Sexually dimorphic interplay between anxiety-related phenotype and fear memory extinction

Maria I. Bonilla,¹ Hae-Lim Lee,² In-Jung Kim,^{3,4,6} and Andrii Rudenko^{1,5,6}

¹Department of Biology, City College, City University of New York, New York, NY 10031, USA

²Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06511, USA ³Department of Ophthalmology and Visual Science, Yale University School of Medicine, New Haven, CT 06511, USA

⁴Department of Neuroscience, Yale University School of Medicine, New Haven, CT 06511, USA ⁵Graduate Program, City University of New York, New York, NY 10031, USA

⁶These authors contributed equally to this work

Abstract

Ability to efficiently extinguish fear memories is critical for individual well-being, and extinction impairments are commonly observed in a variety of neuropsychiatric conditions. Efficient extinction is also a critical component of exposure therapy, the most widely utilized form of treatment for anxiety and trauma/stressor-related disorders. While individual as well as sex-related differences in extinction are recognized, their neurobiological underpinnings remain unclear. To examine neurobehavioral and molecular features that may contribute to the efficacy of fear memory extinction in genetically similar subjects raised in the same conditions, extinction learning was analyzed in the cohorts of inbred wild-type male and female mice. We showed that unlike memory acquisition, anxiety status of the animals

appeared to significantly influence extinction. Interestingly, the effect was sex-specific: the level of anxiety-related behavior inversely correlated with extinction efficacy in male mice only. Significant difference in hippocampal expression of anxiolytic Corticotropin Releasing Hormone Receptor 2 (CRHR2) between the male, but not female animals differing in extinction efficacy, suggests that CRHR2 may serve as a key factor underlying sexually dimorphic interplay between anxiety and fear memory extinction. Our results emphasize the need for tailoring treatment strategies for anxiety and fear-related disorders in accordance with the patient sex and identify specific neurobehavioral and molecular features potentially important for such adjustments.

Significance Statement

Fear memory extinction is a gradual diminishment of fear due to the learning that previously fearful context is now safe. Impaired or inefficient extinction has long been associated with anxiety and trauma/stressor-related disorders. Despite strong interest in therapeutic extinction enhancement, the progress has been limited due to insufficient understanding of the mechanisms regulating extinction efficacy and, especially, their sex-related features. This study showed notable sex differences in relation between fear memory extinction and anxiety-related behavior and identified likely molecular underpinnings of such differences. These findings should significantly improve our understanding of neurobehavioral and molecular factors regulating extinction. Furthermore, they could contribute to novel strategies of extinction augmentation and predictive assessments of extinction efficacy in accordance with the subject's sex.

Introduction

Extinction is commonly defined as a decrease of a conditioned response due to its uncoupling from the repeatedly non-reinforced conditioned stimulus (Pavlov 1927; Bouton, 1988; Myers and Davies, 2002; Bouton, 2004; Myers and Davis, 2007). Memory extinction is thought to be a form of new learning, in which the original memory is not erased, but weakened or diminished due to inhibitory influence of the newly encoded competing memory trace (Myers and Davis, 2007; Quirk and Mueller, 2008; Dunsmoor et al., 2015). Impaired extinction is often observed in the individuals afflicted with anxiety and fear-related disorders (Rosen and Schulkin, 1998; Michael et al., 2007; Milad et al., 2008; Graham and Milad, 2011; McGuire et al., 2016; Wicking et al., 2016). Moreover, extinction deficit is considered to represent an endophenotype for a variety of such disorders (Singewald and Holmes, 2019). Besides the importance of normal memory extinction in psychological well-being, commonly utilized treatments for mental trauma/stressor-related and anxiety disorders involve exposure therapy, a psychotherapeutic intervention conceptually based on the principles of extinction learning (Rothbaum and Schwartz, 2002; Graham and Milad, 2011; Rauch et al., 2012; Morrison and Ressler, 2014). However, despite being one of the most efficient forms of treatment, its success rate can be under 50% (Green, 2013; Cusack et al., 2016; Sippel et al., 2018). Recognition of this problem as well as the centrality of impaired memory extinction in multiple neuropsychiatric conditions (Anderson and Insel, 2006) have produced a strong interest in developing pharmacological and neurobehavioral tools for extinction enhancement and

multicomponent therapy (Ressler et al., 2004; Hofmann, 2007; Fitzgerald, et al., 2014; Kelmendi et al., 2016; Lebois et al., 2019; Coventry et al., 2020). Unfortunately, insufficient understanding of the mechanisms of extinction and the factors that could regulate its efficacy have considerably delayed such developments (Hofmann, 2007; Dunsmoor et al., 2015; An et al., 2017; Carpenter et al., 2019). Another important problem is an unclear mechanistic basis of the sex-related differences in extinction (Anagnostaras et al., 1998; Baran et al., 2010; Huang and Woolley, 2012; Matsuda et al., 2015; Clark et al., 2019; Velasco et al., 2019; Day and Stevenson, 2020; Lebron-Milad et al., 2021).

In order to investigate neurobehavioral factors and underlying molecular components that could influence efficacy of fear memory extinction, we analyzed extinction learning using inbred wild-type young adult (~12–14-week-old) mice. As we were also interested in sex-related features of extinction regulation, all the experiments were performed using cohorts of male and female animals. The study utilized the “good-extinguishing” highly inbred C57BL6/J mouse strain (Camp et al., 2012). Pavlovian, or classical, fear conditioning, a behavioral paradigm, commonly used in associative learning and memory research (Morrison and Ressler, 2014; Bowers and Ressler, 2015), was employed to induce memory acquisition. Contextual conditioning was followed by the fear memory extinction, a paradigm often utilized in the basic and translational research on fear memory processing (Milad and Quirk 2012; Craske et al., 2017; Singewald and Holmes, 2019).

Spaced extinction training (Urcelay et al., 2009; Bahari-Javan et al., 2012) demonstrated considerable sex-independent variability in extinction efficacy. While individual differences in fear memory acquisition did not appear to affect extinction learning in either male or female animals, the relation between anxiety-like behavior and extinction exhibited surprising sex-related dichotomy. Specifically, male mice, exhibiting poor extinction, demonstrated significantly increased anxiety-like behaviors compared to the robust-extinction males. On the other hand, no obvious relation between anxiety and extinction efficacy was detected in female animals. Analysis of the candidate molecular factors that might underlie the observed behavioral phenotypes, uncovered differential expression of the anxiolytic *Corticostimulating Hormone Receptor 2 (CRHR2)* in the hippocampi of the male, but not female mice differing in extinction efficacy. Further investigation of the specific roles of CRHR2 in extinction could provide detailed molecular explanation of the sex-related differences in fear memory extinction. It should also improve our understanding of the mechanisms regulating extinction efficacy and the interplay between extinction, anxiety, and stress.

Experimental strategy

Figure 1. Schematic representation of the experimental strategy. Inbred wild-type (C57BL6/J) young adult mice were used in the experiments. Anxiety-like behavior was tested in the cohorts of 40 male and 40 female animals using light/dark exploration paradigm. A single session Pavlovian contextual fear conditioning was performed on the same groups of mice (training: 3 min of the context/conditioning chamber exploration followed by 2 s, 0.7 mA electric foot shock; testing: assessing freezing behavior (fear responses) during 3 min of chamber exploration). 24 hr after testing fear memory acquisition, spaced extinction training was conducted in the fear conditioned mice (6 days of the conditioning context exploration, 3 min/day). The progress of extinction learning was evaluated daily by assessing freezing behavior during 3 min of non-reinforced chamber exploration. Based on the freezing behavior (%) exhibited during the final extinction training session, all the mice were separated into “poor extinction” (PE) groups (20 PE mice/sex; depicted at the bottom of the schematic with red font) and “robust extinction” (RE) groups (20 RE mice/sex; depicted at the top of the schematic with black font).

Hippocampal dissection, mRNA extraction, and RT-qPCR for the candidate genes were performed following extinction training (2 groups (PE and RE mice)/sex; 3 mice/group).

Materials and Methods

Experimental animals

All the experiments utilized inbred wild-type C57BL6/J male and female mice procured from the Jackson Laboratory (stock #000664). The mice were housed in groups of four to five animals in a pathogen-free facility under standard conditions, on 12/12 light/dark cycle, with food and water available *ad libitum*. The experiments were performed according to the Guide for the Care and Use of Laboratory Animals and were approved by the National Institutes of Health and the Institutional Animal Care and Use Committee at the City College and City University of New York. The experiments were performed in the identical conditions by the researchers blinded to the animal identity and the results of the previous experiments.

Behavioral experiments

The behavioral experiments were performed using groups of ~12-14-week-old C57BL6/J male and female mice (40 animals/sex). All the experiments were conducted following the established protocols with minor modifications. For consistency purposes, all the tests were carried out during the light phase, second part of the day (~1 – 4 pm). Before each experiment, the mice were left to acclimate to the testing rooms for 35 min.

Light/dark exploration

The light/dark exploration tests were performed as previously described (Su et al., 2013; Rudenko et al., 2015). Light/dark set-up consisted of a transparent plexiglass open-field chamber (40 x 40 cm), containing a dark (black) plexiglass box (20 x 40 cm) occupying half of the chamber area. The dark box had a removable dark plexiglass cover and small exit/entrance space. Experiments were conducted in a room with the overhead light turned off and a bright 120 W lamp directed at the open/light part of the chamber (brightly lit area). Each mouse was placed in the black plexiglass box, and its behavior was video recorded for 10 min. The latency of the first exit to the brightly lit area, total number of exits, and total time spent in the light and the dark areas were scored. The chambers were cleaned with 10% ethanol between the trials.

Fear conditioning

The fear conditioning tests were performed as previously described (Rudenko et al., 2013; Rudenko et al., 2015) with minor modifications. The experiments were performed using Noldus/Ugo Basile fear conditioning set-up, containing sound-attenuating conditioning chambers (Ugo Basile SLR, Italy) controlled by EthoVision XT software (Noldus, the Netherlands). Pavlovian contextual fear conditioning was conducted over the course of 2 days. On the first training day, the animals were placed in the conditioning chambers, allowed to freely explore them for 3 min, and then presented with a mild 2 s, 0.7 mA electric foot shock. Each animal's reaction to the shock (jump, vocalization) was visually confirmed.

Post-shock 15 s was given for additional context exploration. After the training, the animals were placed in their home cages for 24 hr. On the second (testing) day, the fear memory acquisition was assessed by quantifying freezing behavior representing mouse fear responses (absence of movement except breathing) during 3 min of the conditioning chamber exploration. The chambers were cleaned with 10% ethanol between the trials.

Fear extinction.

The spaced contextual fear memory extinction was performed as previously described (Agis-Balboa et al., 2011; Bahari-Javan et al., 2012) with minor modifications. The mice were repeatedly exposed to the conditioning chambers (Noldus/Ugo Basile fear conditioning set-up controlled by EthoVision XT software) 3 min daily for the non-reinforced context/chambers exploration. After each extinction session, the animals were returned to the home cages for 24 hr. The spaced extinction training was carried out for 6 subsequent days. Between each trial, the chambers were cleaned with 10% ethanol. The progress of extinction learning was evaluated during each training session by assessing freezing behavior (absence of movement except breathing = fear responses) representing mouse fear responses. Fear memory extinction was determined by quantifying freezing behavior on the final day of the extinction training.

Hippocampal dissection, RNA isolation, quantitative RT-PCR (RT-qPCR)

Hippocampi were dissected on ice, and total RNA was extracted using the RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized using the Superscript III First-Strand system (Thermo Fisher) and equal amount of RNA for each sample. RT-qPCR was performed in duplicate using iTaq Universal SYBR Green Supermix (Bio-Rad) on the CFX96 real-time system (Bio-Rad). The Ct values of samples were normalized to that of GAPDH and expression level of each gene in relation to control were calculated by the $\Delta\Delta C_t$ method.

The following RT-qPCR primers were used:

CRH: Forward 5'-ATCTCTCTGGATCTCACCTTCC-3'
Reverse 5'-CCCGATAATCTCCATCAGTTTCC-3'

CRHR1: Forward 5'-CTCCAGGGTTGTCTTCATCTAC-3'
Reverse 5'-CGGACCTCACTGTTTCAGAAAAC-3'

CRHR2: Forward 5'-TACAGAGAGTGCCTGGAGAAC-3'
Reverse 5'-ATGAGGGCGATTCCGGTAATGC-3'

GAPDH: Forward 5'-GTGGAGTCATACTGGAACATGTAG-3'
Reverse 5'-AATGGTGAAGGTCGGTGTG-3'

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9 (Version 9.1.0) software (GraphPad Software; San Diego, California, USA). Two-tailed unpaired Student's t-test were used when comparing two independent samples. Significant differences were accepted at $p < 0.05$. The normality was assessed using D'Agostino-Pearson test. All the results are presented as mean $\pm$ SEM. All statistical data are shown in the Table 1.

Results

Anxiety-like behavior and fear memory acquisition exhibited by inbred wild-type mice

In order to assess anxiety-like behavior of the animals of different sex, but the same genomic background and age, raised and maintained in similar conditions, we utilized the cohorts of 40 young adult (~12-14-week-old) inbred C57BL6/J mice of each sex (Fig. 1). Relatively large number of subjects was used to increase chances of detecting individual variations in the levels of anxiety-like behavioral features exhibited by the animals. The paradigm of choice was light/dark exploration (see Materials and Methods). In brief, the mice behavior in the light/dark chamber was video recorded for 10 min. The latency of the first exit from the dark chamber, the number of transitions between the dark and brightly lit compartments, and the total amount of time spent in the brightly lit area were quantified following the test (see Extended Data).

Seven days following assessment of anxiety-related behavior, the same cohorts of animals underwent Pavlovian fear conditioning. Fear memory acquisition was induced using a single-session contextual fear conditioning paradigm (see Methods for details). Briefly, the unconditional aversive stimulus involved a weak (2 s, 0.7 mA) electric foot shock, and the visual/tactile details of the conditioning chamber provided the learning context. 24 hours after the training, fear memory acquisition was tested during 3 min of the conditioning chamber explorations by assessing freezing behavior representing mice fear reactions (see Extended Data).

Sex-independent variability in extinction efficacy in inbred wild-type mice

Following fear memory acquisition, the conditioned animals were subjected to the fear memory extinction. To make the timescale of extinction more relevant to human exposure therapy, spaced fear extinction was utilized as a specific paradigm. Additionally, multiple studies have demonstrated that spaced extinction is particularly effective in reducing fear memories (Urcelay et al., 2009; Solomon et al., 2012). The extinction training involved daily exploration of the conditioning chambers without shock reinforcement over the course of 6 days. The progress of extinction learning was assessed at each session by quantifying fear-related freezing events during 3 min of chamber exploration (see Materials and Methods).

We discovered that despite the same genomic background, age, maintenance, and experimental conditions, both male and female animals demonstrated significant variability in fear memory extinction (Fig. 2 and Fig. 3). The fact that the

variability was exhibited by inbred animals prompted us to hypothesize about genomic background-unrelated factors that could underly individual differences in extinction. In order to facilitate characterization of such factors and their effects on extinction efficacy, all the mice were separated into four groups. Using a median split (DeCoster et al., 2011) for the differences between freezing responses exhibited 24 hr after fear conditioning (acquired fear memory) and on the final day of the spaced extinction (post-extinction fear memory) calculated for each mouse, the following groups (20 animals/group) were defined: 1) poor-extinction male mice (PE males); 2) robust-extinction male mice (RE males); 3) poor extinction female mice (PE females); 4) robust-extinction female mice (RE females). Comparison of the fear memory extinction between the PE and RE groups of mice showed highly significant difference in extinction efficacy in the animals of both sexes (Fig. 4).

A

**Spaced fear memory extinction in
PE male mice (20 animals)**

B

**Spaced fear memory extinction in
RE male mice (20 animals)**

Figure 2. Individual variability in fear memory extinction in inbred wild-type male mice. Spaced fear memory extinction was performed over 6 days period in a cohort of 40 inbred C57BL6/J young adult male mice. The progress of extinction learning was assessed daily by quantifying freezing behavior (fear responses) during 3 min of free exploration of the conditioning chambers/context. Based on the results of extinction learning, all the male animals were separated in the poor-extinction (PE; n=20) and robust-extinction (RE; n=20) groups (median split). (**A,B**) The graphs on the left depict complete sequences of extinction training for all PE (**A, left**) and all RE (**B, left**) experimental animals. Each line graph/color represents 6 days of extinction training for each individual mouse. The sequence of the training days is plotted on the X-axis; the freezing (%) is plotted on the Y-axis. The graphs on the right depict fear responses (%) of all PE (**A, right**) and RE (**B, right**) mice only on the first and last days of extinction training. Fear responses of the individual mice are represented by different symbol/color combinations. Black solid arrowhead lines indicate temporal progression of the experiment (days 1 - 6). Black dashed-line quadrilaterals and the dashed red arrowhead lines indicate the range of the fear responses on the first and last days the extinction training.

A

**Spaced fear memory extinction in
PE female mice (20 animals)**

B

**Spaced fear memory extinction training in
RE female mice (20 animals)**

Figure 3. Individual variability in fear memory extinction in inbred wild-type female mice. Spaced fear memory extinction was performed over 6 days period in a cohort of 40 inbred C57BL6/J young adult female mice. The progress of extinction learning was assessed

daily by quantifying freezing behavior (fear responses) during 3 min of free exploration of the conditioning chambers/context. Based on the results of extinction learning, all the female animals were separated in the poor-extinction (PE; n=20) and robust-extinction (RE; n=20) groups (median split). (**A,B**) The graphs on the left depict complete sequences of extinction training for all PE (**A, left**) and all RE (**B, left**) experimental animals. Each line graph/color represents 6 days of extinction training for each individual mouse. The sequence of the training days is plotted on the X-axis; the freezing (%) is plotted on the Y-axis. The graphs on the right depict fear responses (%) of all PE (**A, right**) and RE (**B, right**) mice only on the first and last days of extinction training. Fear responses of the individual mice are represented by different symbol/color combinations. Black solid arrowhead lines indicate temporal progression of the experiment (days 1 - 6). Black dashed-line quadrilaterals and the dashed red arrowhead lines indicate the range of the fear responses on the first and last days the extinction training.

Fear memory extinction in PE and RE mice

Figure 4. The efficacy of the fear memory extinction in the poor-extinction and robust-extinction groups of mice. **(A)** Extinction efficacy in the poor-extinction (PE) and robust-extinction (RE) groups of male mice. **(B)** Extinction efficacy in the poor-extinction (PE) and robust-extinction (RE) groups of female mice. Graphs show the levels (%) of freezing behavior (fear responses) following spaced extinction training (6 days of daily 3 min exploration of the fear conditioning context/chambers; 20 mice/group; red: PE male mice; black: RE male mice; yellow: PE female mice; blue: RE female mice; *** $p < 0.001$; error bars: $\pm$ SEM).

It is feasible that the observed differences in extinction efficacy may reflect corresponding differences in fear memory acquisition. To examine this hypothesis, we analyzed the previously obtained results of Pavlovian fear conditioning for the poor- and robust-extinction mouse groups. Our analysis failed to identify significant differences in fear memory acquisition between PE and RE groups of male as well as female animals (Fig. 5), suggesting that extinction efficacy may be influenced by other neurobehavioral factors.

Fear memory acquisition in PE and RE mice

Figure 5. No significant difference in fear memory acquisition between mice exhibiting poor or robust extinction. **(A)** Poor-extinction (PE) and robust-extinction (RE) groups of male mice did not exhibit significant difference in memory acquisition during Pavlovian contextual fear conditioning. **(B)** PE and RE groups of female mice did not exhibit significant difference in memory acquisition during Pavlovian contextual fear conditioning. Graphs show the levels (%) of freezing behavior (fear responses) exhibited by mice 24 hr following fear conditioning (training: 3 min of context/conditioning chamber exploration followed by 2 s, 0.7 mA foot shock; testing: 3 min of the chamber exploration; 4 groups of mice; 20 mice/group; red: PE male mice; black: RE male mice; yellow: PE female mice; blue: RE female mice; ns: not significant; error bars: $\pm$ SEM). Figure 5-1 and Figure 5-2 support Figure 5.

Anxiety-like behavior is significantly related to the extinction efficacy in the male mice

Fear is typically conceptualized as an organism's response to the objective and clearly present threat. Similar response to the objectless and poorly defined future danger, is often defined as anxiety (Catherall, 2003; Horwitz, 2013). While fear and anxiety are often considered to represent closely corresponding phenomena, there is no clear consensus on their exact relation (Davies, 1998; Tovote et al., 2015; Perusini and Fanselow, 2015).

To examine potential effect of anxiety-like behavior on the ability to extinguish fear memory in inbred wild-type mice, we compared previously obtained findings of the light/dark exploration test between PE and RE groups of animals. The comparison showed that PE male mice clearly exhibited elevation in all the anxiety-related parameters measured, including increased latency of the first exit from the dark chamber, number of light/dark transitions, and the total time spent in the brightly lit compartment (Fig. 6A-C).

Interestingly, the inverse correlation between the elevated anxiety-like behavioral measurements and poor fear memory extinction, was observed in the male animals only. Similar comparisons between the PE and RE groups of female mice failed to show significant differences in any of the anxiety-related parameters (Fig. 6D-F)

Anxiety-like behavior exhibited by PE and RE mice

Light/dark exploration: male mice

Light/dark exploration: female mice

■ PE male mice ■ PE female mice
■ RE male mice ■ RE female mice

Figure 6. Significantly increased anxiety-like behavior in male, but not in female mice exhibiting poor fear memory extinction. The light/dark exploration paradigm was used to assess and compare anxiety-like behavior exhibited by the groups of poor-extinction (PE) and robust-extinction (RE) male (**A-C**) and female (**D-F**) mice. The test showed significant increase in PE compared to RE male mice in all measured anxiety-related parameters, including latency to emerge (**A**), number of dark/light transitions (**B**), and the total time spent in the brightly lit area (**C**). (**D-F**) No significant differences in the anxiety-like

behaviors were detected between PE and RE female animals (10 min of the light/dark chamber exploration; 4 groups of mice; 20 mice/group; red: PE male mice; black: RE male mice; yellow: PE female mice; blue: RE female mice; ns: not significant; * $p < 0.05$; error bars: $\pm$ SEM). Figure 6-1 and Figure 6-2 support Figure 6.

Increased expression of CRHR2 in hippocampus of male mice exhibiting robust fear memory extinction

The discovery of the sexually dimorphic relation between anxiety-like behavior and fear memory extinction, lead us to hypothesize about specific molecular underpinnings of such phenomenon. To test our hypothesis, we chose the candidate gene approach. Specifically, we assessed expression of the corticotropin releasing hormone (CRH), and its two receptors, corticotropin releasing hormone receptor 1 and 2 (CRHR1 and CRHR2), all of which have been implicated in anxiety regulation (Smith et al., 1998; Radulovic et al., 1999; Holsboer and Ising, 2008; Kalin et al., 2016). Both CRHR1 and CRHR2 are heavily expressed in the hippocampus, a brain structure, playing important roles in memory processing, including extinction, as well as in emotional regulation and stress response (Fischer et al., 2004; Milad et al., 2007; Radulovic and Tronson, 2010; Kheirbek et al., 2013; Jin and Maren, 2015; Eichenbaum, 2017; Anacker et al., 2018; Briscione et al., 2014; Lacagnina et al., 2019). Therefore, our analysis of the candidate gene expression focused on the hippocampus.

Expression levels of CRH, CRHR1, and CRHR2 were assessed by RT-qPCR, using total RNA extracted from the hippocampi of the mice exhibiting either poor or robust extinction (see Materials and Methods). 3 PE male, 3 RE male, 3 PE female, and 3 RE female mice, selected among the animals demonstrating the

poorest and the most robust fear memory extinction, were used in the experiment. The results of RT-qPCR demonstrated that while expression levels of CRH as well as CRHR1 were similar in PE and RE male mice, CRHR2 was significantly upregulated in RE compared to PE males (Fig. 7A-C). Analysis of the same genes in the female animals failed to identify any significant expression differences between PE and RE groups (Fig. 7D-F).

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Hippocampal expression of the candidate anxiety-related genes in PE and RE mice

Figure 7. Significantly increased hippocampal expression of CRHR2 in male, but not in female mice exhibiting robust fear memory extinction. Assessing expression of the stress- and anxiety-related CRH, CRHR1, and CRHR2 genes in the hippocampi of the male (**A-C**) and female (**D-F**) mice exhibiting poor (PE) or robust (RE) extinction. RT-qPCR showed no expression differences of the CRH (**A**) and CRHR1 (**B**) between PE and RE males, however, significantly increased expression of CRHR2 (**C**) in the RE males. No differences in expression of CRH, CRHR1, and CRHR2 was found between PE and RE females (**D-F**).

F). Relative mRNA levels are shown (4 groups of mice; 3 mice/group; red: PE male mice; black: RE male mice; yellow: PE female mice; blue: RE female mice; ns: not significant; $*p < 0.05$; error bars: $\pm$ SEM).

Discussion

Here, we analyzed fear memory extinction and potentially relevant neurobehavioral and molecular factors that could affect efficacy of the extinction learning. In order to avoid genomic background-related influences, the study was performed using inbred wild-type (C57BL6/J) mice. As we were also interested in the sex-related features of extinction regulation, the cohorts of male and female animals were utilized in the experiments. Using spaced contextual fear extinction paradigm, we discovered that despite genomic similarity, same age, maintenance, and experimental conditions, both male and female mice exhibit significant individual differences in extinction learning. Such variability allowed us to separate all the experimental animals into 4 groups based on their sex and extinction efficacy. Further analysis involved characterizing potentially relevant neurobehavioral and molecular parameters in the groups of animals exhibiting poor or robust extinction (PE versus RE males and PE versus RE females).

Comparing Pavlovian fear conditioning-based memory scores between PE and RE groups mice, demonstrated the lack of significant effect of memory acquisition on extinction learning in both males and female animals. This finding suggests that extinction efficacy may be influenced by the neurobehavioral features other than ability to encode, consolidate, and retrieve fear memories. It also functionally supports the findings that memory acquisition and extinction utilize at least partially

distinct neural substrates and molecular mechanisms (Cain et al., 2002; Marsicano et al., 2002; Herry et al., 2008; Graham and Milad, 2011; Davis et al., 2017).

Unlike fear memory acquisition, anxiety-like behavior appeared to significantly influence extinction learning. This finding is not completely unexpected, as the comorbidity of high anxiety and impaired fear extinction have been previously reported (Muigg et al., 2008; McGuire et al., 2016; Duits et al., 2016; Prater et al., 2017). Surprising result of our study was the discovery of the sexually dimorphic relation between these neurobehavioral phenomena. Specifically, we found that the PE group of inbred wild-type (C57BL6/J) male mice showed significant increase in all measured anxiety-related parameters compared to the RE males. Contrary to the finding in male animals, there were no significant differences in any of the anxiety-related parameters between PE and RE groups of female mice. We will be careful not to claim that anxiety-like behavior does not have any influence on extinction in females, as potential effects could be subtle. However, our findings suggest that among two sexes, fear memory extinction in males is much more strongly associated with the anxiety-like phenotype of the animal.

Examination of the molecular mechanisms, potentially underlying observed connection between anxiety-like behavior and fear memory extinction, utilized candidate gene approach, focusing on Corticotropin Releasing Hormone (CRH) and its receptors, CRHR1 and CRHR2. While these key stress-response factors, (Chrousos and Zoumakis, 2017), have been linked to anxiety (Holsboer and Ising, 2008; Kalin et al., 2016; Dedic et al., 2018), several studies demonstrated that CRHR1 and CRHR2 may have distinct functional and physiological context-

dependent properties (Radulovic et al., 1999; Todorovic et al., 2007; Donner et al., 2016; Randesia et al., 2018). Lack of clear consensus notwithstanding, there is considerable evidence that CRHR1 and CRHR2 may have, at least in part, antagonistic functions: while CRHR1 is thought to convey anxiogenic effects, CRHR2 appears to play primarily anxiety-attenuating role (Smith et al., 1998; Timpl et al., 1998; Bale et al., 2000; Kishimoto et al., 2000; Takahashi et al., 2001; Randesia et al., 2018). Our discovery that neither CRH, nor CRHR1 or CRHR2 showed expression differences between PE and RE female mice, supports, and likely explains the finding that the female animals differing in extinction learning, demonstrated similar levels of anxiety-like behavioral features. Significant upregulation of hippocampal CRHR2 in RE compared to PE male mice also supports the observed anxiety-like phenotypes and provides at least partial mechanistic explanation of the decreased anxiety-related behavior in the robust-extinction males.

Collectively, our findings demonstrated that despite close similarity in such critical features as genomic background, age, maintenance, and experimental conditions, inbred wild-type mice exhibit significant sex-independent variability in fear memory extinction. Our results also indicate that unlike memory acquisition, anxiety-like behavior appears to influence extinction efficacy. Surprisingly, we found that this effect reaches statistical significance in male mice only: “more anxious” animals were also the ones exhibiting poor extinction. The sexually dimorphic nature of the anxiety/extinction relation could be explained by significant

upregulation of the anxiolytic CRHR2 in the hippocampi of robust-extinction male but not female mice.

Our discovery that anxiety-like phenotype has much stronger effect on fear memory extinction in male compared to female mammalian subjects could suggest specific directions for tailoring extinction-based exposure therapies depending on patient's sex. The evidence that CRHR2 may act as a regulator of fear memory extinction in male subjects touches upon several important points. First, it suggests mechanistic basis for the sex-related differences in the interplay between stress-, anxiety-, and extinction-related neural circuitry (Shin and Liberzon, 2010; Maren and Holmes, 2016; Velasco et al., 2019; Daviua et al., 2019; Ressler, 2020). Second, it provides a promising molecular target (Krystal et al., 2017) for investigating and modulating such interplay. Furthermore, assessing and manipulating levels of CRHR2 and its downstream components could be utilized in biomarker-based evaluations (Yehuda et al., 2013) of the ability to extinguish fear memory and pharmacological strategies (Lebois eta al., 2019) for extinction augmentation.

Author Contributions: AR designed research, MB, H-LL, I-JK and AR performed research, I-JK and AR analyzed data, AR and I-JK wrote the paper.

Acknowledgements: The authors would like to thank Aisa Sabovic for experimental assistance and members of the Rudenko and the Kim labs for helpful

discussions. This work was supported by the Whitehall Foundation grant 2017-08-39 (AR), NIH grants R21EY029820, R21EY031512, and R01EY031751 (I-JK).

Conflict of Interest: authors declare no conflict of interest

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Table 1. Statistical table.

Figure	Data structure	Type of test	Sample size	Statistical data
Figure 4A	Normal distribution	Unpaired t-test, two-tailed	n (PE male mice) = 20 n (RE male mice) = 20	t = 13.09, df = 38, p < 0.0001
Figure 4B	Normal distribution	Unpaired t-test, two-tailed	n (PE female mice) = 20 n (RE female mice) = 20	t = 10.27, df = 38, p < 0.0001
Figure 5A	Normal distribution	Unpaired t-test, two-tailed	n (PE male mice) = 20 n (RE male mice) = 20	t = 0.4320, df = 38, p = 0.6682
Figure 5B	Normal distribution	Unpaired t-test, two-tailed	n (PE female mice) = 20 n (RE female mice) = 20	t = 1.747, df = 38, p = 0.0888
Figure 6A	Normal distribution	Unpaired t-test, two-tailed	n (PE male mice) = 20 n (RE male mice) = 20	t = 2.595, df = 38, p = 0.0134
Figure 6B	Normal distribution	Unpaired t-test, two-tailed	n (PE male mice) = 20 n (RE male mice) = 20	t = 2.707, df = 38, p = 0.0101

Figure 6C	Normal distribution	Unpaired t-test, two-tailed	n (PE male mice) = 20 n (RE male mice) = 20	t = 2.498, df = 38, p = 0.0169
Figure 6D	Normal distribution	Unpaired t-test, two-tailed	n (PE female mice) = 20 n (RE female mice) = 20	t = 1.402, df = 38, p = 0.1690
Figure 6E	Normal distribution	Unpaired t-test, two-tailed	n (PE female mice) = 20 n (RE female mice) = 20	t = 0.1373, df = 38, p = 0.8915
Figure 6F	Normal distribution	Unpaired t-test, two-tailed	n (PE female mice) = 20 n (RE female mice) = 20	t = 0.4088, df = 38, p = 0.6850
Figure 7A	(n too small to test normality)	Unpaired t-test, two-tailed	n (PE male mice) = 3 n (RE male mice) = 3	t = 0.3441, df = 4, p = 0.7481
Figure 7B	(n too small to test normality)	Unpaired t-test, two-tailed	n (PE male mice) = 3 n (RE male mice) = 3	t = 0.4076, df = 4, p = 0.704
Figure 7C	(n too small to test normality)	Unpaired t-test, two-tailed	n (PE male mice) = 3 n (RE male mice) = 3	t = 2.998, df = 4, p = 0.0400

	test normality)			
Figure 7D	(n too small to test normality)	Unpaired t-test, two- tailed	n (PE female mice) = 3 n (RE female mice) = 3	t = 1.470, df = 4, p = 0.2156
Figure 7E	(n too small to test normality)	Unpaired t-test, two- tailed	n (PE female mice) = 3 n (RE female mice) = 3	t = 2.149, df = 4, p = 0.0981
Figure 7F	(n too small to test normality)	Unpaired t-test, two- tailed	n (PE female mice) = 3 n (RE female mice) = 3	t = 0.6379, df = 4, p = 0.5582

Extended Data

Mouse #	Freezing (%)
1	83
2	67
3	67
4	28
5	61
6	56
7	44
8	28
9	44
10	56
11	50
12	89
13	67
14	50
15	33
16	89
17	17
18	50
19	50
20	50
21	67
22	78
23	61
24	61
25	17
26	78
27	22
28	61
29	72
30	50
31	56
32	89
33	50
34	50
35	78
36	56
37	44
38	28
39	50
40	72

Figure 5-1. Fear memory acquisition in the inbred wild-type (C57BL6/J) male mice (n = 40) during Pavlovian contextual fear conditioning. See the Materials and Methods for the test details

Mouse #	Freezing (%)
1	56
2	44
3	56
4	50
5	83
6	61
7	39
8	94
9	44
10	39
11	56
12	78
13	56
14	39
15	83
16	72
17	67
18	39
19	78
20	67
21	50
22	61
23	61
24	61
25	50
26	44
27	28
28	44
29	44
30	56
31	78
32	61
33	39
34	44
35	72
36	50
37	39
38	50
39	56
40	44

Figure 5-2. Fear memory acquisition in the inbred wild-type (C57BL6/J) female mice (n = 40) during Pavlovian contextual fear conditioning. See Materials and Methods for the test details

Mouse # Latency of the first exit (s) Time in the light area (s) # of transitions

1	16	74	12
2	69	44	3
3	10	110	6
4	43	95	11
5	35	129	9
6	34	111	13
7	37	85	11
8	41	85	10
9	20	105	9
10	21	124	10
11	54	29	5
12	170	35	6
13	11	139	12
14	42	71	11
15	34	127	13
16	23	37	4
17	21	186	13
18	37	92	8
19	5	113	10
20	13	100	8
21	186	24	2
22	27	21	3
23	24	98	9
24	16	119	15
25	9	137	13
26	125	61	5
27	9	103	12
28	6	103	19
29	21	187	9
30	32	129	20
31	48	67	4
32	89	24	2
33	32	111	11
34	13	119	10
35	22	92	9
36	14	53	5
37	8	74	10
38	27	84	6
39	19	103	12
40	97	76	10

Figure 6-1. Anxiety-like behavior in the inbred wild-type male mice (n = 40) assessed using light/dark exploration paradigm. See Materials and Methods for the test details.

Mouse # Latency of the first exit (s) Time in the light area (s) # of transitions

1	12	137	3
2	13	60	30
3	12	80	4
4	14	121	1
5	12	74	32
6	16	84	7
7	15	83	7
8	11	70	23
9	3	25	25
10	13	67	71
11	7	52	87
12	11	121	34
13	13	106	48
14	17	131	2
15	8	51	11
16	13	93	4
17	11	129	32
18	19	123	1
19	10	81	3
20	15	112	5
21	14	71	22
22	8	56	120
23	12	119	1
24	6	56	5
25	13	96	37
26	15	120	1
27	7	96	32
28	11	132	9
29	16	128	27
30	12	107	3
31	11	70	18
32	15	105	11
33	11	78	58
34	11	62	45
35	10	64	32
36	14	140	27
37	17	92	43
38	10	75	70
39	10	148	6
40	5	93	36

Figure 6-2. Anxiety-like behavior in the inbred wild-type female mice (n = 40) assessed using light/dark exploration paradigm. See Materials and Methods for the test details.