NFR threshold assessment began with a current intensity set to 1.5 mA. Current intensity was increased in 1.5 mA steps until the NFR was evoked and then decreased in 0.75 mA steps until the NFR was no longer elicited (Fig. 1). Based on prior parametric studies [11,33] an NFR was considered present if the interval z score (NFR interval mean – baseline mean/baseline standard deviation) was equal or greater than 1.38. The up-down staircase procedure was repeated two additional times using 0.5 mA steps. The intensity of the last two peaks and troughs were averaged and multiplied by 120% to obtain the suprathreshold stimulus intensity (mA) level to be used for the remainder of the experiment. Following NFR threshold assessment, two test electrical stimuli set to subject’s suprathres hold stimulus intensity were delivered to the sural nerve and subject’s pain ratings from these test trials were averaged and used as an index of pain at NFR suprathreshold. In the event that a reliable NFR could not be evoked during threshold assessment or if subject rated 100 on the intensity scale prior to NFR threshold detection, subject was given the option of either repeating the threshold work-up procedure or discontinuing the experiment. If upon repeating the procedure, the NFR threshold was still not reached, subject was thanked for participating, debriefed and sent home.

Similar procedures for NFR elicitation and recording employed during threshold assessment were used to probe the NFR during the experiment. Briefly, sural nerve stimulation consisted of a train of 5 rectangular wave pulses (1 ms pulse width, 3 ms ISI) set at subject’s suprathreshold stimulus intensity determined during the NFR threshold assessment procedure. NFR magnitude was calculated by subtracting the mean bicep femoris EMG activity (in μV) occurring in the pre-stimulus baseline interval window (−65 to −5 ms) from the mean bicep femoris EMG activity (in μV) in the 90-150 ms post-stimulus interval window and dividing by the pooled standard deviations to obtain a Cohen’s *d* value [34].

2.6. Experimental design and procedure

Figure 1 illustrates the experimental design and procedures for the ‘unpredictable threat of abdominal shock’ paradigm. Upon arrival, each subject was familiarized with the laboratory environment and given a brief audiometric test wherein a series of pure tones (1 kHz,15 dB) were delivered randomly to the left and right ear using an audiometer (Ambco; Model 650A). Intensity level was increased in 5 dB steps until subject responded to hearing two consecutive tones in both ears or a 40 dB intensity level was reached. This procedure was repeated for frequencies at 2, 3, and 4 kHz. Subjects that failed to respond to two consecutive tones in the left or right ear at or below 40 dB for any of these frequencies were excluded from the study. Sites for electrode placement were then prepared by gently abrading the skin with a mildly abrasive cleansing solution (Nuprep™) and a conductive water-soluble paste was applied between the skin and each electrode disk to achieve impedances below 20 kΩ, or in the case of biceps femoris recording and stimulating electrodes, less than 10 kΩ. Once electrode placement was complete, subject was led to an adjacent, dimly lit, sound-attenuated room and seated in a recliner positioned in front of a 21” computer display monitor placed approximately 2 m from the subject, at eye-level. The subject room was equipped with a voice-activated intercom and closed-circuit video camera allowing for continuous monitoring and communication with subject throughout the experiment. As described previously, each subject underwent NFR threshold assessment prior to the start of the experiment to determine the suprathreshold stimulus current intensity level (mA) that reliably evoked an NFR response. Following NFR threshold assessment, two surface stimulation pads for delivery of aversive electrical abdominal shocks were placed over subject’s lower abdomen, in a region directly overlaying the descending sigmoid colon.

Each subject was then given explicit instructions by the researcher regarding the nature of the experimental paradigm and fitted with a pair of headphones for delivery of acoustic startle probes during the experiment.

Prior to the start of the unpredictable threat of shock experiment but after determination of the NFR threshold, subjects underwent an affective picture viewing task in which ASR and NFR responses were probed while subjects viewed neutral and negatively-valenced pictures displayed on the computer monitor. These data were collected to assess picture based affect modulation of defensive reflexes and autonomic responses. Since they involve a very different independent variable and hypotheses from the results of the current paper the data will be presented in a separate report.

After a brief rest period, the unpredictable threat of shock paradigm began. The experiment consisted of two blocks, each containing 8 separate 30 s periods or trials in which the ASR or NFR was evoked 9-21 s following period onset (ISI = 25-40 s) (Fig. 1). In the first block of the experiment (Block 1), the ASR was probed; in the second block (Block 2) the NFR was probed. As is standard procedure for startle experiments [2], preceding the first block, subjects were given a series of five startle stimuli every 18-25 s to reduce initial startle reactivity (data not shown). For both experimental blocks, threat was manipulated by alternating periods of “safe” and “threat” (Fig. 1). Whether a phase started with a safe or a threat period was counterbalanced across subjects. Subjects were given explicit instructions prior to the start of the experiment regarding the conditions under which they may or may not receive abdominal stimulation. Subjects were instructed that during the ‘safe’ periods the computer would display the text “*SAFE: No abdominal stimulation will be given*” and that no stimulation to their abdomen would occur while this text was displayed. In contrast, for the ‘threat’ periods subjects were told that when the text “*DANGER: abdominal stimulation may occur at anytime*” appeared on the screen, they may or may not receive stimulation to their abdomen. Although subjects were told they may receive abdominal stimulation at any time during each threat period, in actuality subjects only received abdominal shocks during two of the four threat periods (50% of threat trials), once towards the start and middle of each block. For threat periods that contained abdominal shocks, delivery of electrical stimulus was randomly varied between 19-21 s following the onset of the threat period (Fig. 1). Upon completion of the experiment, each subject was asked to rate retrospectively their general level of anxiety during the threat of shock paradigm using a 11-point scale (0 = not at all anxious; 10 = extremely anxious).

2.7. Statistical analyses

For all dependent measures (SCR, ASR, NFR), analyses were conducted with mixed model analysis of variance (ANOVA) using the PROC MIXED procedure in SAS 9.2 (SAS Institute Inc., Carey, NC). Model fitting of covariance structure was accomplished using standard procedures employed in our laboratory [29,47] and described by others [24,44]. Heterogeneous compound symmetry yielded the best fitting covariance structure as indicated by Akaike’s Information Criteria. For the primary analyses, a 2 (Condition: Safe; Threat) by 2 (Sex: Male; Female) mixed model ANOVA was used for each dependent measure. Planned contrasts were adjusted using Bonferroni correction with alpha levels set to $p < 0.05$. In addition, a series of one-way ANOVAs were conducted to test for sex differences in suprathreshold stimulus intensity, BMI, ratings of pain, anxiety and depression. Bivariate correlational (Pearson’s r; two-tailed) analyses and analysis of covariance (ANCOVA) were also conducted to examine the influence of these individual difference variables on ASR and NFR magnitudes across the experimental conditions (safe; threat) for both males and females. For the mixed model ANCOVA analyses, each of these covariates were mean centered before adding to the model.

3. Results

3.1. Subject characteristics

Table 1 presents the descriptive and inferential statistics for subject characteristics obtained prior to and following the experiment, as well as during the NFR threshold assessment procedure. Analyses using a series of one-way ANOVAs revealed no significant sex differences for any of the dependent variables with the exception of the post-experiment anxiety measure [$F(1, 44) = 5.76$ $p = 0.03$]. Visual inspection of estimated means verified that females reported significantly higher levels of anxiety during the unpredictable threat of shock experiment than males.

3.2. Threat modulated SCRs to acoustic startle and noxious sural nerve stimulation

To examine whether baseline SCL differed across the safe and threat conditions, preliminary analyses using mixed model ANOVAs were conducted on average SCL during the 3 s time window preceding stimulus probe onsets. Analyses indicated that that the basal SCLs were not significantly different across conditions for either Block 1 [$F(1, 31) = 1.84$, $p = 0.19$] or Block 2 [$F(1, 41) = 0.109$, $p = 0.74$], nor were there any sex differences in this response [Block 1: $F(1, 31) = 1.84$, $p = 0.19$; Block 2: $F(1, 41) = 1.23$, $p = 0.27$].

A 2 (Condition: Safe; Threat) by 2 (Sex: Male; Female) mixed model ANOVA demonstrated that unpredictable threat significantly altered SCRs to the acoustic startle probe [$F(1, 31) = 24.20$, $p < 0.001$] and noxious sural nerve stimulation (‘pain evoked’ SCR) [$F(1, 41) = 11.99$, $p = 0.001$]. Visual inspection of estimated means (least square means) revealed participants displayed larger startle- and ‘pain-evoked’ SCRs during the threat compared to the safe periods (Fig. 2A and 2C). No significant main effects of Sex for startle-evoked SCRs [$F(1, 31)= 1.83$, $p = 0.18$] or SCRs evoked by noxious sural nerve stimulation [$F(1, 41) = 0.38$, $p = 0.54$] were found (Figs. 2B and 2D). In addition, no significant Condition x Sex interaction was observed for SCRs to acoustic startle probes [$F(1, 31) = 0.01$, $p = 0.91$]. However, there was a significant Condition x Sex interaction [$F(1, 41) = 4.66$, $p = 0.04$] for SCRs to noxious sural nerve stimulation (Fig. 2D). A post-hoc contrast of condition effects (threat – safe) between groups showed that females had significantly larger SCR magnitude changes (difference scores) in their ‘pain-evoked’ SCRs compared to their male counterparts, $t (41) = 3.71$, $p < 0.001$ (Fig. 2D).

3.3. Threat modulated ASR and NFR responses

Analysis using a 2 (Condition: Threat; Safe) by 2 (Sex: Male; Female) mixed model ANOVA on ASR magnitudes showed a significant main effect for condition, $F(1, 39) = 80.23$, $p < 0.001$. As predicted, participants had larger ASR magnitudes during threat compared to safe periods (Fig. 3A). No significant main effect for Sex [$F(1, 40) = 0.04$, $p = 0.84$] or Condition by Sex interactions were found [$F(1, 39) = 0.02$, $p = 0.88$], suggesting that differences in ASR responding during the unpredictable threat paradigm were independent of sex (Fig. 3B).

A 2 (Condition: Threat; Safe) by 2 (Sex: Male; Female) mixed model ANOVA conducted on NFR magnitudes showed significant main effects for both Condition [$F(1, 43) = 6.75$, $p = 0.01$] and Sex [$F(1, 43) = 8.58$, $p = 0.005$], but no significant Condition x Sex interaction [$F(1, 43) = 0.20$, $p = 0.65$]. NFR magnitudes differed significantly between the safe and threat conditions, with subjects having overall larger NFR magnitudes during threat compared to safe periods (Fig. 3C). Moreover, females displayed larger NFR magnitudes across both conditions compared to males (Fig. 3D), indicating an overall enhancement in nociceptive signaling in females.

3.4. Relationship between threat modulated arousal, ASR and NFR responses

To test the relationships among SCR, ASR, and NFR responses during the unpredictable threat of shock paradigm, bivariate correlational analyses were conducted independently on safe and threat trials averaged within conditions and within subjects. Analyses revealed no significant correlations between ASR and NFR responses during either the safe ($r = 0.06$, $p = 0.69$) or threat ($r = 0.12$, $p = 0.47$) periods. In contrast, acoustic startle-evoked SCRs were positively correlated with ASR responses during threat ($r = 0.51$, $p = 0.003$), but not safe periods ($r = 0.14$, $p = 0.43$), whereas SCRs to noxious sural nerve stimulation were positively correlated with NFR magnitudes during both safe ($r = 0.32$, $p = 0.04$) and threat ($r = 0.43$, $p = 0.004$) periods. Thus, while ASR and NFR responses for individual subjects were not correlated, the size of the SCR, representing a SNS response to the acoustic startle probe or to sural nerve stimulation, was positively related to the magnitude of the ASR and NFR.

3.5. Relationships between NFR magnitude, suprathreshold stimulus intensity, and subject characteristics

Bivariate correlational analyses were also conducted between NFR magnitude scores and sural nerve suprathreshold stimulus intensity (120% NFR threshold in mA), pain ratings, BMI, post-experiment anxiety ratings, and HAD anxiety and depression scores. Suprathreshold stimulus intensity was positively correlated with NFR magnitude (Cohen's d) scores ($r = 0.33$, $p = 0.03$); higher stimulus intensities were associated with larger NFR magnitudes. No other significant correlations were found for NFR magnitude scores and pain ratings ($r = 0.01$, $p = 0.93$), BMI ($r = 0.01$, $p = 0.96$), post-experiment anxiety ($r = 0.10$, $p = 0.51$) or HAD anxiety ($r = -0.09$, $p = 0.55$) and depression ($r = 0.07$, $p = 0.66$).

A mixed model ANCOVA using suprathreshold stimulus intensity as the covariate was performed to examine the impact of the sural nerve suprathreshold stimulus intensity used during the experiment on NFR magnitude responses across conditions and between groups. Significant main effects for Condition [$F(1, 266) = 4.42$, $p = 0.04$], Sex [$F(1, 266) = 5.63$, $p = 0.02$], and the covariate [$F(1, 266) = 7.30$, $p = 0.007$] were found. Similar to the primary analysis of NFR magnitude, mean comparisons suggested that NFR magnitude was larger during the threat compared to the safe periods, with females showing greater NFR magnitude scores than males. Therefore, the significant main effect for Sex endured even after inclusion of the covariate suggesting that the primary group differences in NFR magnitude responses were not due to variations in suprathreshold stimulus intensity between males and females. The ANCOVA analysis did not yield any significant Condition x Sex interactions [$F(1, 266) = 0.21$, $p = 0.65$] when the suprathreshold stimulus intensity covariate was added to the model.

A second mixed model ANCOVA specifying post-experiment anxiety as the covariate was performed to examine whether anxiety ratings covaried with NFR magnitude responses differentially in males compared to females for the safe and threat conditions. Analysis revealed that the significant main effect for Condition [$F(1, 266) = 4.44$, $p = 0.04$] endured even after including anxiety in the model, whereas the main effect for Sex [$F(1, 266) = 1.92$, $p = 0.17$] no longer reached significance. No significant interaction for Sex x Condition was found [$F(1, 266) = 0.43$, $p = 0.51$], nor were there any significant interactions with the covariate for Sex [$F(1, 266) = 0.44$, $p = 0.51$], Condition[$F(1, 266) = 0.63$, $p = 0.43$], or Sex x Condition [$F(1, 266) = 0.10$, $p = 0.75$].

4. Discussion

4.1. Affective modulation of acoustic startle

The present findings are consistent with previous reports demonstrating threat-induced potentiation of acoustic startle, with heightened startle reactivity observed during anticipatory threat of an aversive stimulus compared to periods signifying safety or the absence of threat [5,15-16]. Grillon and colleagues showed facilitation of acoustic startle during periods signaling the possibility of a mild, but sufficiently aversive electric shock to the wrist compared to periods that denoted safety and thus, no possibility of shock [15]. Bradley et al. also found enhanced acoustic startle and heightened autonomic arousal, indexed by skin conductance and heart rate deceleration, for periods signaling threat compared to safe periods [5]. These findings parallel our current results demonstrating heightened startle reactivity and startle-evoked SCRs during threat of an abdominal shock relative to safe periods and provide further empirical support for the premise that the defensive system is upregulated during anticipation of a moderately aversive, unpredicatable threat.

Based on a prior study showing greater ASR during an unpredictable threat of shock paradigm in women compared to men [14], we also predicted sex differences in ASR reactivity during anticipatory threat with women showing greater potentiated ASRs than men. However, we found no sex differences in ASR during the threat or safe periods. Differences in experimental design may underlie the discrepancy in findings observed across the two studies. Grillion and colleagues found sex differences when a relatively long 150 s threat period was used, but no sex difference when comparing brief, 8 s threat and safe periods [14]. The added length of the threat period employed by Grillon may have contributed to a greater degree of uncertainty and perhaps greater sex differentiation than seen in the current study which used relatively brief, 30 s threat periods. Alternative explanations for the lack of sex differences in ASR could be attributed to variations in sample characteristics and/or menstrual cycle effects in females which were not controlled for in either study.

4.2. Threat modulation of the nociceptive flexion reflex

The current study demonstrated that anticipation of an aversive, unpredictable threat significantly enhanced the nociceptive reflex, with subjects showing greater NFR responses during threat compared to periods signaling safety. This suggests anticipatory anxiety enhanced nociception presumably via withdrawal of descending inhibition and/or an increase in descending facilitation, by supraspinal centers involved in threat detection, arousal, and affect-driven responses. Willer et al. in an early NFR study also reported potentiated NFRs during anticipation of high intensity sural nerve stimulations, but specific procedures in terms of predictability of the stimulus were omitted [50]. Moreover, our findings are also consistent with motivational priming theory which postulates that activation of the defensive motivational system primes defensive responses such as escape or attack and leads to increased vigilance and pain sensitivity [22]. Rhudy and Meagher have refined some aspects of this theory by hypothesizing that pain facilitation is maximum at moderate levels of threat intensity, whereas at higher and more imminent degrees of danger, pain may in fact be inhibited [28,36]. The threat of shock procedure in the current study represents a moderate and somewhat unpredictable danger, thus the increase in pain sensitivity fits these predictions.

4.3. Sex differences in NFR responses

Although there were no sex differences in ASR response we did find a sex difference in nociceptive responding, with females showing larger NFRs during both safe and threat

periods compared to males. We hypothesize that the larger NFR responses in females may reflect sex-related differences to the overall experimental context as opposed to the threat versus safe periods per se. Under conditions associated with an unpredictable, moderately aversive threat, females may show greater engagement of emotional-arousal circuits [21], resulting in facilitation of endogenous pain modulatory systems. This interpretation is supported by several findings: a) no sex-related differences in NFR suprathreshold stimulus intensity were observed when assessed prior to the threat of shock paradigm; b) females reported higher levels of anxiety following the threat of shock paradigm than males; and c) the sex difference in NFR was abolished when differences in anxiety were controlled statistically. Additionally, the experiment included repeated unpredictable noxious stimulations both to the abdomen and to the sural nerve which may have differentially impacted males and females through a process of sensitization [32].

There is a large body of evidence demonstrating greater clinical pain reports in women compared to men, and a parallel, but less extensive experimental literature showing increased pain sensitivity (lower thresholds and tolerance) in women for pressure and electrical stimuli [8,37,49]. A few studies conducted in healthy volunteers have shown significant sex differences in NFR threshold characteristics, with females generally displaying lower NFR thresholds than males [12,27,30]. However, an absence of significant sex differences in NFR thresholds has also been reported [9,10]. Studies examining pain modulation using diffuse noxious inhibitory control (DNIC) or temporal summation procedures have also shown decreased pain inhibition and increased pain facilitation in women, respectively, although the results are not entirely consistent [8]. Sex-related differences in pain perception are multi-determined, and may include hormonal effects, differences in structure and function of the peripheral and central nervous systems, and differences in psychological and cultural variables related to gender [28]. Interestingly, the most robust sex differences in experimental pain are seen when the stimulus and/or the experimental procedure involves strong negative affect [8,37,40]. Rhudy and Williams have suggested that the increased responsiveness of women to affective stimuli may be a significant contributor to endogenous pain modulation, especially under conditions of mild to moderate stress [37]. However, while SNS activity as measured by SCRs to the sural nerve stimulus was positively associated with NFR magnitude, it did not follow the pattern of overall increased responsiveness in females during both the safe and threat periods. Females appeared to have lower SCRs during the safe condition compared to males but significantly greater SCR increases under conditions of threat, whereas males failed to show any threat-induced augmentation of their SCRs. Thus, these data suggest there are sex-related differences in nociceptive signaling and pain-evoked arousal responses under conditions associated with anticipation of a moderately aversive threat. This is seen in the potentiated NFR response across both safe and threat conditions in females and the concomitant threat-induced increase in SCRs to noxious sural nerve stimulation.

4.4. Limitations

It should be noted that since we did not control for women's menstrual phase it is possible that differences in circulating hormones may have influenced our findings. By self-report, the majority of female subjects were naturally cycling in various phases of their menstrual cycle, whereas a small percentage was on birth control. Few studies have investigated menstrual cycle effects on NFR and of these studies, results are mixed. For example, one study found NFR threshold was lower during the luteal compared to the follicular phase in 14 healthy women [46], whereas another found no effect of menstrual cycle on NFR in a sample of 41 healthy women [31]. Another limitation is that the post-experiment anxiety ratings were retrospective and therefore may include memory bias. Furthermore, these ratings were derived from a single subjective average across conditions which may further

limit their validity. It should also be noted that a significant number of subjects (18.6%; 4 males, 7 females) were excluded from the analysis due to reaching pain tolerance prior to showing an NFR. This percentage is similar to that of other NFR studies [7,30,39] but nevertheless may have resulted in some selection bias towards subjects with lower pain tolerance.

5. Conclusion

The present study investigated sex differences in the modulation of two defensive reflexes by an unpredictable, aversive threat. Two new findings extend the current literature on pain processing. First, we show that anticipatory anxiety induced by threat of shock leads to an upregulation of the NFR. This was accompanied by a potentiation in the ASR and increased sympathetic nervous system responses. Secondly, females showed greater NFRs across safe and threat conditions compared to males. This may reflect greater responding to the experimental context associated with anticipation of a pain threat in women and suggests that context-related pain facilitation may be an important mechanism underlying the greater pain reports in females.

SUMMARY
Unpredictable threat enhanced arousal and affect-related defensive responses across males and females, whereas nociceptive and sympathetic responses to noxious sural nerve stimulation showed sex-dependent effects.

Acknowledgments

Special thanks to Bradford Lubell, Cathy Liu, Byron Alexander, and Melissa Alberto for their assistance. This research was supported by NIH Grants P50-DK64539, R24-AT002681, NIH GI Training Grant T32-DK07180-34, VA Medical Research, and a gift from the Virginia Friedhofer Charitable Trust.

References

[1]. Arnold BS, Alpers GW, Suss H, Friedel E, Kosmutzky G, Geier A, Pauli P. Affective pain modulation in fibromyalgia, somatoform pain disorder, back pain, and healthy controls. Eur J Pain. 2008; 12:329–338. [PubMed: 17723312]

[2]. Blumenthal TD, Cuthbert BN, Filion DL, Hackley S, Lipp OV, Van Boxtel A. Committee report: guidelines for human startle eyeblink electromyographic studies. Psychophysiology. 2005; 42:1–15. [PubMed: 15720576]

[3]. Bobey MJ, Davidson PO. Psychological factors affecting pain tolerance. J Psychosom Res. 1970; 14:371–376. [PubMed: 5495257]

[4]. Bolles RC, Fanselow MS. A perceptual–defensive–recuperative model of fear and pain. Behav Brain Sci. 1980; 3:291–323.

[5]. Bradley MM, Silakowski T, Lang PJ. Fear and defensive activation. Pain. 2008; 137:156–163. [PubMed: 17904289]

[6]. Davis MC, Zautra AJ, Reich JW. Vulnerability to stress among women in chronic pain from fibromyalgia and osteoarthritis. Ann Behav Med. 2001; 23:215–226. [PubMed: 11495222]

[7]. Dowman R. A noninvasive strategy for identifying and quantifying innocuous and nociceptive peripheral afferent activity evoked by nerve stimulation. Physiol Behav. 1993; 53:1163–1169. [PubMed: 8346300]

[8]. Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL. Sex, gender, and pain: a review of recent clinical and experimental findings. J Pain. 2009; 10:447–485. [PubMed: 19411059]

[9]. France CR, France JL, al'Absi M, Ring C, McIntyre D. Catastrophizing is related to pain ratings, but not nociceptive flexion reflex threshold. Pain. 2002; 99:459–463. [PubMed: 12406521]

[10]. France CR, Froese SA, Stewart JC. Altered central nervous system processing of noxious stimuli contributes to decreased nociceptive responding in individuals at risk for hypertension. Pain. 2002; 98:101–108. [PubMed: 12098621]

[11]. France CR, Rhudy JL, McGlone S. Using normalized EMG to define the nociceptive flexion reflex (NFR) threshold: further evaluation of standardized NFR scoring criteria. Pain. 2009; 145:211–218. [PubMed: 19595510]

[12]. France CR, Suchowiecki S. A comparison of diffuse noxious inhibitory controls in men and women. Pain. 1999; 81:77–84. [PubMed: 10353495]

[13]. Gannon LR, Haynes SN, Cuevas J, Chavez R. Psychological correlates of induced headaches. J Behav Med. 1987; 10:411–423. [PubMed: 3669073]

[14]. Grillon C. Greater sustained anxiety but not phasic fear in women compared to men. Emotion. 2008; 8:410–413. [PubMed: 18540756]

[15]. Grillon C, Ameli R, Woods SW, Merikangas K, Davis M. Fear-potentiated startle in humans: effects of anticipatory anxiety on the acoustic blink reflex 1991. Psychophysiol. 1991; 28:588–595.

[16]. Grillon C, Davis M. Acoustic startle and anticipatory anxiety in humans: effects of monaural right and left ear stimulation. Psychophysiol. 1995; 32:155–161.

[17]. Grillon C, Morgan CA, Davis M, Southwick SM. Effects of experimental context and explicit threat cues on acoustic startle in Vietnam veterans with posttraumatic stress disorder. Biol Psychiatry. 1998; 44:1027–1036. [PubMed: 9821567]

[18]. Jones A, Zachariae R. Gender, anxiety, and experimental pain sensitivity: an overview. JAMWA. 2002; 57:91–94. [PubMed: 11991428]

[19]. Kessler RC, McGonagle KA, Zhao S, Nelson CB, Hughes M, Eshleman S, Wittchen HU, Kendler KS. Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. Arch Gen Psychiatry. 1994; 51:8–19. [PubMed: 8279933]

[20]. King TE, Crown ED, Sieve AN, Joynes RL, Grau JW, Meagher MW. Shock-induced hyperalgesia in rats: evidence that forebrain systems play an essential role. Behav Brain Res. 1999; 100:33–42. [PubMed: 10212051]

[21]. Labus JS, Naliboff BD, Fallon J, Berman SM, Suyenobu B, Bueller JA, Mandelkern M, Mayer EA. Sex differences in brain activity during aversive visceral stimulation and its expectation in patients with chronic abdominal pain: a network analysis. Neuroimage. 2008; 41:1032–1043. [PubMed: 18450481]

[22]. Lang PJ, Bradley MM, Cuthbert BN. Emotion, attention, and the startle reflex. Psychol Rev. 1990; 97:377–395. [PubMed: 2200076]

[23]. Lang PJ, Greenwald MK, Bradley MM, Hamm AO. Looking at pictures: affective, facial, visceral, and behavioral reactions. Psychophysiol. 1993; 30:261–273.

[24]. Littell RC, Pendergast J, Natarajan R. Modelling covariance structure in the analysis of repeated-measures data. Stat Med. 2000; 19:1793–1819. [PubMed: 10861779]

[25]. Malow RM. The effects of induced anxiety on pain perception: a signal detection analysis. Pain. 1981; 11:397–405. [PubMed: 7329704]

[26]. Meagher MW, Ferguson AR, Crown ED, McLemore S, King TE, Sieve AN, Grau JW. Shock-induced hyperalgesia: IV. Generality. J Exp Psychol Anim Behav Process. 2000; 27:219–238. [PubMed: 11497322]

[27]. Mylius V, Kunz M, Schepelmann K, Lautenbacher S. Sex differences in nociceptive withdrawal reflex and pain perception. Somatosens Mot Res. 2005; 22:207–211. [PubMed: 16338828]

[28]. Naliboff, BD.; Rhudy, JL. Anxiety and functional pain disorders. In: Mayer, EA.; Bushnell, MC., editors. Functional pain syndromes: presentation and pathophysiology. IASP Press; Seattle, WA: 2009. p. 185-213.

[29]. Naliboff BD, Waters AM, Labus JS, Kilpatrick L, Craske MG, Chang L, Negoro MD, Ibrahimovic H, Mayer EA, Ornitz E. Increased acoustic startle responses in IBS patients during abdominal and nonabdominal threat. Psychosom Med. 2008; 70:920–927. [PubMed: 18842745]

[30]. Page GD, France CR. Objective evidence of decreased pain perception in normotensives at risk for hypertension. Pain. 1997; 73:173–180. [PubMed: 9415503]

[31]. Rhudy JL, Bartley EJ. The effect of the menstrual cycle on affective modulation of pain and nociception in healthy women. Pain. 2010; 149:365–372. [PubMed: 20304557]

[32]. Rhudy JL, Bartley EJ, Williams AE. Habituation, sensitization, and emotional valence modulation of pain responses. Pain. 2010; 148:320–327. [PubMed: 20022696]

[33]. Rhudy JL, France CR. Defining the nociceptive flexion reflex (NFR) threshold in human participants: a comparison of different scoring criteria. Pain. 2007; 128:244–253. [PubMed: 17070999]

[34]. Rhudy JL, France CR, Bartley EJ, McCabe KM, Williams AE. Psychophysiological responses to pain: further validation of the nociceptive flexion reflex (NFR) as a measure of nociception using multilevel modeling. Psychophysiol. 2009; 46:939–948.

[35]. Rhudy JL, Meagher MW. Fear and anxiety: divergent effects on human pain thresholds. Pain. 2000; 84:65–75. [PubMed: 10601674]

[36]. Rhudy JL, Meagher MW. Role of emotion in pain modulation. Curr Opinion Psychiatr. 2001; 14:241–245.

[37]. Rhudy JL, Williams AE. Gender differences in pain: do emotions play a role? Gend Med. 2005; 2:208–226. [PubMed: 16464733]

[38]. Rhudy JL, Williams AE, McCabe KM, Nguyen MA, Rambo PL. Affective modulation of nociception at spinal and supraspinal levels. Psychophysiol. 2005; 42:579–587.

[39]. Rhudy JL, Williams AE, McCabe KM, Rambo PL, Russell JL. Emotional modulation of spinal nociception and pain: the impact of predictable noxious stimulation. Pain. 2006; 126:221–233. [PubMed: 16890356]

[40]. Riley JL, Robinson ME, Wise EA, Myers CD, Fillingim RB. Sex differences in the perception of noxious experimental stimuli: a meta-analysis. Pain. 1998; 74:181–187. [PubMed: 9520232]

[41]. Sandrini G, Serrao M, Rossi P, Romaniello A, Cruccu G, Willer JC. The lower limb flexion reflex in humans. Prog Neurobiol. 2005; 77:353–395. [PubMed: 16386347]

[42]. Sheehan DV, Lecrubier Y, Sheehan KH, Amorim P, Janavas J, Weiller E, Hergueta T, Baker R, Dunbar GC. The Mini-International Interview (M.I.N.I.): The development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. J Clin Psychiatry. 1998; 59:22–33. [PubMed: 9881538]

[43]. Skljarevski V, Ramadan NM. The nociceptive flexion reflex in humans. Pain. 2002; 96:3–8. [PubMed: 11932055]

[44]. Singer J. Using SAS PROC Mixed to fit multilevel models, hierarchical models, and individual growth models. J Educat Behav Stat. 1998; 24:323–355.

[45]. Smith JC, Bradley MM, Lang PJ. State anxiety and affective physiology: effects of sustained exposure to affective pictures. Biol Psychol. 2005; 69:247–260. [PubMed: 15925028]

[46]. Tassorelli C, Sandrini G, Cecchini AP, Nappi RE, Sances G, Martignoni E. Changes in nociceptive flexion reflex threshold across the menstrual cycle in healthy women. Psychosom Med. 2002; 64:621–626. [PubMed: 12140352]

[47]. Twiss C, Kilpatrick L, Craske M, Buffington T, Ornitz E, Rodríguez LV, Mayer EA, Naliboff BD. Increased startle responses in interstitial cystitis: evidence for central hyperresponsiveness to visceral related threat. J Urolog. 2009; 181:2127–2133.

[48]. Walters ET. Injury related behavior and neural plasticity: an evolutionary perspective on sensitization, hyperalgesia, and analgesia. Int Rev Neurobiol. 1994; 36:325–426. [PubMed: 7822120]

[49]. Wiesenfeld-Hallin Z. Sex differences in pain perception. Gen Med. 2005; 2:137–145.

[50]. Willer JC, Boureau F, Albe-Fessard D. Supraspinal influences on nociceptive flexion reflex and pain sensation in man. Brain Res. 1979; 179:61–68. [PubMed: 509233]

[51]. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. Acta Psychiatry Scand. 1983; 67:361–370.

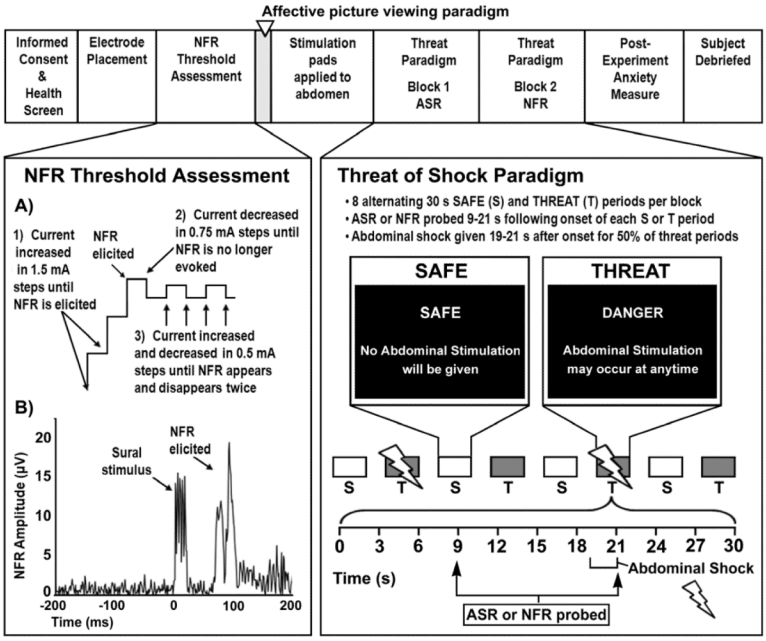

Fig. 1.

Schematic depicting experimental design and procedures. Left panel shows (A) the NFR threshold detection procedure and (B) an example trace of an evoked NFR response. Right panel shows the “threat of abdominal shock” paradigm.

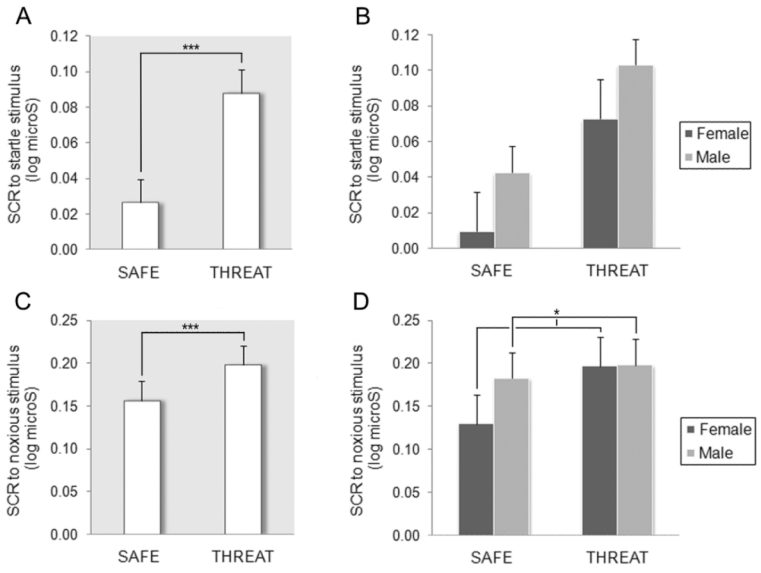

Fig. 2.

Top panel illustrates the means and standard errors (±1 SE) for skin conductance responses (log μS) to acoustic startle probe during the safe and threat periods for all subjects combined (A) and by sex (B). Bottom panel illustrates the means and standard errors (±1 SE) for skin conductance responses to noxious sural nerve stimulation during the safe and threat periods for all subjects combined (C) and by sex (D). *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$

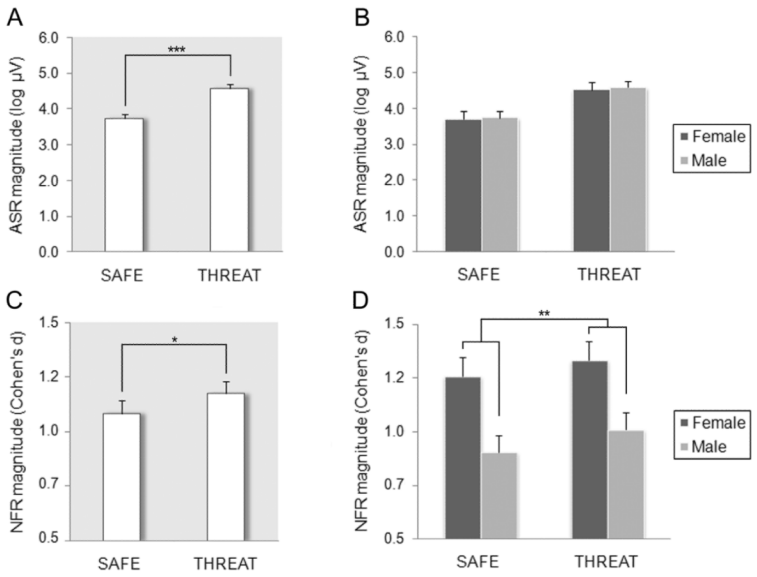

Fig. 3.

Top panel displays the means and standard errors (±1 SE) for ASR magnitudes (log uS) during the safe and threat periods for all subjects combined (A) and by sex (B). Bottom panel shows the means and standard errors (±1 SE) for NFR magnitudes (Cohen's d) during the safe and threat periods for all subjects combined (C) and by sex (D). *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$

Table 1

Means, standard errors, and significance levels for subject characteristics prior to and following threat of shock paradigm and during NFR threshold work-up procedure

Dependent measure	All Subjects (N = 45)	Males (n = 25)	Females (n = 20)	*p*-value
Age	22.18 ± 0.60	22.96 ± 1.01	21.2 ± 0.37	.144
BMI	23.89 ± 0.60	24.50 ± 0.49	23.14 ± 1.20	.264
Anxiety	4.08 ± 0.38	4.28 ± 0.57	4.00 ± 0.47	.718
Depression	1.49 ± 0.24	1.84 ± 0.35	1.10 ± 0.33	.142
Post-Experiment Anxiety	5.54 ± 0.46	4.60 ± 0.61	6.72 ± 0.63	.021
Pain Ratings	60.63 ± 3.74	61.12 ± 5.17	60.03 ± 5.85	.889
Suprathreshold Stimulus Intensity (mA)	17.30 ± 1.27	17.69 ± 8.41	17.18 ± 8.96	.853

* $p < .05$