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Caloric Restriction Enhances Fear Extinction Learning in Mice

Megan C Riddle[1,2], Morgan C McKenna[3], Yone J Yoon[3], Siobhan S Pattwell[3,4], Patricia Mae G Santos[5], B J Casey[2,3,4] and Charles E Glatt*[,3]

[1]Weill Cornell/Rockefeller/Sloan Kettering Tri-Insitutional MD-PhD Program, Weill Cornell Medical College, New York, NY, USA; [2]Department of Neurology and Neuroscience, Weill Cornell Medical College, New York, NY, USA; [3]Department of Psychiatry, Weill Cornell Medical College, New York, NY, USA; [4]Sackler Institute for Developmental Psychobiology, Weill Cornell Medical College, New York, NY, USA; [5]Brown University, Providence, Rhode Island

Fear extinction learning, the ability to reassess a learned cue of danger as safe when it no longer predicts aversive events, is often dysregulated in anxiety disorders. Selective serotonin reuptake inhibitors (SSRI's) enhance neural plasticity and their ability to enhance fear extinction learning may explain their anxiolytic properties. Caloric restriction (CR) has SSRI-like effects on neural plasticity and anxiety-related behavior. We implemented CR in mice to determine its effects on conditioned-fear responses. Wild type and serotonin transporter (SERT) knockout mice underwent CR for 7 days leading to significant weight loss. Mice were then tested for cued fear learning and anxiety-related behavior. CR markedly enhanced fear extinction learning and its retention in adolescent female mice, and adults of both sexes. These effects of CR were absent in SERT knockout mice. Moreover, CR phenocopied behavioral and molecular effects of chronic fluoxetine, but there was no additive effect of CR in fluoxetine-treated mice. These results demonstrate that CR enhances fear extinction learning through a SERT-dependent mechanism. These results may have implications for eating disorders such as anorexia nervosa (AN), in which there is a high prevalence of anxiety before the onset of dietary restriction and support proposals that in AN, CR is a motivated effort to control dysregulated fear responses and elevated anxiety.
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INTRODUCTION

The ability to differentiate cues of danger and safety is critical for survival (LeDoux, 2012). Fear learning is an evolutionarily conserved process that enables an individual to learn danger signals, and then reassess these signals over time to adapt to a changing environment. In cued fear conditioning studies, an association is formed between an aversive, unconditioned stimulus (US) and a co-occurring, neutral conditioned stimulus (CS). Over repeated pairings, the CS takes on the aversive properties of the US such that it can independently elicit a fear response. Fear extinction occurs when, after fear conditioning, the CS alone is presented repeatedly and fear responses to the CS decrease. The ability to reassess cues of danger as safe has been associated with lower subjective experiences of anxiety, and inefficient fear extinction learning is present in a number of anxiety disorders (Graham and Milad, 2011). Because fear extinction learning is an active learning process that requires neural plasticity (Kaplan and Moore, 2011; Sotres-Bayon *et al*, 2007), genetic and pharmacologic factors that increase neural plasticity, can enhance fear extinction learning (Mao *et al*, 2006; Soliman *et al*, 2010; Sotres-Bayon *et al*, 2007).

The serotonin transporter (SERT) can modulate neural plasticity including adaptive fear learning. These properties may explain its role in mood and anxiety disorders. Mice with genetic knockout of SERT have impaired retention of fear extinction, as well as increased depression- and anxiety-like behavior (Wellman *et al*, 2007). Reduced expression of SERT is seen in humans with anxiety disorders (Maron *et al*, 2004; Murrough *et al*, 2011; Reimold *et al*, 2007), and a polymorphism affecting SERT expression in humans has been associated with fear extinction retention, and risk for panic disorder (Gyawali *et al*, 2010; Hartley *et al*, 2012). Moreover, the SERT-targeting selective serotonin reuptake inhibitors (SSRI's) enhance fear extinction retention (Karpova *et al*, 2011).

CR has SSRI-like anxiolytic properties in mice that may also be due to enhanced neural plasticity and fear extinction learning (Yamamoto *et al*, 2009). In the visual system CR enhances neural plasticity in a manner similar to SSRI's. Both CR and fluoxetine allow adult mice to reestablish binocular vision following early-life monocular deprivation even after the sensitive period for visual plasticity has closed (Maya Vetencourt *et al*, 2008; Spolidoro *et al*, 2011).

To assess the effects of CR on adaptive fear responses ,we implemented a cued fear learning paradigm in mice. We found that CR enhances fear extinction learning and

*Correspondence: Dr CE Glatt, Department of Psychiatry, Weill Cornell Medical College, LC929A, 1300 York Avenue, New York, NY 10065, USA, Tel: +212 746 6723, Fax: +212 746 8529, E-mail: ceg2004@med.cornell.edu
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retention. In contrast, mice lacking SERT do not display enhanced fear extinction learning with CR, suggesting SERT has a crucial role in mediating this effect of CR. We also found that CR phenocopies the effects of fluoxetine on fear extinction learning, and the expression of a SERT mRNA species that we previously associated with decreased anxiety states and enhanced fear extinction retention (Gyawali *et al*, 2010; Hartley *et al*, 2012). We discuss how these results may be relevant to anorexia nervosa, an eating disorder characterized by both high levels of anxiety and caloric restriction (CR).

MATERIALS AND METHODS

Animals

Pregnant C57BL/6 females were purchased from Charles River, and offspring used for studies with adolescent mice. Adult C57BL/6 mice were purchased from Charles River at postnatal day (P) 60. Breeding pairs of SERT knockout B6.129(Cg)-*Slc6a4*^{tm1Kpl}/J mice (Bengel *et al*, 1998) were obtained from the Jackson Laboratory's repository. All B6.129(Cg)-*Slc6a4*^{tm1Kpl} mice used for testing were from heterozygous crosses, allowing for comparison of wild-type and knockout littermates. Knockout mice were genotyped by Mouse Genotype. Mice received *ad libitum* (AL) access to food until assignment to diet regimen. Mice were weighed and fed daily within 2 h of onset of dark cycle. Mice undergoing CR received 60% of the AL group's previous day's consumption as described (Yamamoto *et al*, 2009). Adolescent mice began CR between days P36 and P38. Adult mice began CR when 2–4 months old. Mice remained on the feeding regimen until the termination of testing. Mice were handled following the guidelines of Weill Cornell Medical College's Institutional Animal Care and Use Committee and the National Institutes of Health.

Fluoxetine Treatment

Fluoxetine dissolved in tap water (160 mg/l) was provided in the drinking water in light-protected bottles, and changed every 3 days. AL intake led to a dose of ~18 mg/kg per mouse, reported to produce anxiolytic levels (Chen *et al*, 2006). Mice began fluoxetine 2 weeks before CR and remained on the drug until study completion.

Fear Conditioning and Extinction

On day 8 of CR, mice were fear conditioned using a standard mouse shock-chamber (Coulbourn Instruments) in a sound-attenuated box scented with peppermint odor. Mice received three trials of CS tone (30 s, 70 dB, and 5 kHz) coterminating with an US footshock (1 s, 0.7 mA) with 30 s between each CS-US pairing (Soliman *et al*, 2010). Extinction training occurred 24 and 48 h after conditioning. In a novel context, a silver metal cylinder scented with limonene (0.1% limonene), mice were exposed to 18 unreinforced presentations of the tone at 3 min intervals (Monfils *et al*, 2009). Trials were recorded and freezing behavior assessed with FreezeFrame and FreezeView software (Coulbourn Instruments). This protocol resulted in submaximal levels of extinction in AL animals, making the paradigm sensitive to manipulations that would enhance extinction learning.

Freezing behavior was compared between groups for within session extinction learning on day 1 and retention of extinction learning over 2 days of extinction training. For within session extinction, the difference was calculated between tones 2–5 and 15–18. The first tone was excluded because many mice displayed escape behavior during this tone, attempting to jump out of the chamber. Although this represents a fearful response, it lowered apparent freezing, thus this time point was eliminated from analysis. For extinction retention, the difference was calculated between tones 2–5 on day 1 and 1–4 on day 2. On day 2 of extinction, mice froze in response to the first tone, so this tone was included in analysis. Female animals were randomly tested across the estrous cycle.

Elevated Plus Maze

Anxiety-like behavior was measured using the elevated plus maze 2 days after the final session of extinction training. For each trial, the mouse was placed on the central platform facing an open arm and behavior recorded for 5 min and analyzed using Ethovision XT software (Noldus) (Yamamoto *et al*, 2009).

Object Placement

Mouse behavior was recorded with a digital camera and analyzed with EthoVision XT tracking system (Noldus). On day 8 of CR, adult female mice that had not undergone fear conditioning training were placed in an open field arena, and behavior recorded for 5 min to determine there was no preference for one specific region. The next day, two identical Lego trees were placed in the northeast and northwest quadrants of the arena; the mouse was placed in the center of the arena and behavior recorded for 5 min, trial 1 (T1). The mouse was placed in a holding cage for 5 min and the arena and objects cleaned. One object was returned to its previous location, the other moved to the southern end of the box, diagonally opposite the northern object. The mouse was returned to the open field for 5 min (T2). Mice failing to explore the objects were eliminated ($n = 2$). The percent time spent exploring the novel object was calculated for T2 (((time novel)/(time familiar + time novel)) × 100). Successful spatial learning was defined as a mouse spending over 50% of its exploration with the novel placement object.

Monitoring Activity

Two days after the completion of object placement testing, mice were placed in the center of an open field Med Associates. Distance traveled was recorded for 60 min (Runker *et al*, 2011).

Estrous Cycling

Estrous cycling was conducted on a subset of adult female mice. Vaginal swabs were taken daily (0800–1000 hours) for 6 days, and stained with the Hema 3 Stain Set (Fisher

Scientific). Estrous stage was determined following published criteria (Caligioni, 2009).

Quantitative PCR

Total brain RNA was prepared with a Trizol protocol and reverse transcribed using Moloney Murine Leukemia virus reverse transcriptase and oligo dT primer (New England Biolabs). Total SERT, distal SERT, and control gene (glyceraldehyde 3-phosphate dehydrogenase, Beta-glucuronidase, hypoxanthine phosphoribosyltransferase 1, and TATA binding protein) mRNA levels were measured in separate reactions via an ABI 7900HT real time-PCR thermal cycler using SYBR green detection of amplified fragments (Gyawali *et al*, 2010). Samples were reverse transcribed twice and each cDNA sample analyzed in quadruplicate. Details of primer sequences and cycling parameters are in Supplementary material.

Statistics

Comparisons of AL *vs* CR mice were performed using Student's *t*-test. ANOVA was used for analysis of diet by SERT genotype or diet by fluoxetine treatment comparisons with.

Additional details are available in the Supplementary material online.

RESULTS

CR Enhances Fear Extinction Learning

To assess whether CR altered fear learning and extinction during adolescence, postnatal day (P)36–38 female mice were diet restricted for one week (Figure 1a). CR reduced the body weight to about 80% of AL-fed mice (CR mean 13.0 ± 0.3, AL mean 16.2 ± 0.8, $p < 0.01$). Behavioral testing was begun after 8 days of CR as previous work has reported maximal anxiolytic effect of CR at this time (Yamamoto *et al*, 2009). Mice were fear conditioned followed by 2 days of fear extinction training. Groups did not show differences in fear acquisition (Figure 1b). CR mice showed enhanced fear extinction learning, with a greater reduction in freezing within session on day 1 of training (Figure 1c and d, $p < 0.01$) and increased fear extinction retention over 2 days (Figure 1e, $p < 0.01$).

Anxiety-like behavior was assessed using an elevated plus maze EPM 2 days after the mice completed fear extinction training. Consistent with studies in adult male mice (Yamamoto *et al*, 2009), CR adolescent females had decreased anxiety-like behavior, spending more time in the open arms than their AL littermates (Figure 1f, $p < 0.05$).

CR Enhances Extinction Learning in Adult Mice

We tested adult mice to determine if the fear extinction-enhancing properties of CR were limited to adolescent females, and might explain the epidemiological distribution of anorexia nervosa (AN) (Hudson *et al*, 2007). Adults of both sexes showed enhanced fear extinction learning on the first day of fear extinction training with CR (Figure 2a and b, $p < 0.01$ females; $p < 0.01$ males). Females showed significant effects of CR on fear extinction retention (Figure 2c, $p < 0.01$), while males showed a nonsignificant trend towards enhanced fear extinction retention (Figure 2d, NS).

CR does not Increase General Activity

To ensure differences in freezing patterns were not confounded by increased locomotor activity in CR mice, we assessed overall movement. In an activity box, CR mice did not show increased distance traveled per minute over a 1 h period (AL = 111.1 ± 2.8 cm, CR 111.8 ± 2.4 cm, and $n = 15,15$, NS). These results are consistent with previous studies showing no increase in activity with CR in C57BL/6 mice (Gelegen *et al*, 2007; Yamamoto *et al*, 2009).

CR does not Improve Performance on an Object Placement Task

To assess whether CR enhanced cognition in nonemotional contexts, we used an object placement task of spatial memory (Ennaceur and Aggleton, 1997). CR adult female mice did not spend significantly more time with the novel object than AL mice, meaning they did not display enhanced spatial learning (AL = $61.1 \pm 4.5\%$, CR = $49.4 \pm 7.7\%$, and $n = 15$, 13, NS).

The Effects of CR on Estrous Cycles

Ovarian hormones affect fear extinction learning, with increased estrogen enhancing consolidation of fear extinction learning (Lebron-Milad and Milad, 2012). We found CR treatment increased time spent in diestrus (AL = $6.7\% \pm 2.7$, CR = $23.3\% \pm 6.2$, $n = 10,10$, $p < 0.05$), as reported previously (Nelson *et al*, 1985), consistent with amenorrhea seen in AN as both are low estrogen states (Bailer and Kaye, 2003).

CR does not Enhance Fear Extinction Learning in SERT KO Mice

Because of the role of SERT in anxiety and fear extinction learning (Hartley *et al*, 2012; Wellman *et al*, 2007), we assessed whether the CR effect on extinction learning seen in wild-type mice would be altered in SERT knockout mice (Wellman *et al*, 2007). There was a significant effect of diet ($F_{1,156} = 15.74$, $p < 0.001$) and a trend towards and effect of SERT genotype ($F_{1,156} = 2.74$, $p = 0.10$) on fear extinction learning. There was an interaction between SERT genotype and dietary status ($F_{1,156} = 6.71$, $p = 0.01$). There were no differences in fear extinction learning between CR and AL female knockout mice for fear extinction learning on the first day (Figure 3a) or retention (Figure 3b). Although wild-type littermates that underwent CR continued to show enhanced fear extinction learning (Figure 3a, $p < 0.001$), CR in SERT knockout mice did not enhance fear extinction learning ($p = 0.56$). The results were similar for fear extinction retention (Figure 3b). There was a main effect of diet ($F_{1,156} = 3.74$, $p = 0.05$) but not SERT genotype ($F_{1,156} = 0.82$, $p = 0.37$) on fear extinction retention with a diet × genotype interaction ($F_{1,156} = 10.33$, $p = 0.001$). CR increased extinction retention in wild-type mice ($p < 0.001$), but had no effect in SERT knockout mice ($p = 0.52$).

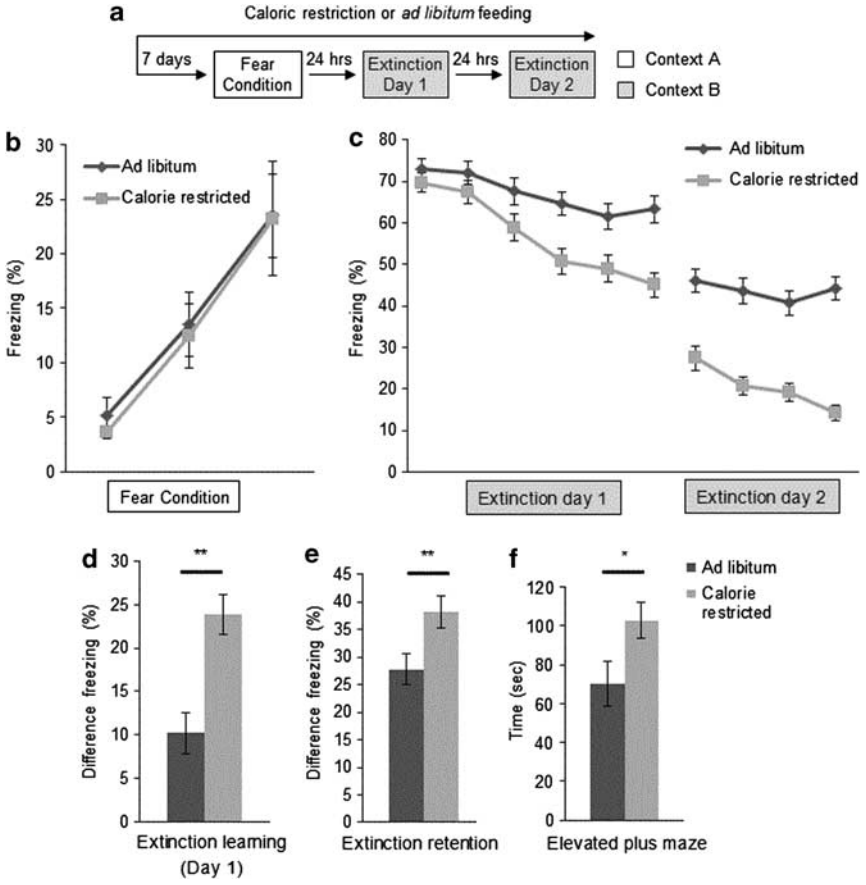

Figure 1 (a) Fear learning paradigm. CR began 1 week before fear conditioning, and was maintained throughout behavioral testing. (b) Adolescent female mice showed similar levels of freezing during acquisition during each of three tone-shock pairings ($n = 10, 10$). (c) CR improved fear extinction learning on both days of extinction training in adolescent female mice. Each block represents three tones. Extinction day 1 shows all 6 bins; extinction day 2 shows first 4 bins, at which point the groups plateaued. (d) The degree of extinction on day 1, as defined by the difference in freezing between tones 2–5 and 15–18, was significantly enhanced by CR ($n = 22, 25$) as was (e) extinction retention, defined as the difference in freezing between tones 2–5 on extinction day 1 and tones 1–4 on extinction day 2 ($n = 22, 25$). (f) CR also increased time spent in the open arm of the elevated plus maze ($n = 28, 28$). Significance determined with Student's *t*-test. All results are presented as means ± SEM. $^{*}p < 0.05$, $^{**}p \leqslant 0.01$.

CR Increases the Distal Polyadenylation Fraction of SERT mRNA

The SERT mRNA occurs in two alternative polyadenylation forms that differ by the presence or absence of a conserved sequence element in the 3′ untranslated region (Battersby *et al*, 1999; Gyawali *et al*, 2010). Increased expression of SERT mRNA containing the distal polyadenylation sequence appears associated with decreased anxiety. In particular, the distal polyadenylation fraction, the relative amount of total SERT mRNA that contains the distal polyadenylation sequence, is increased in mouse brain by chronic fluoxetine treatment (Hartley *et al*, 2012). To determine if CR induces similar changes in SERT expression, we quantified the SERT distal polyadenylation fraction using quantitative PCR. CR increased the distal polyadenylation fraction in a manner similar to our earlier fluoxetine results (Figure 4a, $p < 0.01$). CR did not change the level of total SERT mRNA relative to a panel of control genes (Figure 4b) consisting of glyceraldehyde 3-phosphate dehydrogenase, TATA binding protein, Beta-glucuronidase, and hypoxanthine phosphoribosyltransferase 1. There were no significant differences in SERT expression between AL and CR mouse brain using any of the control genes to normalize for total mRNA or when all control genes were averaged.

Fluoxetine Enhances Fear Extinction Retention in AL Fed Mice only

SSRIs are anxiolytic drugs that improve fear extinction retention (Karpova *et al*, 2011). Given that fluoxetine and CR have similar effects on the expression of SERT mRNA species, and that the effects of CR on fear extinction appear to act through SERT, we hypothesized that fluoxetine would increase extinction retention in AL-fed mice, but not in CR mice. We compared the effects of these two treatments on fear extinction learning (Figure 5a) and retention (Figure 5b). Consistent with our *a priori* hypothesis, fluoxetine enhanced fear extinction retention in AL-fed mice but not CR mice ($p < 0.05$).

DISCUSSION

Adaptive fear responses are critical to the survival of organisms, allowing them to predict and avoid danger. Fear extinction is an active learning process that allows

reassessment of cues of danger in response to a changing environment. In the absence of effective fear extinction, cues of safety that once predicted danger continue to elicit a fear response, and fearful associations can accumulate. In humans, inefficient fear extinction or inadequate retention of extinction learning are associated

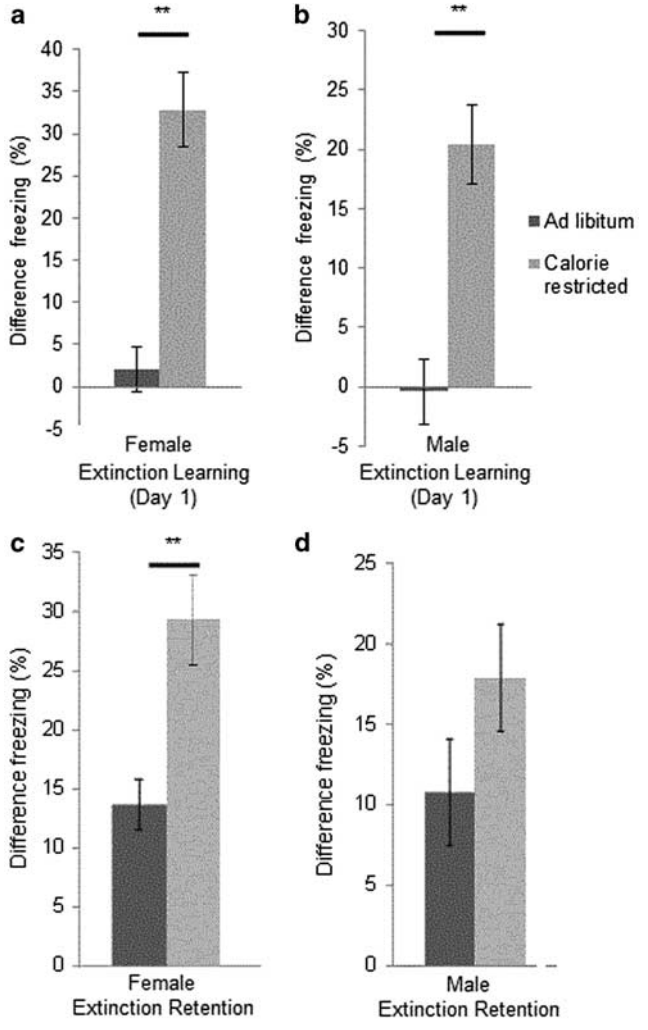

Figure 2 Effect of CR on the first day of extinction learning in adult mice. Extinction learning is improved in adult (a) females ($n=17$, 17) and (b) males ($n=10,11$). Significance analyzed by Student's *t*-test. All results are presented as means ± SEM. $^{**}p \leqslant 0.01$.

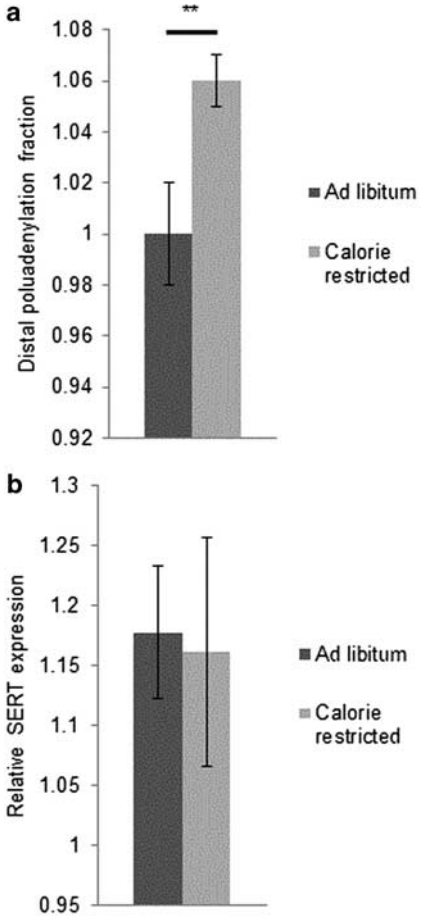

Figure 4 The effect of CR on SERT expression in adolescent female mouse brain. (a) CR increases the distal polyadenylation fraction (DPF) Brains of mice that had undergone CR ($n=4$) had elevated levels of SERT mRNA containing the distal polyadenylation sequence than those of control animals ($n=4$). DPF = relative amount of distal sequence containing SERT mRNA/Total SERT mRNA. (b) CR does not alter the expression of total SERT mRNA. Expression of the coding region of SERT mRNA was compared with a panel of four control genes in the brain from AL mice ($n=4$) or CR mice ($n=4$). Data presented is for the average of all four control genes. Differences assessed using Student's *t*-test. All results are presented as means ± SEM. $^{**}p \leqslant 0.01$.

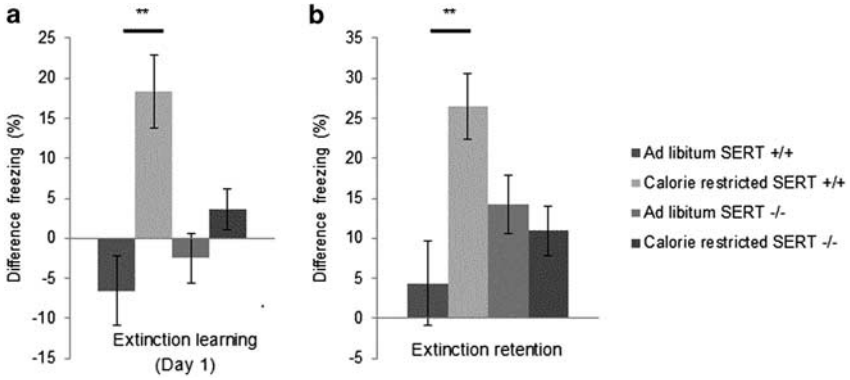

Figure 3 Effect of CR on extinction learning and retention in mice lacking SERT. Neither (a) Day 1 extinction learning ($n=11$, 12) or (b) extinction retention over 2 days ($n=11$, 12) are improved in female SERT $-/-$ mice. Wild-type (SERT $+/+$) littermates showed improvements on both measures ((a) $n=8$, 9; (b) $n=8$, 9). Differences analyzed by ANOVA. All results are presented as means ± SEM. $^{**}p \leqslant 0.01$.

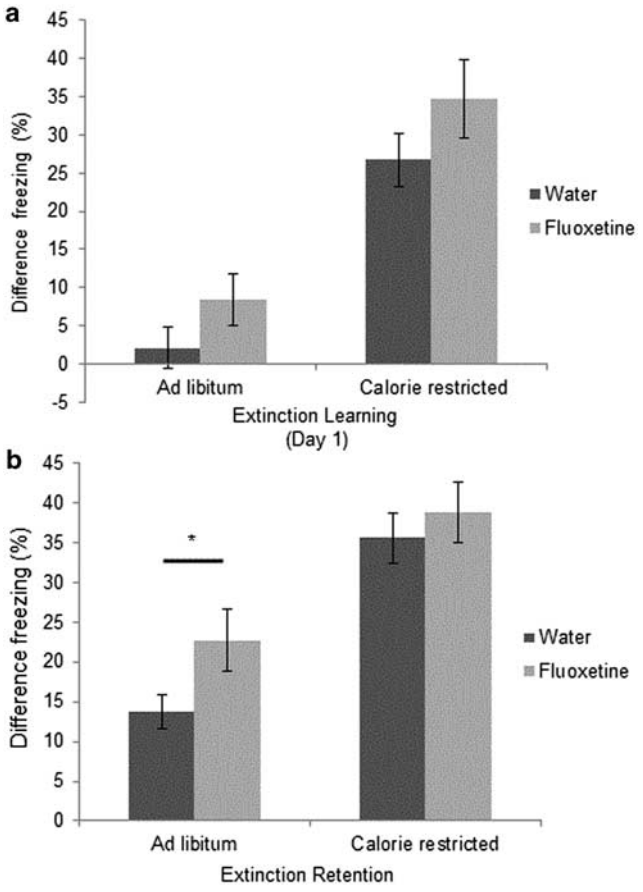

Figure 5 Effects of fluoxetine and CR on extinction learning and retention. (a) Fluoxetine does not significantly improve extinction learning on day one of extinction training in either AL ($n = 17, 8$) or CR ($n = 17, 7$) mice. (b) Fluoxetine improves extinction retention in female AL mice ($n = 17, 8$), but does not significantly increase extinction retention in CR mice ($n = 17, 7$). Statistical significance analyzed using ANOVA with Fisher's least significant difference procedure. All results are presented as means ± SEM. *$p < 0.05$.

with avoidance, trait anxiety, and risk for anxiety disorders (Graham and Milad, 2011). Because adaptive fear responses are so central to survival, and reproductive success their neural substrates are highly conserved from rodent to human (LeDoux, 2012). This phylogenetic conservation means that studies of fear extinction are a useful translational approach to gain insight into human psychopathology. In this study we have implemented cued fear learning in calorie restricted and AL fed mice to determine the role of metabolic status in regulating adaptive fear responses. CR substantially enhances fear extinction learning and the ability of mice to retain extinction learning. These effects of CR are dependent on SERT as they are absent in knockout mice. SERT is further implicated in these effects of CR because individually SSRI's and CR induce similar enhancement of extinction retention, but when combined do not produce an additive effect. Finally, CR induces expression of a species of the mRNA for SERT that is associated with enhanced extinction retention and is also induced by chronic fluoxetine treatment.

SERT is a key molecule in regulating serotonergic neurotransmission that may represent a mechanistic link between anxiety, fear extinction learning, and CR. Mice lacking SERT display elevated anxiety-like behaviors and impaired fear extinction retention (Wellman *et al*, 2007). Decreased expression of SERT has been reported in individuals with anxiety disorders (Kang *et al*, 2010), which may contribute to impaired fear extinction learning reported in these disorders (Graham and Milad, 2011). Similarly, alterations in the serotonergic circuitry is seen in individuals with AN, a disorder characterized by CR (Kaye *et al*, 2003, 2009). Chronic treatment with fluoxetine enhances extinction learning and retention in mice, and these effects have been proposed to explain its anxiolytic properties (Karpova *et al*, 2011). In our studies, CR displayed SSRI-like effects on extinction learning and retention that were absent from mice lacking SERT. These results strongly implicate SERT as a mediator of the effects of CR on fear extinction. The underlying mechanism by which SERT regulates fear extinction learning is unclear, but the amygdala receives dense innervation from serotonergic raphe neurons, and iontophoretically applied serotonin reduces excitatory responses to glutamate in the lateral amygdala through the activation of GABAergic interneurons (Stutzmann and LeDoux, 1999). In addition, fluoxetine has also been shown to enhance neural plasticity through enhanced expression of BDNF (Karpova *et al*, 2011).

Both CR and chronic fluoxetine induce expression of the distal polyadenylation form of the SERT mRNA. The distal polyadenylation form of SERT is associated with enhanced fear extinction learning and decreased risk for panic disorder through a common polyadenylation polymorphism in the 3′ untranslated region of the human SERT gene (*SLC6A4*) (Hartley *et al*, 2012). In our studies there was no additive effect of CR and fluoxetine on fear extinction learning further suggesting that these two treatments act through similar mechanisms involving SERT. This is consistent with clinical data suggesting a limited role of SSRI treatment in individuals with AN at a low-weight state (Kaye *et al*, 2001).

Estrogen enhances fear extinction learning, which may explain its effects on emotion regulation. In our studies, CR increased the time mice spent in diestrus, a low estrogen phase of the mouse estrus cycle. At the same time, CR enhanced fear extinction learning suggesting that CR does not act primarily through its effects on the estrus cycle. Furthermore, male mice showed enhanced fear extinction learning within session with CR (Figure 2b).

The marked effects of CR on fear extinction learning may inform the pathophysiology of AN, in which there is both a high level of premorbid anxiety and subsequent weight loss. Lifetime comorbid anxiety disorders are as high as 83%, and, in 75% of these individuals, anxiety precedes the onset of diet restriction (Godart *et al*, 2000). Individuals with AN have decreased tolerance for uncertainty and are thought to attempt to mitigate this perceived lack of control over life stressors through excessive control of food and weight as a way to reduce their anxiety (Frank *et al*, 2012). Furthermore, previous literature suggests that, in individuals with AN, eating may induce dysphoria while dietary restriction decreases anxiety (Kaye *et al*, 2009). Thus, CR is seen as a form of emotional regulation in AN (Espeset *et al*, 2012; Wildes *et al*, 2010). Treatment of the underlying anxiety as a central component to AN has gained increasing interest clinically. Recently, it was proposed that the therapeutic

success of family-based therapy, which has shown promising results in the treatment of adolescents, centers on the use of exposure therapy, shown to be efficacious in the treatment of other anxiety disorders (Hildebrandt *et al*, 2012). Premeal anxiety has been seen to be inversely correlated with caloric intake in acutely weight restored individuals with AN (Steinglass *et al*, 2010), with meal-based exposure therapy being associated with a change in anxiety significantly associated with caloric intake (Steinglass *et al*, 2007, 2012).

Anxiety is accompanied by sensitization of fear circuitry in AN. Functional MRI studies using disorder-relevant stimuli such as high calorie foods and distorted body images induce elevated amygdala reactivity in subjects with AN relative to healthy controls (Ellison *et al*, 1998; Joos *et al*, 2011); positron emission tomography studies show hypoperfusion of structures associated with fear extinction—namely the medial prefrontal cortex—and hyperperfusion of the amygdala – hippocampal complex in AN (Takano *et al*, 2001). Consistent with this clinical perspective, our results suggest that CR may normalize dysregulated fear responses in AN, which in combination with trait or acquired alterations in cortico-striatal circuit function that have been described in AN, may help explain why dieting behavior is so vigorously reinforced as well as the high comorbidity of anorexia with obsessive-compulsive disorder (Milad and Rauch, 2012; Wagner *et al*, 2007; Zheng *et al*, 2012).

Our present findings provide motivation to study conditioned-fear responses directly in individuals with AN as an important factor in the rapid, tenacious, and potentially dangerous reinforcement of dieting behavior seen in the disorder. Further studies of conditioned-fear responses in nonhuman systems may lead to a more precise mechanistic understanding of the biological effects of CR in AN and potentially lead to improved treatments.

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DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)