TEXAS TECH UNIVERSITY HEALTH SCIENCES CENTER
School of Medicine

DEPARTMENT OF PHARMACOLOGY & NEUROSCIENCE

Inter-individual and sex differences in pain-related vocalizations and anxiety-like behaviors

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Introduction

Inter-individual and sex differences have been well documented with regard to anxiety- and depression-like conditions and in pain. However, neural mechanisms and biomarkers related to pain vulnerability and resilience, including potential sexual dimorphisms, have yet to be fully elucidated. Fear learning and extinction networks have been implicated in neuropsychiatric disorders such as anxiety disorders, post-traumatic stress disorder (PTSD), and obsessive compulsive disorder (OCD). Vulnerability to these disorders has been predicted using fear extinction (FE) learning ability as a biomarker for inter-individual differences in the preclinical and clinical settings. Behavioral studies are a crucial tool for the validation of pain mechanisms and for the assessment of potential pharmacological therapies. Higher integrated pain behavior at supraspinal levels has been assessed using vocalizations, an important method of communication among rodents. Inter-individual and sex differences in audible and ultrasonic vocalizations, particularly in the context of pain and fear interactions, have not been determined. The purpose of this study was to examine the predictive value of fear extinction (FE) learning ability for inter-individual differences in pain-related behavioral responses, particularly emotional-affective pain aspects, with regard to sex. We subjected adult male and female rats to cued fear learning and FE tests and correlated inter-individual differences with pain responses in models of acute arthritis pain and chronic neuropathic pain. We also investigated sex differences in FE phenotypes for measures of sensory (mechanical withdrawal thresholds) and emotional-affective (open field tests for anxiety-like behaviors and audible and ultrasonic components of vocalizations) pain-related behaviors.

Methods

Animals:
Male and female Sprague–Dawley rats (150-350g) were housed in a temperature-controlled room and maintained on a 12-hour day/night cycle with unrestricted access to food and water.

Experimental protocol:
Naïve rats were subjected to fear conditioning and FE trials. Rats were then randomly assigned to the acute arthritis pain model or the chronic neuropathic pain model (spinal nerve ligation, SNL). Behavioral studies were performed 4 weeks after surgery or 6 hours after arthritis induction.

Fear conditioning and extinction:
Auditory fear conditioning and fear extinction (FE) learning were tested using two chambers of a Video Fear Conditioning System (Med Associates Inc.). On day 1, rats were habituated to context A followed by fear conditioning by 2 CS-US pairings, inter-tone interval, ITI 240 sec (CS: 80 db, 4.5 kHz, 30s white noise, US: 0.7 mA foot shock, 2s). On day 2, rats were habituated to context B followed by extinction training (30 CSs).

Arthritis pain model:
Rats were briefly anesthetized with isoflurane (2-3%; precision vaporizer, Harvard Apparatus) and a kaolin suspension (4% in sterile saline, 100 µL) was slowly injected into the left knee joint cavity followed by repetitive flexions and extensions of the leg for 15 min. A carrageenan solution (2% in sterile saline, 100 µL) was then injected into the knee joint cavity and the leg was flexed and extended for another 5 min. Naïve rats that underwent similar handling but did not receive intraarticular injections were used as a control group.

Neuropathic pain model:
The well-established SNL model of neuropathic pain was used. Rats were anesthetized with isoflurane (2-3%; precision vaporizer, Harvard Apparatus) and underwent sterile surgery where the left L5 spinal nerve was exposed and tightly ligated using 6-0 sterile silk. This was compared to a sham-operated control group where the nerve was exposed but not ligated.

Pain-related behavioral tests:
Behavioral assays were performed 6 h after arthritis induction in the acute pain group or 4 weeks after SNL or sham surgery in the chronic neuropathic pain group.

•Mechanical withdrawal thresholds were measured by briefly anesthetizing rats (isoflurane, 2-3%) and placing them in a slightly restrained recording chamber that permitted access to the hindlimbs. Hindlimb withdrawal thresholds were evaluated using calibrated forceps with a force transducer whose output was displayed in grams on an LED screen. The calibrated forceps were used to gradually compress the left knee joint (arthritis pain model) or the left hindpaw (neuropathic pain model) with a continuously increasing intensity until a withdrawal reflex was evoked.

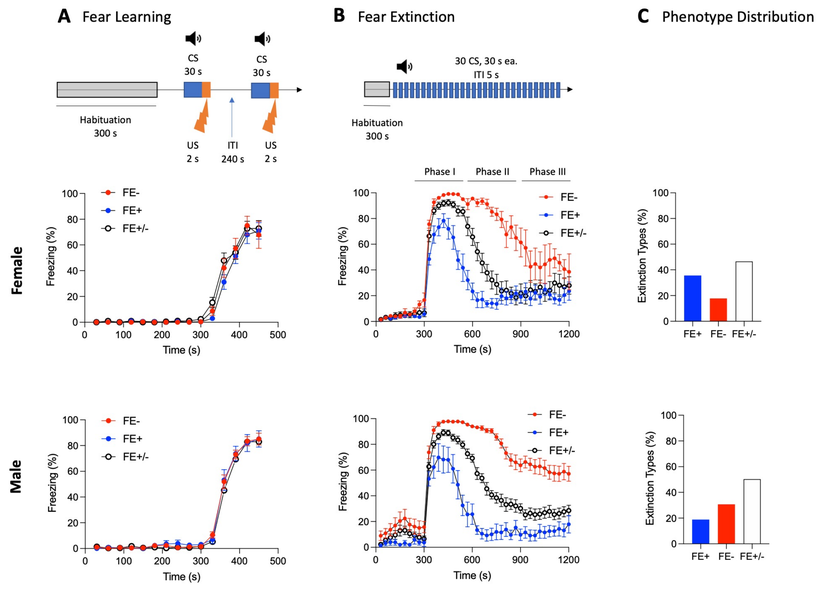
•Vocalizations in the audible (20Hz-16kHz; supraspinally organized nocifensive responses) and ultrasonic (22kHz; limbic-driven negative emotional responses) were measured with our patented computerized recording system. Brief (10 s) noxious stimuli were applied to the left knee joint (arthritis pain model, stimulus: 1500 g/30 mm^2) or to the left hind paw (neuropathic pain model, stimulus: 500 g/6 mm^2) of rats using a calibrated forceps. Vocalizations were recorded for 1 min and analyzed using Ultravox XT 3.2 software (Noldus).

•Anxiety-like behavior was measured using the open field test as decreased number of entries in an illuminated arena (70 cm x 70 cm) for 15 min with a computerized videotracking system (Noldus, EthoVision XT).

Statistical analysis:
Significance was accepted at the level P<0.05. All averaged values represent means ± SEM.

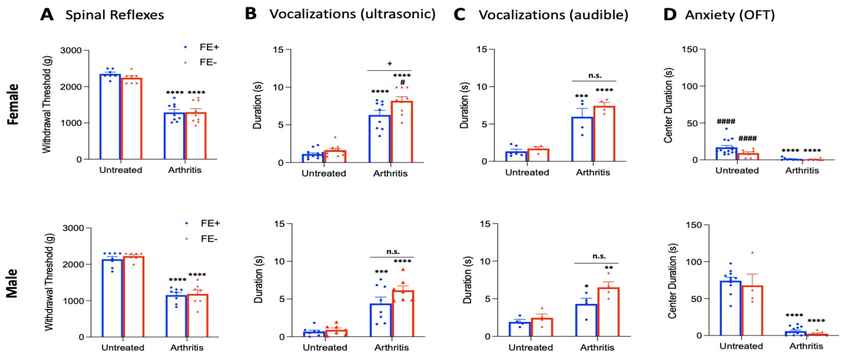
Results

Figure 1 FE phenotypes



Inter-individual and sex differences in fear extinction learning ability in naïve female and male rats. Fear conditioning on Day 1 **(A)** and extinction **(B)** tests were conducted using two distinct context chambers. **(A)** Fear conditioning on Day 1—rats were habituated to context A followed by fear conditioning (2 CS-US pairs). The diagram illustrates the experimental protocol. Symbols in the line graph show freezing responses expressed in percent per 30 s segment during fear conditioning with 2 CS-US pairings. **(B)** Fear extinction learning on Day 2—rats were habituated to context B followed by extinction training (30 CSs, no US). The diagram illustrates the experimental protocol. Symbols in the line graph show freezing responses to tone (CS) expressed in percent per 30 s segment. **(C)** Bar histograms show the distribution of rats with strong (FE+), "normal" (FE+/−), and weak (FE−) fear extinction. The population (%) of FE+ was larger in female rats compared to male rats. CS: conditioned stimulus; US: unconditioned stimulus; ITI: intertone interval; FE: fear extinction.

Figure 2 Arthritis pain behaviors



Inter-individual and sex differences in arthritis pain-related behaviors of FE+ and FE− rats. **(A)** Mechanical thresholds tested in untreated control rats and arthritic rats (6 h post-induction) showed no significant differences between FE− and FE+ untreated rats or between FE− and FE+ arthritic rats, but arthritic FE− and FE+ rats had significantly lower withdrawal thresholds than their untreated controls. ****P<0.0001, ANOVA with Bonferroni post hoc tests. **(B,C)** Duration (s) of ultrasonic and audible vocalizations, respectively, evoked by a brief (10 s) noxious (1500g/30mm^2) mechanical compression of the knee. Significant differences in ultrasonic (but not audible) vocalizations were found between FE- and FE+ female arthritic rats but not between FE- and FE+ male arthritic rats or between untreated FE- and FE+ rats. For both sexes, arthritic rats had significantly increased vocalizations compared to their untreated controls. n.s.: non-significant; $^{+}$P<0.05; $^{\#}$P<0.05; *P<0.05; ** <0.01; ***P<0.001; ****P<0.0001, ANOVA with Bonferroni post hoc tests. **(D)** Center duration (s) in the OFT was significantly lower in arthritic FE− and FE+ rats compared to the untreated FE− and FE+ control rats. No differences were found between FE− and FE+ rats in the untreated control or arthritic groups for either sex. ####P<0.0001; ****P<0.0001, ANOVA with Bonferroni post hoc tests. FE: fear extinction; OFT: open field test. Asterisk (*) indicates comparison to untreated group; plus sign (+) indicates comparison between phenotypes; pound sign (#) indicates comparison between sexes.

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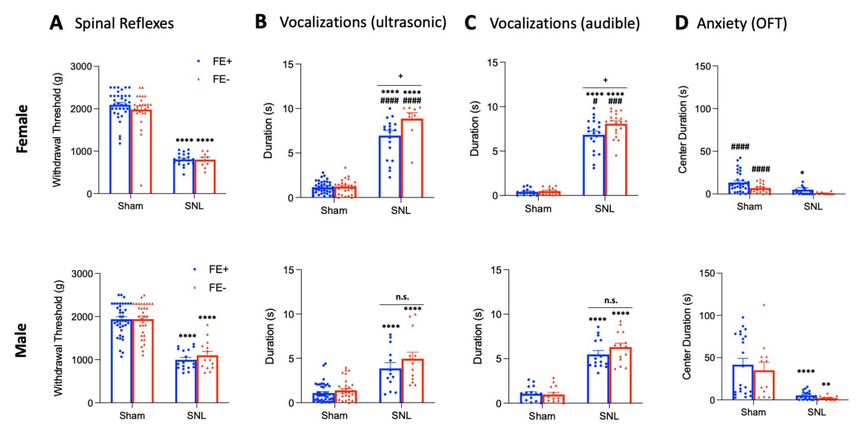
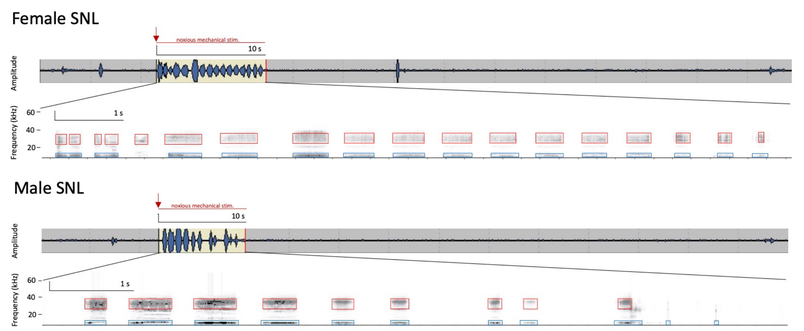
Figure 3 Neuropathic pain behaviors



Inter-individual and sex differences in neuropathic pain-related behaviors of FE+ and FE- rats. (A) Mechanical thresholds tested in sham and chronic neuropathic SNL rats (4 weeks post-induction) showed no significant differences between FE- and FE+ sham rats or between FE- and FE+ SNL rats, but SNL FE- and FE+ rats had significantly lower withdrawal thresholds than their sham controls. ****P<0.0001, ANOVA with Bonferroni post hoc tests. **(B and C)** Duration (s) of ultrasonic and audible vocalizations, respectively, evoked by a brief (10 s) noxious (1500 g/6 mm^2) mechanical compression of the affected hindpaw. Significant differences in ultrasonic and audible vocalizations were found between female FE- and FE+ SNL rats but not between male FE- and FE+ SNL rats or between FE- and FE+ sham rats. For both sexes, SNL rats had significantly increased vocalizations compared to their sham controls. n.s.: non-significant; $^{+}$P<0.05; ****P<0.0001, ANOVA with Bonferroni post hoc tests. **(D)** Center duration (s) in the OFT was significantly lower in FE- and FE+ SNL rats compared to their FE- and FE+ sham controls. No differences were found between FE- and FE+ rats in the sham or SNL groups for either sex. *P<0.05; **P<0.01;****P<0.0001, ANOVA with Bonferroni post hoc tests. FE: fear extinction; OFT: open field test; SNL: spinal nerve ligation. Asterisk (*) indicates comparison to untreated group; plus sign (+) indicates comparison between phenotypes; pound sign (#) indicates comparison between sexes.

Figure 4 Representative vocalizations



Representative audible and ultrasonic vocalizations from female and male rats in the SNL model of neuropathic pain. Original real-time waveform and spectrogram recordings of vocalizations evoked in response to brief (10 s) noxious (1500 g/6 mm^2) mechanical stimulation of the affected hindpaw 4 weeks after SNL surgery in phenotyped female and male rats. Mechanical stimuli were applied to the hindpaw in each recording period as indicated by the highlighted yellow section of the waveform (upper panel, red arrow indicates initiation of noxious stimulus application); total duration of the recording is 1 min. Boxes (events) in the spectrogram (lower panel) represent the presence of audible (blue; 20 Hz – 16kHz) and ultrasonic (red; 25 ± 4 kHz) vocalizations during the 10 s application of mechanical stimuli. Female rats showed more vocalization events in response to noxious stimulus than male rats.

Conclusions

Inter-individual and sex differences in FE learning

- The population of the FE+ phenotype was larger and the population of the FE- phenotype was smaller in female compared to male rats.

Inter-individual and sex differences in pain behaviors

- Emotional responses to arthritis and neuropathic pain developed in all groups but emerged most prominently for female FE- rats.
- Females of both phenotypes showed greater baseline anxiety levels than males.

The data may suggest that sexual dimorphisms in FE learning ability have a predictive value for pain-related behavioral changes, particularly among emotional-affective pain aspects, in both an acute and a chronic pain model. The increased correlation between FE learning ability and affective pain-related behaviors in female compared to male rats warrant further investigation into sex-specific synaptic and cellular neurobiological mechanisms.