Hunger promotes fear extinction by activation of an amygdala microcircuit

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Abstract

Emotions control evolutionarily conserved behavior that is central to survival in a natural environment. Imbalance within emotional circuitries, however, may result in malfunction and manifestation of anxiety disorders. Thus, a better understanding of emotional processes and in particular the interaction of the networks involved is of considerable clinical relevance. Although neurobiological substrates of emotionally controlled circuitries are increasingly evident their mutual influences are not. To investigate interactions between hunger and fear, we performed Pavlovian fear conditioning in fasted wildtype mice and in mice with genetic modification of a feeding-related gene. Furthermore we analyzed in these mice the electrophysiological microcircuits underlying fear extinction. Short-term fasting before fear acquisition specifically impaired long-term fear memory, while fasting before fear extinction facilitated extinction learning. Furthermore, genetic deletion of the Y4 receptor reduced appetite and completely impaired fear extinction, a phenomenon that was rescued by fasting. A marked increase in feed-forward inhibition between the basolateral and central amygdala has been proposed as a synaptic correlate of fear extinction and involves activation of the medial intercalated cells. This form of plasticity was lost in Y4KO mice. Fasting before extinction learning, however, resulted in specific activation of the medial intercalated neurons and re-established the enhancement of feed-forward inhibition in this amygdala microcircuit of Y4KO mice. Hence, consolidation of fear and extinction memories is differentially regulated by hunger suggesting that fasting and modification of feeding-related genes could augment the effectiveness of exposure therapy and provide novel drug-targets for treatment of anxiety disorders.

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Introduction

Emotions, motivations and reinforcement are closely related, evolutionarily conserved phenomena maintaining the integrity of an individual and promoting survival in a natural environment (Atasoy et al., 2012; LeDoux, 2012; Sternson, 2013). However, if such survival circuits are out of balance, maladaptation may arise and result in the development of anxiety disorders. Thus, a better understanding of fear and anxiety includes also the interaction with other life-sustaining brain circuitries and their reciprocal integration.

We therefore hypothesized that modulation of one survival circuit will provoke a significant impact on the other survival circuits. For instance, if a decrease in blood glucose signals that energy homeostasis is out of balance, release of hormones and activation of hypothalamic nuclei will be initiated (Balleine, 2005; Sohn et al., 2013; Williams and Elmquist, 2012). As a consequence food intake and search for food will prevail and emotional behavior will be adapted accordingly. Indeed, recent experiments in Drosophila suggest a close relation between food intake and the formation of aversive memories (Hirano et al., 2013; Placais and Preat, 2013).

Here, we investigated the neurobiological effects of fasting on the different phases of mammalian fear processing in mice, in particular fear acquisition, consolidation and most importantly fear extinction. The amygdala complex, located in the temporal lobe, is centrally involved in coordinating fear-related processes. It receives information about fear provoking stimuli via thalamic and cortical afferents and regulates the resulting fear response by efferent projections targeting hypothalamus and brain stem (Ehrlich et al., 2009). The central amygdala (CEA) represents the major output station for generating an adaptive fear response. It consists of a highly elaborate micro-network that receives afferent projections from the adjacent basolateral amygdala (BLA), intercalated cell masses, and also from more distant brain areas such as hypothalamus and brain stem. Recent evidence suggests that the CEA is also essential for suppression of food intake (Cai et al., 2014; Carter et al., 2013) and for directing motivational behavior (Robinson et al., 2014), providing hence a possible hub for integrating survival circuits for fear and hunger. Interestingly, among the multiple neuromodulators that shape amygdala output, the neuropeptide Y system is involved in both, feeding and fear. In particular, central NPY Y4 receptors that are targeted by peripherally released pancreatic polypeptide (PP) may link peripheral feeding-related signals to emotional processes in the brain (Holzer et al., 2012).

Experimentally, amygdala functioning and related fear learning can be tested by Pavlovian fear conditioning, in which a subject learns to associate an initially neutral stimulus, such as a tone (conditioned stimulus, CS) with an aversive stimulus, typically a mild electric food shock (unconditioned stimulus, US) (LeDoux, 2000). As a consequence, the presentation of the CS alone or the context (consisting of the conditioning environment, such as light, texture or odor of the chamber) in which the fear memory was acquired will result in a species-specific fear reaction. Repetitive presentations of the CS in the absence of a shock, however, result in a reduction of the acquired fear reaction. This learning process is termed fear extinction and is the underlying principle of exposure therapy in human patients.
suffering from anxiety disorders (Davis, 2011; Herry et al., 2010; Myers and Davis, 2007; Ricardo and Koh, 1978).

Here, we provide evidence that the fear-related mechanisms controlled by amygdala circuitries are strongly correlated with those regulating food intake and energy balance. We further demonstrate that short-term fasting results in suppression of fear by enhancing feed-forward inhibition in an amygdala microcircuit whereas genetic deletion of the Y4 receptor reduced appetite and impaired fear extinction.

**Methods and Materials**

**Animals**

Wilttype (WT) and Y4KO mice, both on a C57BL6/NCrl background were bred at the Institute of Pharmacology, Medical University of Innsbruck. All procedures involving animals and animal care were conducted in accordance with international laws and policies (Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 2011) and were approved by the Austrian Ministry of Science. All effort was taken to minimize the number of animals used and their suffering.

**Behavioral experiments**—Experiments were performed in adult male C57Bl/6NCrl mice (10-12 weeks old, weighing 22-28g) during the light phase of the light/dark cycle. Y4KO mice were backcrossed for at least 10 generations to a C57Bl/6NCrl background. They were housed in groups of 3 to 5 animals under standard laboratory conditions (12h/12h light/dark cycle, lights on: 07:00, food and water ad libitum). Generation of Y4KO mouse has been described in detail previously (Sainsbury et al., 2002; Tanan et al., 2009).

**Fear conditioning**

Fear acquisition, context testing and reinstatement were performed in context A consisting of a transparent acrylic rodent conditioning chamber with a metal grid floor that was enclosed by a sound attenuating chamber. Illumination for Context A was 80 lux and chambers were cleaned with 70% ethanol. Fear recall, fear extinction, extinction recall and reinstatement testing were performed in a different context consisting of a dimly illuminated (10 lux) chamber with black walls and cleaned with 1% acetic acid (context B). On day 1, mice were fear conditioned in context A by repetitive pairing of an auditory stimulus (CS, 30s white noise, 80 dB) with a mild electric foot-shock (US, 2s, 0.5 mA). All animals received 5CS each of them co-terminating with a US. On day 2, mice were tested for their context fear memory in context A (15 min). Fear extinction (15CS presentations, each 30s, inter-stimulus interval 5s) and extinction recall (5CS presentations, each 30s, inter-stimulus interval 5s) were performed in context B on day 3 and 4, respectively. As Y4KO mice did not show fear extinction, we performed an extensive extinction protocol, consisting of 5 extinction sessions, with 15CS each (30s, inter stimulus interval 5s) and extinction recall was tested the following day (5CS).
Fasting procedures

Mice were fasted overnight for 16h before fear acquisition, for fear conditioning experiments and starting immediately after or 5h after fear acquisition for 16h, for extinction experiments. For fasting, mice were single housed; food and bedding was removed but water was available ad libitum. Control mice were also single housed for the same time without bedding but with access to food and water ad libitum. Bedding was removed because mice that have no access to food tend to eat the bedding of the cage, which may trigger the activation of feeding-related neuronal circuits and release of hormones.

Electrophysiology

Slice electrophysiology was performed on acute slices 24h after the final behavioral experiment. See Fig.4H and supplemental information for details.

Immunohistochemistry

cFos, cFos/GABA, cFos/FOX2 and cFOS/PC1 were performed as described previously in detail (Tasan et al., 2011). See supplemental information for details.

Statistical Analysis—Data are presented as means ± SEM and were analyzed for normal distribution and equal variances using GraphPad-Prism 5 software (San Diego, USA). Fear conditioning experiments were analyzed by repeated two-way ANOVA (time, genotype/treatment, interaction) and Bonferroni post-hoc test for selected comparisons. One-way ANOVA with Bonferroni post-hoc test was used to analyze changes in bodyweight and Mann-Whitney test for analyzing US sensitivity-threshold.

Results

Short-term fasting specifically impairs long-term memory but not short-term memory of cued and context fear

To investigate the role of short-term fasting on the acquisition of conditioned fear, male C57BL/6N mice were fasted overnight for 16 hours before fear conditioning and their performance was compared to non-fasted littermates (Figure 1A). Short-term fasting resulted in a mean reduction of body weight by 17.4% (Supplementary figure 1).

Acquisition of conditioned fear was unchanged in fasted and non-fasted mice (Figure 1B, two-way ANOVA for repeated measurements: time: F(4,80)=60.74, P<0.0001, treatment: F(1,20)=0.24, P>0.05, interaction F(4,80)=2.51, P>0.05). Context fear memory, however, (Figure 1C, 3min context testing on day 2: t(20)=3.21, P<0.01), tested 24h after fear acquisition and re-feeding by exposing the mice to the original conditioning chamber (context A), was significantly reduced, while context fear acquisition was unchanged (Supplementary figure 2). Due to reduced context fear expression, context extinction appeared to be facilitated in fasted mice compared to fed mice (Supplementary figure 2, two-way ANOVA for repeated measurements: time: F(14,280)=6.77, P<0.0001, treatment: F(1,20)=9.84, P<0.01, interaction F(14,280)=0.45, P>0.05). Similarly, freezing to the CS under fed conditions, tested 48h after fear acquisition by exposing mice to the auditory stimulus alone (CS test, long-term memory, LTM, Figure 1A+D) in a different chamber (context B) was significantly lower in mice that were fasted before fear acquisition (Figure 1D,
However, short-term memory tested (Figure 1A+D, CS test STM, under fed conditions) in separate groups of mice (Figure 1A) was similar in fasted and non-fasted mice, as demonstrated by CS induced freezing 30 min and 150 min after fear acquisition (Figure 1D, t\(_{(10)}\)=0.67, P>0.05 and t\(_{(10)}\)=1.26, P>0.05, respectively). As the effect of fasting on fear memory developed slowly, we performed fear acquisition after 16 h of fasting and 24 h of re-feeding; further demonstrating that fasting did not affect fear acquisition (Supplementary figure 3). Collectively, these data suggest impaired consolidation or recall of conditioned fear in mice that were fasted before fear acquisition, while learning and short-term memory remain unaffected.

**Short-term fasting facilitates extinction learning and promotes extinction recall**

To understand if fasting-induced inhibition of fear depends on memory consolidation and to investigate the effect of fasting on fear extinction, a separate group of mice was tested for fear recall after 16 h of fasting. Fasting was initiated here either immediately or 24 h after fear acquisition (Figure 2A). There was no change in CS induced freezing during fear recall in mice that were fasted immediately or 24 h after fear acquisition compared to non-fasted controls, suggesting that fasting induced changes develop slowly, over time (e.g. 16 h) and have to be present within the consolidation window to affect long-term fear memory. Interestingly, freezing to the CS upon fear recall was similar in animals that were fasted after fear acquisition and non-fasted controls (Figure 2C, t\(_{(16)}\)=0.38, P>0.05), suggesting equal expression of fear in fasted and non-fasted mice. Fear extinction learning, however, was significantly facilitated in fasted mice (Figure 2D, two-way ANOVA for repeated measurements, time: F\(_{(24,432)}\)=9.03, P<0.0001, treatment: F\(_{(1,18)}\)=5.44, P<0.05 and interaction F\(_{(24,432)}\)=3.55, P<0.0001). More importantly, extinction recall, tested under fed conditions 24 h after fear extinction training, by exposing the mice to 5 CS in context B was still reduced in those mice that were fasted before extinction learning (Figure 2E, t\(_{(16)}\)=3.61, P<0.01). Together, these data indicate that 16 h of acute fasting does not alter learning in general, but rather modulates fear memory by specifically influencing the emotional valance of learning processes. Thus, fasting inhibits the consolidation of an acquired fear memory, but promotes the acquisition and consolidation of fear extinction.

**Genetic deletion of the Y4 receptor reduces appetite and impairs fear extinction**

If survival circuits, such as feeding and fear, were indeed influencing each other, we hypothesized that mice with altered feeding behavior or genetic ablation of feeding-related genes would also display specific changes in fear extinction behavior (Gutman et al., 2008; Verma et al., 2012). Y4 receptors are expressed in the CNS and are activated by PP that is released from pancreas in response to feeding. Interestingly, Y4KO mice display decreased body weight and reduced food intake, both suggesting chronic suppression of the hunger circuit (Lin et al., 2004; Sainsbury et al., 2002). To investigate the relation of a feeding-related gene and satiety to fear extinction we subjected Y4KO mice to Pavlovian fear conditioning (Figure 3A). Acquisition (Figure 3B, two-way ANOVA for repeated measurements, time: F\(_{(4,48)}\)=27.29, P<0.0001, genotype: F\(_{(1,12)}\)=2.54, P>0.05, interaction F\(_{(4,48)}\)=1.28, P>0.05) and recall of conditioned fear (Figure 3C, t\(_{(13)}\)=0.54, P>0.05) were unchanged in Y4KO mice compared to controls. Fear extinction, however, was significantly impaired in Y4KO mice (Figure 3D, E; two-way ANOVA for repeated measurements, time:
F(14, 182) = 0.82, P > 0.05, genotype: F(1, 13) = 26.90, P < 0.001, interaction F(14, 182) = 3.08, P < 0.001 and fear recall: t(13) = 3.86, P < 0.01), suggesting that genetic alterations in the feeding circuit considerably alter fear behavior. Sensitivity to the electric foot-shock was not different from controls (Supplementary figure 4). These results suggest that modification of the feeding circuit has a significant impact on fear processing. In particular dys-regulated appetite correlated with impaired fear extinction.

**Short-term fasting rescues impaired fear extinction in Y4KO mice**

Next, we tried to rescue the specifically impaired fear extinction in Y4KO mice by subjecting them to three cycles of extinction-recall sessions, each consisting of 16h fasting before extinction training followed by extinction recall tested under fed conditions 24h later (Figure 3F, Supplementary figure 1 for reduction of body weight). CS induced freezing in context B on the testing day was similar in fasted and fed Y4KO mice (Figure 3H, t(16) = 0.20, P > 0.05), indicating equal acquisition and expression of conditioned fear. Extinction learning, however, was significantly enhanced in Y4KO mice that were subjected to 16h fasting compared to fed Y4KO mice (Figure 3I, two-way ANOVA for repeated measurements, time: F(24, 168) = 0.72, P > 0.05, treatment: F(1, 7) = 7.46, P < 0.05, interaction F(7, 168) = 1.01, P > 0.05). More importantly, this extinction memory was preserved upon re-feeding, as demonstrated by the reduced freezing behavior during extinction recall in context B (Figure 3J, t(7) = 1.63, P > 0.05; t(7) = 2.55, P < 0.05; t(7) = 4.84, P < 0.01 for extinction recall 1, 2 and 3, respectively), suggesting that impaired fear extinction can be rescued by modulation of the feeding circuit.

To investigate whether this reduction of fear was permanent, we subjected these mice two weeks after the last extinction trial to a re-instatement paradigm, consisting of one unsignaled foot-shock in context A and testing of CS-induced freezing in context B on day 20 and 21, respectively (Figure 3F). Importantly, CS-induced freezing during re-instatement testing was still reduced in Y4KO mice that were repetitively fasted before extinction learning (Figure 3J, t(7) = 3.89, P < 0.01), suggesting a long-lasting, stress-resistant suppression of fear.

**Short-term fasting specifically activates medial intercalated neurons and facilitates feed-forward inhibition from the basolateral to the centromedial amygdala**

We next investigated the underlying synaptic correlates linking feeding and fear extinction circuits. Following fear acquisition Y4KO mice were fasted for 16h, subjected to extinction training and brains were processed for immunohistochemistry 90 min after the end of fear extinction training. Compared to non-fasted Y4KO mice (Figure 4A-C), expression of the immediate early gene cFos was increased in those Y4KO mice that were fasted before extinction training (Figure 4D-F) specifically in the medial intercalated cells (mITC), a brain nucleus associated with fear extinction (Busti et al., 2011; Likhtik et al., 2008) (Figure 4G, t(14) = 2.60, P < 0.05). Thus, short-term fasting activates specific neuronal populations in extinction-related brain areas.

As shown previously in rats, fear extinction results in enhanced feed-forward inhibition from the BLA to the CEm, mediated by increased activity of mITC neurons (Amano et al., 2010).
To determine whether facilitated fear extinction of fasted mice corresponded to alterations in synaptic neurotransmission we performed whole-cell patch clamp recordings to measure BLA to CEm feed-forward inhibition in acute amygdala slices of mice 24h after two extinction trainings. Stimulation of the BLA at intensities ranging from 100-500 μA consistently evoked an IPSP in CEm neurons with an initial, brief EPSP component (Figure 4L). Compared to untrained C57BL/6N mice (WT homecage), the amplitude of IPSPs was increased 24h after successful fear extinction (Figure 4I, two-way ANOVA F(2,36) = 4.93, P<0.01). Bath application of the AMPA receptor antagonist, DNQX, abolished both the IPSP and EPSP (Supplementary figure 5A), while application of GABA receptor antagonist, picrotoxin, completely blocked the IPSP. These data confirm that fear extinction results in enhanced feed-forward inhibition in an amygdala microcircuit connecting the BLA with the CEm.

In Y4KO mice (homecage), similar as in WT mice, electrical stimulation of the BLA consistently evoked a brief EPSP followed by a larger IPSP in CEm neurons (Figure 4L). The amplitude of evoked IPSPs did not significantly differ between untrained fed or fasted Y4KO mice (both homecage), or Y4KO mice that underwent fear acquisition (Supplementary figure 5B-D). But in contrast to wildtype mice, and in line with the impaired fear extinction of Y4KO mice, feed-forward inhibition from BLA to CEm neurons remained also unchanged in Y4KO mice 24h after two consecutive fear extinction trainings (Figure 4J). However, CEm neurons recorded from Y4KO mice that were fasted for 16h prior to the two fear extinction trainings, and thus had successfully acquired extinction memory, exhibited larger IPSPs in response to BLA stimulation (Figure 4K, two-way ANOVA F(4,60) = 2.90, P<0.05). As fasted Y4KO mice displayed reduced freezing levels already after two extinction sessions (Figure 3J), we decided to perform electrophysiological recordings after the second extinction. No changes in general membrane properties were observed (Supplementary figure 6). These data suggest that the impaired fear extinction in Y4KO mice is due to lack of inhibitory synaptic plasticity in BLA to CEm projections and that fasting before fear extinction rescues fear extinction by specifically activating medial ITC neurons, leading to a marked increase in BLA to CEm feed-forward inhibition.

**Discussion**

Here, we demonstrated that short-term fasting differentially affects fear and fear extinction learning. This differential effect on two fear-related learning processes suggests that the effect of acute fasting on fear was not a uniform inhibition or promotion of learning and memory *per se*. It rather indicates that short-term fasting manipulates these memories towards a reduction of fear. This is interesting in the light of an evolutionary conserved mechanism that adapts explorative behavior depending on internal homeostatic demand. Thus, in situations of increased hunger a subject will be prepared to take a higher risk, whereas in a saturated state safety concerns will predominate.

Furthermore, we demonstrated that genetic deletion of the Y4 receptor impaired fear extinction, an effect that was rescued by short-term fasting. PP that is released peripherally upon food intake serves as the main ligand for Y4 receptors (Holzer *et al.*, 2012). In the brain Y4 receptors are localized in specific areas that are in part open to the blood-brain...
barrier, such as hypothalamus and brain stem (Tasan et al., 2009). These brain regions are tightly integrated into the ascending reticular activating system that may provide the necessary arousal for successful fear extinction. In particular, beta-adrenoreceptor-dependent activation of the nucleus tractus solitarii (NTS) is essential for amygdala activation and respective memory formation (McGaugh, 2004; Mueller et al., 2008). Interestingly, Y4 receptors are highly expressed in the NTS and peripheral PP injection results in fast activation of NTS neurons (Tasan et al., 2009). Furthermore, a reduction of adrenergic tonus and sympathetic activity has been reported in Y4KO mice (Smith-White et al., 2002). Thus, deletion of Y4 receptors in the NTS may inhibit extinction learning by dampening the necessary arousal. On the other hand, short-term fasting may have rescued fear extinction in Y4KO mice by alternative activation systems and release of stress hormones (McGaugh, 2004).

Besides PP, also NPY displays affinity for central Y4 receptors at nanomolar concentrations (Bard et al., 1995; Gehlert et al., 1996), suggesting that central Y4 receptors may be either targeted by peripherally released PP or by central NPY. Expression of the immediate early gene cFos demonstrated that short-term fasting specifically activates AGRP/NPY neurons in the arcuate nucleus of the hypothalamus (Supplementary figure 7). While activation of some arcuate AGRP/NPY neuron projections promote food intake others do not (Betley et al., 2013; Wu et al., 2009). In particular AGRP/NPY neurons that are projecting to limbic areas, such as the amygdala may be crucial for adapting emotional behaviors to the internal homeostatic situation. This could also be achieved by NPY release that is involved in both, regulating feeding and reducing anxiety (Bacchi et al., 2006; Kask et al., 2002; Tasan et al., 2010). Recent evidence demonstrated that NPY suppresses the expression of conditioned fear and promotes fear extinction (Fendt et al., 2009; Gutman et al., 2008), extending the anxiolytic properties of NPY to models of learned fear. In fact, similar to Y4KO mice also NPYKO mice fail to extinguish learned fear (Verma et al., 2012), further emphasizing the central role of feeding-related genes in the modulation of fear extinction. In our experiments, the extensive activation of AGRP/NPY neurons and the consecutive release of NPY upon fasting may have significantly contributed to the facilitated extinction learning. Furthermore, NPY is released from a considerable number of amygdala interneurons, and injection of NPY into the BLA may facilitate fear extinction (Gutman et al., 2008).

The amygdala complex is crucially involved in mediating fear- and anxiety-related behaviors (Pape and Pare, 2010; Quirk and Mueller, 2008). This is achieved by extensive reciprocal connections with hypothalamus, brain stem and cortical areas. Short-term fasting results in a drop of glucose levels, activation of the autonomic nervous system and release of stress-hormones. This general activation is important, as a certain degree of arousal is essential for successful fear extinction (McGaugh, 2004). For instance, targeting the catecholaminergic system by yohimbine or L-DOPA but also release of stress hormones, such as glucocorticoids, have been shown to facilitate fear extinction learning in rodents and humans (Fitzgerald et al., 2014; Holmes and Quirk, 2010; McGuire et al., 2014; Soravia et al., 2006). The duration of the fasting period may be a crucial factor. Although prolonged fasting (24h and more) resulted in facilitated extinction it also increased freezing behavior to the first CS, probably by augmenting stress levels. On the other hand, shorter fasting periods
may not provide the necessary motivation to reduce fear. Thus, fasting may trigger the synchronous release of different neuromodulators and hormones that ultimately promote synaptic plasticity in the amygdala as the central stage for fear and extinction learning. Specifically, using cFos mapping our experiments indicate that the interaction of fear and hunger takes place on the level of the amygdala and more precisely in the medial intercalated neurons. These neurons are extensively activated after successful extinction learning (Busti et al., 2011) while ablation of the mITC completely abolishes consolidation of fear extinction (Likhtik et al., 2008). Recently, the electrophysiological correlate of fear extinction has been pinpointed to an amygdala microcircuit connecting the BLA with the CEm via mITC (Amano et al., 2010). Here, we investigated this microcircuit in the mouse brain and demonstrated that short-term fasting before extinction training activates inhibitory neurons in the mITC projecting to the main output nucleus of the amygdala, the CEm, with a concomitant increase in feed-forward inhibition from the BLA and improved extinction memory. Together with the reduced freezing behavior during extinction recall tested under fed conditions, these results confirm that short-term fasting does not unspecifically increase locomotion, but rather promotes extinction learning by activating mITC in an extinction-relevant amygdala microcircuit.

It is important to note that fasting affects both, context fear and fear extinction, two phenomena that are sensitive to environmental encoding and hippocampal damage (Corcoran et al., 2005; Hobin et al., 2006). The hippocampus modulates fear behavior by direct connections to the amygdala or indirectly via activation of the prefrontal cortex (Maren, 2005). Interestingly, the infralimbic region of the prefrontal cortex activates inhibitory intercalated neurons (Amir et al., 2011; Pinard et al., 2012), a population of neurons that were activated in our experiments by short-term fasting and consequently reduced CEm activation by feed-forward inhibition (Supplementary figure 8).

Fear memories are strong and often persist lifelong whereas extinction memories are rather labile and transient, resulting in relapse of fear in particular under stressful situations (Ji and Maren, 2007; Quirk and Mueller, 2008). Here, we demonstrate that short-term fasting before fear extinction not only rescues impaired fear extinction in Y4KO mice, but more importantly results in a permanent suppression of fear, even under stressful situations. Given that treating human anxiety disorders by exposure therapy is not equally effective in all patients and relieves symptoms only temporarily, an efficient supportive treatment is required. Our results indicate that genetic deletion of genes that reduce appetite may also impair fear extinction and that bypassing the circuit, e.g. by increasing appetite through periods of mild fasting can rescue impaired fear extinction. Thus, fasting before exposure therapy may be a valuable supportive therapeutic option with fast translation into clinics and may be well accepted by patients. Furthermore, the molecular machinery of the feeding circuit may provide novel targets for pharmacological treatment of anxiety disorders.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

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Fig. 1.
Acute short-term fasting before fear conditioning inhibits formation of context and CS
induced long-term fear memory (LTM) while not affecting short-term memory (STM). (A)
Fasting started 16h before and continued during fear acquisition. All mice had access to food
*ad libitum* after fear acquisition and before and during fear testing. (B) Both, fasted and fed
mice exhibited similar baseline freezing and acquisition of conditioned fear. (C) Reduced
context freezing of mice that were fasted before and during fear acquisition. (D) Mice that
were fasted before and during fear acquisition displayed reduced CS-induced freezing 48h
after fear conditioning, while short-term memory tested 30min and 150min after fear
acquisition was similar to controls (repeated two-way ANOVA for acquisition, Student’s t-
test for context and CS testing. **P<0.01; LTM: Food: n= 11, Fasting: n=11; STM 30min:
Food: n= 6, Fasting: n=6; STM 150min: Food: n= 6, Fasting: n=6).
Fig. 2.
Short-term fasting before fear extinction improves extinction learning. (A) Following fear conditioning mice were fasted for 16h before and during extinction training. Extinction recall was tested 24h later with food available *ad libitum*. (B) Following fear acquisition mice were divided into two equal groups, one that was fasted and one with food available, (C) no difference in CS induced freezing was observed between fasted and non-fasted mice, (D) but facilitated fear extinction in mice that were fasted 16h before and during fear extinction learning and (E) reduced CS induced freezing in extinction recall of mice that were fasted before and during extinction learning (repeated two-way ANOVA for acquisition and extinction learning, Student’s t-test for CS-induced freezing and extinction recall testing, *P*<0.05, **P**<0.01; Food: n= 10, Fasting: n=10).
Fig. 3.
Impaired fear extinction in Y4 receptor KO mice was rescued by repeated fasting episodes before extinction learning. (A) Y4KO mice and wildtype controls were subjected to fear acquisition, CS testing 24h later and to a total of 4 extinction sessions on day 2-6 followed by extinction recall on day 7. (B) No difference in fear acquisition and (C) fear recall, but (D) impaired extinction learning (note the apparent reduction of freezing levels in Y4KO mice during CS1 and 3, that was, however, due to jumping behavior, thus probably indicating an increased stress reaction) and (E) extinction recall in Y4KO mice compared to wildtype controls. (F) After fear acquisition, one group of Y4KO mice was subjected to repeated cycles of fasting before and during extinction learning followed by extinction recall under fed conditions. (G) Following fear acquisition Y4KO mice were divided into two equal groups, one that was fasted before extinction training and one that was not, (H) no difference in fear expression between fasted and non-fasted Y4KO mice, (I) rescued fear extinction in fasted Y4KO mice compared to fed Y4KO controls, (J) successive reduction of freezing in extinction recall on testing day 3, 5 and 7 tested under fed conditions and reduced re-instatement on day 21 in Y4KO mice that were fasted before extinction training, suggesting permanent suppression of fear (repeated two-way ANOVA for acquisition and extinction learning. Student’s t-test for CS-induced freezing and extinction recall and re-instatement testing, *P<0.05, **P<0.01, ***P<0.001; WT vs. Y4KO: WT: n= 9, Y4KO: n=6 and Y4KO fasted vs. non-fasted: Y4KO fasting: n=8, Y4KO food: n=8).
Fig. 4.
Fasting in Y4KO mice results in activation of the mITC and enhanced BLA to CEm feed forward inhibition. (A-C) Expression of immediate early gene cFos in the ITC of a fasted Y4KO compared to (D-F) a non-fasted Y4KO control reveals (G) increased activation of ITC neurons, (H) experimental setup for ex vivo electrophysiology indicating home cage controls, WT and Y4KO undergoing fear acquisition on day 1, extinction on day 2 and electrophysiology on day 3 under fed conditions and experimental group with a 16h fasting period before and during extinction training, (I) enhanced feed-forward inhibition from BLA to CEm via mITC in WT mice after successful fear extinction, (J) lack of increased feed-forward inhibition in fed Y4KO after extinction training corresponding to impaired fear extinction and (K) rescue of impaired fear extinction in Y4KO mice by fasting facilitates enhanced feed-forward inhibition between BLA and CEm. (L) Example traces from wildtype and Y4KO mice of the individual groups with increasing stimulation intensities. (n = WT Homecage 4 mice, 12 cells; WT Food+Ext 4 mice, 15 cells; Y4KO Homecage 5 mice, 13 cells; Y4KO Food+Ext 4 mice, 14 cells; Y4KO Fasting+Ext 4 mice, 12 cells; *P<0.05, two way ANOVA for repeated measurements for electrophysiology, and Y4KO Food: n=8, Y4KO Fasting: n=8; Student’s t test for c-Fos immunohistochemistry).