Supplementary Figure Legends

Supplementary Figure 1. Chronic stress does not alter fear extinction or expression. Rats in the SHAM groups from Figure 1 received either daily handling (NS) or immobilization stress (STR). Rats received Pavlovian fear conditioning 24h after the last stress or handling session. Fear memory was assessed by placing the animals in a novel context 48h after the conditioning session and measuring conditional freezing following tone presentation. (a) An extinction test was performed 48h after the fear memory test during which animals were returned to the extinction context and presented with additional tones. Extinction retention measures the memory strength for the extinction memory acquired during the first extinction session. It is calculated as the difference in initial freezing levels between the first and the second extinction sessions. This value was normalized for each rat to the NS-SHAM group average. (b) In a second group, intact animals received auditory Pavlovian fear conditioning 24h before beginning daily handling (Post FC: NS) or immobilization stress (Post FC: STR) for 14d. Fear memory was assessed by placing the animals in a novel context and measuring conditional freezing following tone presentation 24h after the last stress or handling session. All data are mean ±s.e.m.

Supplementary Figure 2. Adrenalectomy and immobilization stress do not alter locomotion or spontaneous freezing levels. Rats from Figure 1 received either daily handling (NS) or immobilization stress (STR). Locomotion (a) and spontaneous freezing (b) in a novel environment were assessed prior to fear conditioning. All data are mean ±s.e.m.

Supplementary Figure 3. Stress-induced enhancement of fear arises after five consecutive days of stress exposure. (a) Rats received either daily handling (30s/d, 14d, NS) or immobilization stress (4h/d, STR) for 1, 2, 4,7 or 14 days. Animals received auditory Pavlovian fear conditioning 24h after the last stress or handling session. Fear to the tone was assessed 48h later. (b) In a second group of animals, rats received a single handling (NS) or immobilization stress (Acute STR) session. They were then returned to the vivarium for 14d before receiving auditory Pavlovian fear conditioning. Fear to the tone was assessed 48h later. (c) In a third group of animals, rats received either daily handling (30s/d, 5d, NS) or immobilization stress (4h/d, STR) for 5 days. Animals received auditory Pavlovian fear conditioning 24h after the last stress or handling session. Fear to the tone was assessed 48h later. (All data are mean ±s.e.m. * $p<0.05$, ~ $p<0.10$ in planned comparisons.

Supplementary Figure 4. Repeated water stress increases circulating acylated ghrelin levels. Rats received either daily handling (30s/d, 14d, NS) or water stress (1h/d, 14d, Water Stress). Trunk blood was collected 24h after the last stress or handling session. Plasma acylated ghrelin was measured by ELISA. All data are mean ±s.e.m. **** $p<0.0001$, in planned comparisons.

Supplementary Figure 5. Circulating acylated ghrelin levels are not altered by acute immobilization stress. Rats received either handling (No Stress, or NS group) or a single session of immobilization stress (4h; ACUTE group). Twenty-four hours later, trunk blood was collected and plasma acylated ghrelin was measured by ELISA. All data are mean ±s.e.m.

Supplementary Figure 6. Chronic ghrelin receptor agonism does not alter locomotion, innate anxiety, or the expression of previously acquired fear memories. Rats received a daily systemic injection of MK-0677 (MK: 5d), an agonist of GHSR-1a, or vehicle (VEH: 5d) for five days at the endogenous ghrelin signaling nadir. Spontaneous freezing (a) and locomotion (b) were assessed in a novel context 24h after the last injection. A separate group of rats was assessed on the elevated plus maze in a single 8 minute session 24h after the final injection. Total arm entries (c) and open arm time (d) were measured to assess exploratory behavior and innate anxiety, respectively. (e) Animals received Pavlovian fear conditioning 24h before beginning daily handling injections of MK-0677 (Post FC MK:5d) or vehicle (Post FC VEH: 5d) for 5d. Fear memory was assessed by placing the animals in a novel context and measuring conditional freezing following tone presentation 24h after the last injection. All data are mean ±s.e.m.

Supplementary Figure 7. A single injection of ghrelin receptor agonist is not sufficient to enhance fear. (a) Rats received a single injection of saline (vehicle, VEH: 1d) or MK-0677 (MK: 1d) and received auditory Pavlovian Fear Conditioning 24h later. Fear to the tone was assessed 48h after fear conditioning. (b) Rats received a single injection of saline (vehicle, VEH) or MK-0677 (MK) and returned to the vivarium for 14d before receiving auditory Pavlovian fear conditioning. Fear to the tone was assessed 48h later. All data are mean ±s.e.m. ~ $p<0.10$ in planned comparisons.

Supplementary Figure 8. Prolonged ghrelin receptor agonism does not alter adrenal weights. Rats received a daily injections of saline (vehicle, VEH: 14d) or MK-0677 (MK: 14d) for 14 days. Adrenal glands were dissected perimortem 24h after the last injection. All data are mean ±s.e.m.

Supplementary Figure 9. Repeated intra-amygdala ghrelin infusions enhance fear memory. Rats were implanted with bilateral cannulae aimed at the basolateral amygdala (BLA). The arrow indicates the tip of the injector within the coronal brain section. Following recovery, intra-BLA infusions of either acylated ghrelin (GHR-Inf: 5d) or artificial cerebrospinal fluid (vehicle, VEH-Inf: 5d) were administered daily for five consecutive days and, 24h following the final infusion, auditory fear conditioning was administered. Fear memory was assessed during tone presentation in a novel context. Brain illustration adapted from (1) . All data are mean ±s.e.m. * $p<0.05$ in planned comparisons.

Supplementary Figure 10. Food restriction to 90% body weight increases exploratory locomotion in a novel context. Rats were food deprived for 15 days at 4g chow per 100g rat. When rats reached 90% of their initial body weight, they were placed in a novel context with a 3x3 grid floor. The number of border crossings was recorded. All data are mean ±s.e.m. * $p<0.05$ in planned comparisons.

Supplementary Methods

Food

A standard rat chow (Prolab RMH 3000, Purina, LabDiet, Richmond, Indiana, USA) was provided *ad libitum*. This high protein (25%): carbohydrate (52%) chow has a metabolizable energy content of 3.20 kcal/g. Food hoppers were maintained with at least 200g of chow and rats consumed up to 30g per day.

Adrenalectomy

Rats that were adrenalectomized were allowed to recover 1 week before being subjected to experimentation. Briefly, bilateral adrenalectomy was carried out through lateral incisions under 3.5% isoflurane anesthesia. Sham adrenalectomy was performed by making the incision through the skin and muscle and locating the adrenal glands. Muscle incisions were closed with chromic gut sutures and skin incisions were closed with wound clips. Some adrenalectomized rats were purchased from Taconic (Germantown, NY). Gland amputation was verified by dissection after removal and further verified by plasma corticosterone analysis.

Cannulae implants

Rats were anesthetized with a cocktail of 10mg/kg acepromazine, 100mg/kg xylazine, and 100mg/kg ketamine (1ml/kg; i.p.). Rats were mounted into a dual arm stereotaxic frame (Kopf Instruments; Tujunga, CA). The rats were then bilaterally implanted with 23-gauge stainless steel guide cannulae aimed 1mm above the lateral amygdala: A/P -2.0, M/L +/-5.3, D/V -5.4, relative to brain surface and bregma (1). The cannulae were secured by the placement of three jeweler

screws in the skull and dental acrylic. Dummy cannulae extending 1mm past the tip of the guide cannulae were placed into the guide cannula after surgery and changed every other day. Rats received 0.03mg/kg of Buprenex (1ml/kg; s.c.) as post-operative pain management every 12h for at least 24h and up to 3 days. All rats recovered for a minimum of 5 days before experimentation commenced.

Virus Preparation

For HSV-based viruses, plasmids were amplified to generate endotoxin-free DNA, which was transfected using Lipfectamine (Invitrogen; Carlsbad, CA) into 2-2 helper cells. The next day, GFP expression was verified and 2-2 cells were superinfected with 5*dl*1.2 helper virus. After two days, cells and media were harvested. The solutions were frozen and thawed three times, sonicated to release infectious viral particles, and centrifuged to clear the media of cell debris. The resulting supernatant was passaged onto 2-2 cells. This amplification procedure was repeated twice. After the final sonication and centrifugation, the supernatant was purified on a sucrose gradient, viral bands were centrifuged, and viral pellets were resuspended in 10% sucrose in D-PBS.

For AAV-based viruses, two cassettes were synthesized (Epoch Life Science; Missouri City, TX). The first was a 2657bp cassette containing the 650bp coding region of the rat GH gene (GenBank Accession number U62779.1) with a single amino acid substitution at position 120 (rGH-G120R) to create a mutant GH protein with antagonist activity at the GH receptor, termed rGHA (2, 3), followed by an IRES and enhanced green florescent protein (GFP). The second 1961bp cassette contained the IRES and GFP only. Each cassette was flanked by EcoRI and BglII sites. Following digestion, each cassette was subcloned into the pFB-AAV-CMV-

SV40pA (V032) AAV gateway plasmid from Virovek digested with EcoRI and BamHI. This yielded two constructs: pAAV-CMV-rGHA-IRES-GFP and pAAV-CMV-IRES-GFP.

Virus Infusions

For HSV amplicons, pulled glass pipettes were backfilled with silicone oil and the appropriate virus solution. The pipettes were mounted in stereotaxic barrel holder and the pipette plunger was placed against a custom-made apparatus designed to control the plunger via a syringe pump (Harvard Apparatus, Hollison, MA). Rats were anesthetized and mounted in a stereotaxic frame as described for cannulae implants. Small holes were drilled for intra-cranial placement of a pulled glass pipette aimed within the lateral amygdala: A/P -2.0, M/L +/-5.3, D/V -6.4, mm relative to brain surface and bregma (1). Virus was infused at 0.1µl/min for 20min (2µl total volume per side). The glass pipette remained in the brain for 10min before being withdrawn. Incisions were closed with wound clips and Buprenex was administered as for cannulae implants.

For AAV infusions, sterile microsyringes (Hamilton Company; Reno, NV) were backfilled with silicone oil and the appropriate virus solution. Rats were prepared for surgeries as described for HSV infusions. Each rat received two infusions of virus targeting each BLA (0.7µl: A/P -2.4, M/L +/-5.1, D/V -7.0, and 0.5µl: A/P -2.4, M/L +/-5.1, D/V -6.7) for a total of 1.2µl per BLA infused at 0.1µl/min (10min between infusions, and 10min after the second infusion). Incisions were closed with wound clips and Buprenex was administered.

Immobilization Stress

Immobilization stress was administered 4h per day for 1-14 consecutive days, depending on the experiment. Animals were placed in Decapicone plastic bags (Braintree Scientific; Braintree, MA), which were secured at the tail. Stress occurred in a lab room used for no other procedures. All stress sessions were performed between 10AM and 4PM. Unstressed control rats were handled daily for 30s. For further parallel control parameters, food was removed from the unstressed rats daily for the same 4h period in which stressed animals were immobilized and, therefore, without food.

Water Stress

Water stress was administered 1h per day for 14 consecutive days. Animals were placed in cages with room temperature water 3.8 to 5.1cm deep. All sessions were performed between 12pm and 2pm. Unstressed control rats were handled daily for 30s. For further parallel control parameters, food was removed from the unstressed rats daily for the same 1h period as above.

Pavlovian Fear Conditioning

Fear conditioning experiments were conducted in a modified chamber (MED Associates; St. Albans, VT) housed in a sound-attenuated cubicle. The animals were placed in individual chambers and infrared video of each session was recorded. Each experiment used auditory fear conditioning wherein rats received 3-5 tone (2kHz, 85dB, 10-16s)-footshock (1-2s, 0.4-0.7mA) pairings in a unique context (metal shock grid floors, chamber fan on, 0.3% Pine Sol odor, house and room lights on). Animals were allowed 2-3min to habituate to the chamber before tone-footshock pairings were given at intervals of 1-4min. Fear memory was tested 24-72h later by placing the animals in a novel context (white Plexiglas plastic floors, curved Plexiglas wall

inserts, fans off, 1% acetic acid odor, house and room lights off). One to 5min after placement in the novel context, fear to the tone was assessed either by presenting a continuous tone (2kHz, 85dB, 8min) or several discrete tones (fifteen 10-16s tones with 1-4min ISI). Freezing was measured using commercial software (VideoFreeze, Med Associates, St. Albans, VT).

Elevated Plus Maze

Rats were tested for anxiety using an elevated plus maze (Hamilton Kinder; Poway, CA). The maze had two open arms (51cm x 12cm each) and was located in a moderately lit room. Open arms consisted of black Plexiglas floors and no walls. The closed arms had black Plexiglas walls 40cm high. Animals were placed on an open arm 88cm above the ground facing away from the center of the maze. Automated software (Motor Monitor 4.14) recorded the second by second movements of the animals while the experimenter made observations in an obscured corner. Each session lasted 8min and both the observer and the software recorded the latency to exit the first arm and the number of entries into the open and closed arms. Additionally, the software recorded time spent in the each region of the maze. The maze was cleaned with 70% ethanol after each animal's session.

Trunk blood collection

Perimortem blood was collected from the trunk after decapitation in a tube which contained 1:100 v/v 0.5 M EDTA and 1:100 v/v HALT (Pierce; Rockford, IL). Immediately after collection, plasma was extracted by centrifugation (2,100g at 4°C for 10-15min). The plasma layer was then collected and half the volume treated with 10% v/v 1M HCl in order to stabilize the acylated form of ghrelin. Samples were stored at -20°C or -80°C.

Amygdala Cultures

Adult rats were anesthetized with an overdose of isoflurane. The brain was removed, briefly chilled in ice cold PBS, and 2mm coronal sections were obtained using a brain matrix (Braintree Scientific; Braintree, MA). A tissue punch (3mm diameter) was used to remove a circular section of each BLA, and each punch was coarsely minced. Each hemisphere of BLA mince was placed in 450µl of oxygenated artificial cerebrospinal fluid at 37ºC. From each rat, one hemisphere was treated with 50µl of sterile saline and the other hemisphere was treated with 50µl of 0.5mg/ml MK-0677. After one hour, the contents of each well were collected and spun briefly (4000rpm for 4min at 4 ºC). The supernatant was collected and stored at -80ºC.

Hormone Assays

For corticosterone, non-acidified plasma was diluted 1:25 in assay buffer 15 (Enzo Life Sciences; Farmingdale, NY). For acylated ghrelin, the acidified sample was used for the active ghrelin ELISA (Millipore; Billerica, MA) and processed according to the manufacturer's protocol. Samples were excluded from analysis if they displayed signs of hemolysis or lipemia. For growth hormone in brain tissue, brain tissue was homogenized 1:6 in lysis buffer and was assayed as per manufacturer's protocol (Millipore; Billerica, MA). For growth hormone released by short-term neuronal cultures, 10µl of the clarified supernatant was assayed as per manufacturer's protocol (Millipore; Billerica, MA). For CRF, brain tissue collected by micro-dissection was homogenized 1:30 w/v in lysis buffer and assayed per manufacturer's protocol (Kamiya Biomedical Company, Seattle, WA).

Supplementary References

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