Orexigenic response to tail pinch: role of brain NPY1 and corticotropin releasing factor receptors

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1CURE Digestive Diseases Research Center, Center for Neurobiology of Stress, Digestive Diseases Division, Department of Medicine, at University of California Los Angeles and Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California; 2Department of Internal Medicine and Institute for Neurogastroenterology at Martin-Luther Krankenhaus Berlin, Germany; and 3Charité Center for Internal Medicine and Dermatology, Division of General Internal and Psychosomatic Medicine and Charité-Universitätsmedizin Berlin, Berlin, Germany

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Goebel-Stengel M, Stengel A, Wang L, Taché Y. Orexigenic response to tail pinch: role of brain NPY1 and corticotropin releasing factor receptors. Am J Physiol Regul Integr Comp Physiol 306: R164–R174, 2014. First published December 11, 2013; doi:10.1152/ajpregu.00335.2013.—Tail pinch stimulates food intake in rats. We investigated brain mechanisms of this response and the influence of repeated exposure. Sprague-Dawley rats received acute (5 min) or repeated (5 min/day for 14 days) tail pinch using a padded clip. Acute tail pinch increased 5-min food intake compared with control (0.92 ± 0.2 vs. 0.03 ± 0.01 g, P < 0.01). This response was inhibited by 76% by intracerebroventricular injection of BIBP-3226, a neuropeptide Y1 (NPY1) receptor antagonist, increased by 48% by astressin-B, a corticotropin-releasing factor (CRF) receptor antagonist, and not modified by 5-406-028, a somatostatin subtype 2 antagonist. After the 5-min tail pinch without food, blood glucose rose by 21% (P < 0.01) while changes in plasma acyl ghrelin (+41%) and adrenocorticotropic hormone (+37%) were not significant. Two tail pinches (45 min apart) activate pontine and hindbrain catecholaminergic and hypothalamic paraventricular CRF neurons. After 14 days of repeated tail pinch, the 5-min orexigenic response was not significantly different from days 2 to 11 but reduced by 50% thereafter (P < 0.001). Simultaneously, the 5-min fecal pellet output increased during the last 5 days compared with the first 5 days (+58%, P < 0.05). At day 14, the body weight gain was reduced by 22%, with a 99% inhibition of fat gain and a 25% reduction in lean mass (P < 0.05). The orexigenic response to acute 5-min tail pinch is likely to involve the activation of brain NPY1 signaling, whereas that of CRF tends to dampen the acute response and may contribute to increased defecation and decreased body weight gain induced by repeated tail pinch.

body weight; corticotropin releasing factor; fecal pellet output; food intake; Fos; ghrelin; neuropeptide Y; stress-induced eating; somatostatin receptor 2 antagonist; tail pinch

THE IMPACT OF STRESS on eating behavior has recently received growing attention (43). Acute exposure to various stressors suppresses food intake of regular chow in rodents. For instance, we previously reported that acute intraperitoneal (ip) injection of the immune stressor lipopolysaccharide at a low dose (100 μg/kg) reduced the food intake response to a fast in rats (7). Similarly, a visceral stressor (abdominal surgery) or psychological stressors (restraint or novel environment) reduced the refeeding response to an overnight fast in rats (8, 45, 55). The underlying mechanisms of acute stress-related reduction of food intake are largely linked to the activation of corticotropin releasing factor (CRF) signaling pathways. It is well documented that various stressors including wrap restraint stress, low dose of lipopolysaccharide, or abdominal surgery activate the hypothalamic/CRF-pituitary-adrenal axis as shown by Fos or Fos/CRF colabeling in stress-responsive hypothalamic regions and the related elevation of circulating adrenocorticotropic hormone (ACTH) and corticosterone (8, 19). Moreover, CRF and the related peptides urocortin 1 or urocortin 2 injected centrally or peripherally reduce feeding via activation of brain CRF receptor subtypes 1 (CRF1) and 2 (CRF2) and peripheral CRF2 receptor in rats (48, 67, 71). Importantly, the blockade of CRF signaling pathways by CRF receptor antagonists inhibits stressors-induced reduction of food intake (10, 29, 48, 50). There is also evidence that acute stress inhibits gastric motility and emptying through CRF2 receptor-dependent central autonomic and peripheral myenteric systems contributing to gastric fullness and decreased food intake (59, 72).

While short exposure to various stressors typically suppresses food intake, acute (2–5 min) mild tail pinch reliably induces an immediate eating response in satiated rats and mice (4, 20, 46). The underlying mechanisms of tail pinch-induced prophagic effect have received attention primarily in relation with the role of brain dopamine and opiate pathways and their interactions (4, 20, 46). Other investigations on the influence of brain CRF signaling pathways in the tail-pinching feeding response have led to contradictory results (21, 46). By contrast, the possible involvement of interconnected brain orexigenic signalings namely those involving neuropeptide Y (NPY)/Y1 receptors (49, 54) or recently demonstrated somatostatin receptor 2 (sst2)/NPY1 (57) have not been explored. With regard to the sst2, we recently showed that intracerebroventricular (icv) injection of a sst2 agonist in satiated rats induces a rapid and robust food intake response through activation of NPY1 receptors (57). In addition, the intracerebroventricular injection of a selective sst2 antagonist in the dark phase increases food intake, suggesting that this pathway may participate in the nocturnal feeding in rats (57). Another approach to gain insight to brain mechanisms is the knowledge of specific nuclei that are activated by mild tail pinch. However, so far little is known except under conditions of tail pinch inducing vocalization (51), reflecting pain/distress (5).

Similar to acute stress, chronic stress can either reduce ingestive behavior and weight gain or increase caloric dense “comfort foods” and obesity in humans or experimental animals (12). Conditions of chronic unpredictable stress decreased
total caloric intake and body weight gain while maintaining adiposity in mice (39). In another report, chronic social defeat stress increased daily food intake in wild-type mice but not in ghrelin receptor knockout mice indicative of a key role played by the activation of ghrelin receptors in the orexigenic response to this psychological stress paradigm (35, 40). Although less studied, repeated tail pinch also resulted in either an increased or decreased food intake in rats (32, 44).

In the present study, we first investigated the molecular substrates and neurocircuits involved in the acute 5-min mild tail pinch-induced feeding response focusing on the orexigenic pathways, namely, the ghrelin-NPY-opioid circuit and sst2 pathways (54, 57, 66). This was achieved using selective antagonists of NPY1, BIBP-3226 (38), and sst2 receptor S-406-028 (9) injected intracerebroventricularly and the μ-opiate receptor antagonist naltxone along with the measurement of circulating ghrelin levels and blood glucose. We also assessed whether brain CRF signaling under the acute condition of tail pinch would modulate the 5-min feeding response using the intracerebroventricular injection of the potent peptide CRF antagonist astressin-B (42). In addition, we measured endocrine and neuronal markers of stress such as ACTH plasma levels and double labeling of Fos/CRF in the brain. Finally, we addressed the consequences of repeated daily 5-min tail pinch over a period of 14 days on food intake, body weight gain, body composition, and fecal pellet output as an index of stress-related visceral response (59).

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Harlan, San Diego, CA) weighing 280–350 g were group housed in pairs under controlled illumination (0600–1800 h) and temperature (21–23°C) until the start and between experiments. Animals had free access to standard rodent chow (Prolab RMH 2500; LabDiet, PMI Nutrition, Brentwood, MO) and tap water. Protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Administration (no. 99127-07). All experiments started between 0900 and 1000 h except when otherwise stated.

Substances

The μ-opiate receptor antagonist naltxone hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in saline. The NPY1 receptor antagonist BIBP-3226 (Sigma-Aldrich) and the CRF receptor (CRF1,CRF2) antagonist astressin-B [J. Rivier, Clayton Foundation Laboratories, Salk Institute, La Jolla, CA, synthesized as previously described (42)] were kept in powder form at −80°C and dissolved in pyrogen-free distilled water immediately before the experiment. The peptide sst2 antagonist H2N-pnO-Phe-DCys-Tyr-DAPh(Chm)-Lys-Thr-Cys-2Nal-NH2 (S-406-028) compound no. 4 in (9) [J. Rivier, Clayton Foundation Laboratories, Salk Institute, synthesized as previously described (9)] was stored in powder form at −80°C until dissolved in saline containing 0.1% bovine serum albumin immediately before use.

Procedures

Intracerebroventricular cannulation. Procedures for implanting the guide cannula into the right lateral cerebroventricle, intracerebroventricular injections of 10 μl and assessment of correct guide cannula placement were as detailed previously (55). Stereotaxic coordinates were based on Paxinos and Watson’s brain atlas (41). After surgery, animals were housed individually and allowed to recover for 7 days.

Tail pinch. The protocol was adapted from that initially described by Antelman et al. (4, 5). In a separate room, one rat at a time was placed in a single cage without bedding with access to four pre-weighed food pellets and given 10 min to accustom to the new environment. During that time, no feeding was observed. Thereafter, the rat was gently restrained by one investigator while a second investigator secured to the tail a padded small black metal binder clip (19 mm, Item 831594, model 10667-CC, Staples, Boston, MA) attached to a hand-held string. The size of the paper clip was chosen based on pilot experiments to exert moderate pressure on the tail that did not induce pain (assessed by the lack of vocalization, escape, or attack behaviors). The clip was positioned at a previously set mark located ~2.5 cm proximal to the tail tip. The rat with tail clip was immediately placed back in the cage and the timer was set to 5 min. Response to tail pinch was defined by induction of food licking, gnawing, shredding, and eating as previously described (37). Only rats that had been once identified as responders were included in the study. The ratio of responders to nonresponders was ~9:1 based on our observations of ~140 rats.

Transcardial perfusion and brain processing. Brains were harvested from rats deeply anesthetized with sodium pentobarbital (70 mg/kg ip, Nembutal, Abbott) after transcardial perfusion and processed as detailed previously (18). Coronal sections (25 μm) from the bregma level to the end of the medulla [+2.04 to −15.96 mm according to Paxinos and Watson’s atlas, (41)] were cut using a cryostat (Microm International, Walldorf, Germany).

Fos immunohistochemistry and double labeling for CRF. All brain sections were processed at the same time to assess immunoreactivity of Fos and CRF by two consecutive cycles of immunohistochemical staining. Both anti-Fos (Catalog No. PC 38, Oncogene, Cambridge, MA) and the previously validated anti-CRF serum (CURE 200101) (65) were both diluted at 1:10,000. The procedures were the same as described previously (65). Fos and CRF-immunoreactive (ir) cells were observed by light microscopy (Axioskop II, Carl Zeiss, Jena, Germany). Cells with dark blue nuclear staining were Fos-ir and cells with strong brown cytoplasmic staining were CRF-ir. For quantitative assessment, the number of immunoreactive cells was counted unilaterally using the following coordinates (mm from bregma) in the supraoptic nucleus (11 sections from −0.72 to −1.56), parvicellular subdivision of the paraventricular nucleus of the hypothalamus (pvPN, 5 sections from −1.32 to −1.92), central and medial amygdaloid nucleus (10 sections from −2.16 to −2.92), lateral hypothalamus (8 sections from −1.8 to −2.52), arcuate nucleus (5 sections from −2.16 to −3.24), dorsomedial hypothalamus (8 sections, from −2.92 to −3.48), locus coeruleus (7 sections, from −9.48 to −10.08), raphe pallidus (10 sections, from −11.64 to −12.36), medial nucleus of the solitary tract (8 sections, from −13.56 to −14.16), and ventrolateral medulla (8 sections, from −13.68 to −14.16). Images were acquired by a digital camera (Hamamatsu, Bridgewater, NJ) using the image acquisition system SimplePCI (Hamamatsu, Sewickley, PA). The average number of single- or double-labeled Fos-ir and CRF-ir cells/section of each animal was calculated unilaterally as described before (65). Since no consecutive sections were used for the detection of the same neuronal marker, no corrections for double counting were applied. The investigator was blinded to the treatment.

Measurements

Plasma acyl and total ghrelin. Immediately after decapitation, blood was processed according to the recently developed RAPID method as detailed before (58). The lyophilized sample was stored at −80°C until further processing. Samples were resuspended in double-distilled water (ddH2O) immediately before radioimmunoassay according to the original plasma volume of 260 μl, and duplicates were used to determine total and acyl ghrelin levels using specific radioimmunoassays (Catalog No. GHRT-89HK and GHRA-88HK, respectivley, 100% cross reactivity with rat acyl and total ghrelin, respec-
tively, Millipore, Billerica, MA). Radioimmunoassay was performed in one batch and the intra-assay variability was 2%. Desacyl ghrelin was calculated as the difference from total minus acyl ghrelin values for each individual sample.

**Plasma ACTH.** Immediately after decapitation, trunk blood was processed according to standard protocols as described in our previous studies (19). The resulting lyophilized peptide power was dissolved in radioimmunoassay buffer according to the original plasma volume of 260 μl immediately before measurements. Duplicates were used to determine ACTH levels using a specific radioimmunoassay (Catalog No. RK-001-21, 100% cross reactivity with rat, mouse, and human ACTH, Phoenix Pharmaceuticals). Intra-assay variability was 5%. The detection range of the assay was 10–1,280 pg/ml and the lowest limit 34.1 pg/ml.

**Blood glucose.** Glucose levels were measured with commercial test strips (One-Touch Ultra; LifeScan, Milpitas, CA) immediately after collecting one drop from trunk blood after decapitation without anesthesia.

**Body composition by magnetic resonance imaging.** Conscious rats were placed lightly restrained (<1 min) in the holder tube of the quantitative nuclear magnetic resonance analysis apparatus (ECHO MRI, 3-1 Composition Analyzer; 150 Echo Medical Systems, Houston, TX). The body composition (fat mass, lean mass, and total water) was measured as described before (53).

**Experimental Protocols**

Except otherwise stated, all experiments were performed in freely fed rats pair-housed and trained for single housing (2 h per day) once daily for 10 days before experiments. On the day of the experiment, animals were separated in single cages for 15 min before the tail pinch to get them accustomed to the new environment.

**Effect of intracerebroventricular injection of NPY₁ antagonist BIBP-3226, sst₂ antagonist S-406-028, CRF antagonist astressin-B, and intraperitoneal injection of naloxone on acute tail pinch-induced food intake.** Rats with a chronically implanted intracerebroventricular cannula and single-housed postsurgeries were accustomed to the intra-cerebroventricular injection procedure by light hand restraint for 5 days. Rats without intracerebroventricular cannula that received intraperitoneal injections were handled daily for 10 days before experiments to practice dummy injections in the backward position. Rats were injected intracerebroventricularly (5 μl) with BIBP-3226 (10 or 30 μg/rat), S-406-028 (1 μg/rat), astressin-B (30 μg/rat), or the respective vehicle. Naloxone (5 mg/kg body wt) or vehicle (saline) was injected intraperitoneally (300 μl). After injection, rats were placed back in a single cage, and at 15 min after BIBP-3226 and astressin-B, 30 min after S-406-028, or 20 min after naloxone, they were subjected to a 5-min tail pinch or left undisturbed. Food was measured by calculating the difference between the preweighed food pellets before and after the 5-min tail pinch. The injection times and injection doses of antagonists were based on previous studies showing the blockade of intracerebroventricular pan-oligosomatostatin agonist, ODT8-SST-induced orexigenic response by intracerebroventricular S-406-028, or BIBP-3226 and intraperitoneal naloxone (54), and intracerebroventricular CRF-induced delayed gastric emptying by intracerebroventricular astressin-B in rats (36). The blockade of intracerebroventricular NPY₁-induced feeding by intracerebroventricular BIBP-3226 and the reduction of dark phase food intake by naloxone at such dose have been previously demonstrated by others (26, 38).

**Effect of acute tail pinch on blood glucose and ghrelin and ACTH plasma levels.** At the start of the tail pinch, rats had no access to water but were given food pellets that were immediately removed after the first bite. This was done to ensure that the tail pinch would induce an eating response while avoiding food interference with hormone or blood glucose levels. Control animals were left in pairs undisturbed in their home cages except for a reduced amount of bedding with free access to food and water (control animals did not ingest food and water during the 5-min experimental period). Animals were decapi
tated immediately after the 5-min tail pinch, and trunk blood was collected. Control animals were euthanized at the same time. Blood was processed for radioimmunoassay measurements of plasma ACTH, acyl, and total ghrelin levels except one drop that was taken to measure blood glucose.

**Effect of acute tail pinch on Fos and CRF immunoreactivity in brain nuclei.** Rats were subjected to two 5-min acute tail-pinch sessions at a 45-min interval and had no access to water but to food pellets that were immediately removed after the first bite. This was done to avoid Fos induction in brain centers connected to the feeding response or stomach distension. As described originally by Antelman et al. (3), tail-pinched rats after removal of food pellets showed agitation reminding of food seeking behavior that lasted as long as the tail-pinch stimulus was in place. In between tail pinches, rats were placed back together with their mates with access to water but not food. Control animals were left undisturbed in pairs in their home cages except for a reduced amount of bedding with free access to food and water (control animals did not ingest food during the experimental period). Hundred minutes from the beginning of the first tail pinch, brains were harvested and processed for Fos/CRF immunohistochemistry. The time point of Fos monitoring was based on the established peak of Fos protein response occurring between 90 and 120 min after the start of the stimulus (28), and the two tail-pinch sessions were selected to enhance Fos expression (51).

**Effect of daily tail-pinch session for 14 days on food intake, body weight, and composition and fecal pellet output.** Rats were subjected to a 5-min tail pinch per day for 14 consecutive days. Control animals underwent the same procedure (15 min single housing in a new cage without tail pinch). During the daily 5-min pinch, food intake and fecal pellet output were measured. Rats had no access to water but to food pellets during the tail-pinch period. Food intake was determined by calculating the difference between the preweighed food pellets before and after tail pinch. Animals were subjected to magnetic resonance imaging before the first and after the last tail pinch on day 14. Body weight and cumulative food intake was recorded daily.

**Statistical Analysis**

Data are expressed as means ± SE and analyzed by one-way ANOVA followed by Tukey post hoc test or two-way ANOVA followed by Holm-Sidak method. Correlations were assessed by linear regression analyses. P < 0.05 was considered significant.

**RESULTS**

**Effects of Intracerebroventricular Injection of NPY₁, sst₂, and CRF Receptor Antagonists and Intraperitoneal Naloxone on 5-min Tail Pinch-Induced Eating**

The 5-min food intake in rats injected intracerebroventricularly with vehicle during the light phase was low (0.03 ± 0.01 g, n = 7, Fig. 1) and not significantly different after the intracerebroventricular injection of NPY₁ receptor antagonist BIBP-3226 (30 μg; 0.0 ± 0.0 g, n = 8, Fig. 1A), CRF receptor antagonist astressin-B (0.03 ± 0.02 g, n = 6, Fig. 1B), or sst₂ antagonist (0.02 ± 0.01 g, n = 7, Fig. 1C) and the intraperitoneal injection of naloxone (0.0 ± 0.0 g, n = 5 vs. intraperitoneal vehicle, 0.3 ± 0.1 g, n = 7, Fig. 1D).

The 5-min tail pinch in intracerebroventricular vehicle-injected rats induces a robust increase in food intake compared with intracerebroventricular vehicle-injected undisturbed rats (0.92 ± 0.2 vs. 0.03 ± 0.01 g, n = 12–16; P < 0.01, Fig. 1, A–D). The feeding response to tail pinch was significantly inhibited by 76% by intracerebroventricular BIBP-3226 (0.22 ± 0.09 g, n = 7, Fig. 1A), increased by 48% by intracerebroventricular astressin-B
Effects of Acute-Tail Pinch on Levels of Blood Glucose and Plasma Ghrelin and ACTH

Immediately after the end of the 5-min tail pinch without food, blood glucose was significantly elevated (131.6 $\pm$ 5.3 mg/dl, $n = 5$ group, $P < 0.01$; Fig. 2A), whereas plasma levels of ACTH (Fig. 2B), acyl ghrelin (Fig. 2C), and desacyl ghrelin (Fig. 2D) showed a nonsignificant trend to increase by 37%, 41%, and 35%, respectively, at 5 min.

Acute Tail Pinches Induce Fos Immunoreactivity in Distinct Fore- and Hindbrain Nuclei and in CRF-ir Neurons of the pPVN

Fos immunostaining was low in the forebrain and hindbrain of undisturbed freely fed control rats (Fig. 3, A, D, G, and K; 4, A, D, and G; Fig. 5A, and Supplemental Table S1). Acute tail-pinch

(1.72 $\pm$ 0.36 g, $n = 6$, Fig. 1B), while intracerebroventricular sst2 antagonist had no effect (0.83 $\pm$ 0.25 vs. 0.68 $\pm$ 0.16 g, $n = 8$, Fig. 1C). Tail pinch-induced eating showed a trend toward reduction by intraperitoneal naloxone ($-35\%$: 0.64 $\pm$ 0.31 vs. 0.99 $\pm$ 0.33 g, $n = 7$, $P = 0.23$; Fig. 1D), which did not reach significance.

Acute 5-min tail pinch-induced eating is blocked by intracerebroventricular (icv) injection of neuropeptide Y1 (NPY1) antagonist BIBP-3226 (A), and enhanced by icv corticotropin releasing factor (CRF) antagonist astressin-B (B), whereas intraperitoneal (ip) opiate antagonist naloxone (C) or icv somatostatin 2 receptor (sst2) antagonist (D) have no effect in rats. The 5-min food intake in undisturbed rats during the light phase is low. The 5-min mild tail pinch strongly increases the 5-min food intake compared with undisturbed rats (A–D). Data are means $\pm$ SE of number of rats/group indicated at the bottom of the columns. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. vehicle/undisturbed; #|$P < 0.05$, ##|$P < 0.01$ and ###|$P < 0.001$ vs. antagonist/undisturbed; †|$P < 0.05$ vs. antagonist high dose/tail pinch, ‡|$P < 0.05$ vs. vehicle/tail pinch.

Fig. 1. Acute 5-min tail pinch-induced eating is blocked by intracerebroventricular (icv) injection of neuropeptide Y1 (NPY1) antagonist BIBP-3226 (A), and enhanced by icv corticotropin releasing factor (CRF) antagonist astressin-B (B), whereas intraperitoneal (ip) opiate antagonist naloxone (C) or icv somatostatin 2 receptor (sst2) antagonist (D) have no effect in rats. The 5-min food intake in undisturbed rats during the light phase is low. The 5-min mild tail pinch strongly increases the 5-min food intake compared with undisturbed rats (A–D). Data are means $\pm$ SE of number of rats/group indicated at the bottom of the columns. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. vehicle/undisturbed; #|$P < 0.05$, ##|$P < 0.01$ and ###|$P < 0.001$ vs. antagonist/undisturbed; †|$P < 0.05$ vs. antagonist high dose/tail pinch, ‡|$P < 0.05$ vs. vehicle/tail pinch.

Fig. 2. Acute tail pinch increases blood glucose (A) while not significantly influencing plasma adrenocorticotropic hormone (ACTH) (B), acyl ghrelin (C), and desacyl ghrelin (D) levels in rats monitored at the end of the 5-min tail pinch. Data are means $\pm$ SE of number of rats/group indicated at the bottom of the columns. **$P < 0.01$ vs. control.
sessions (5-min twice at a 45-min interval) markedly increased the number of Fos-positive cells monitored at 100 min after the beginning of the first tail-pinch exposure in several specific brain sites, namely, in the lateral hypothalamus (34.3 ± 9.2 vs. 2.4 ± 1.0, P < 0.05; Fig. 3, B and C), arcuate nucleus (57.9 ± 7.9 vs. 8.3 ± 1.1, P < 0.001; Fig. 3, E and F), medial amygdaloid nucleus (120.1 ± 6.4 vs. 5.7 ± 1.6, P < 0.001; Fig. 3, H and I), dorsomedial hypothalamus (68.3 ± 9.0 vs. 5.1 ± 2.5, P < 0.001; Fig. 3, D, G, and K).

Fig. 4. Acute tail pinch induces Fos immunoreactivity in distinct hindbrain nuclei. Rats were subjected to two 5-min tail-pinch sessions 45 min apart from each other or left undisturbed and 100 min after the beginning of the stress transcardially perfused. Fos immunostaining was low in the hindbrain of undisturbed freely fed control rats (A, D). Tail pinch increased Fos expression in the locus coeruleus (LC, B) and ventrolateral medulla (VLM, E). Unilateral Fos ir cell count/section showed differences in the LC (C) and VLM (F). Data are means ± SE of 4 rats/group. ***P < 0.001 vs. control. The scale bar represents 100 μm. 3V, 3rd brain ventricle; f, fornix; ME, median eminence; opt, optic tract; BLA, basolateral amygdaloid nucleus; CeA, central amygdaloid nucleus.
Fig. 3, L and M), locus coeruleus (48.5 ± 8.0 vs. 0.4 ± 0.3, P < 0.001; Fig. 4, B and C) and ventrolateral medulla, also known as A1/C1 (13.6 ± 1.5 vs. 1.3 ± 0.4, P < 0.001; Fig. 4, E and F). In the supraoptic nucleus (32.0 ± 13.8 vs. 6.0 ± 1.6, P > 0.05), raphe pallidus (8.3 ± 2.7 vs. 2.5 ± 0.7, P > 0.05), and medial division of the nucleus of the solitary tract (30.3 ± 3.9 vs. 14.4 ± 7.8, P > 0.05) there was a nonsignificant trend toward increased Fos expression in tail-pinched rats (Supplemental Table 1). There is no Fos induction in the nucleus accumbens, striatum, or the ventral tegmental area (Supplemental Table 1).

CRF-ir neurons (number/section) were prominently localized in the pPVN (101.4 ± 10.8; Fig. 5A) and central amygdaloid nucleus (36.3 ± 1.6; Fig. 5E). Rats exposed to tail pinches had a similar number of CRF-positive neurons in the pPVN and central amygdaloid nucleus to that of the control group (Fig. 5, A and B and E and F). Tail pinches significantly increased the number of Fos-ir cells in the pPVN (69.1 ± 11.5 vs. 3.7 ± 1.9, P < 0.01; Fig. 5, B–D) but not in the central amygdaloid nucleus (2.0 ± 0.7 vs. 0.2 ± 0.1, P = 0.05; Fig. 5, F–H). Double-labeled cells for Fos and CRF were significantly increased in the pPVN (26.9 ± 3.4 vs. 0.1 ± 0.1, P < 0.001; Fig. 5, B–D) but not in the CeA (0.3 ± 0.1 vs. 0.0 ± 0.0, P = 0.05; Fig. 5, F–H).

Repeated Tail Pinches Blunt the Orexigenic Response and Decrease Body Weight Gain Associated With Reduced Lean and Fat Mass

There was no difference in the first 24-h cumulative food intake between undisturbed and 5-min tail-pinched animals (19.9 ± 0.4 vs. 19.7 ± 0.4 g, P > 0.05). When rats were pinched daily for 14 days, the 5-min food intake response was similar during the first 5 days. This was followed by a linear decrease that reached significance on day 12 with a 50% reduction of the food intake response versus day 1 that was maintained at this level up to day 14 (P < 0.001, Fig. 6, A and B). Repeated tail pinches over 14 days did not result in behavioral conspicuous (assessed by the lack of vocalization, escape, or attack behaviors). The tail form and skin also appeared normal. Repeated tail pinches did not change the 14-day cumulative food intake, although a trend toward a reduction was noted (283.5 ± 5.4 vs. 291.3 ± 1.7 g, Fig. 7A) while reducing body weight gain by 22% (33.9 ± 3.2 vs. 43.6 ± 3.2 g, P < 0.05, Fig. 7, B and C). This was associated with the inhibition of fat gain (0.02 ± 0.52 vs. 1.62 ± 0.43 g, P < 0.05), reduction of lean mass (24.3 ± 2.5 vs. 32.3 ± 2.5 g, P < 0.05), and no significant change in total water compared with undisturbed controls as monitored on day 14 (Fig. 7C).

Repeated Tail Pinches Increase Fecal Pellet Output

When rats were pinched 5 min daily for 14 days, the 5-min fecal pellet output increased during days 10–14 compared with days 1–5 (+58%, P < 0.05, Fig. 8A) with a negative correlation of 5-min food intake and fecal pellet output (r = −0.56, P < 0.05, Fig. 8B).

DISCUSSION

In the present study, acute mild tail pinch robustly increases food intake with an immediate onset of action in freely fed rats consistent with previous reports in rats showing facilitation of behavior related to the incentive present in the environment (2, 4, 21, 46, 51). NPY neurons located in the arcuate nucleus are a major hypothalamic component mediating the signals of orexigenic peptides (47). Opioid receptors have also been implicated in the central regulation of feeding (31), especially the hedonic rather than nutritional drive to ingest food, indicating a role in the rewarding aspect of eating and the maintenance of NPY-induced feeding (17). We show that intracebroventricular pretreatment with the NPY1 antagonist BIBP-3226, inhibited the 5-min tail pinch-induced increase in food intake by 76%. BIBP-3226 used at the same dose was shown previously to have no effect on 2-h food intake while com-
completely preventing intracerebroventricular NPY-induced stimulation of light phase feeding in rats (39). These data indicate that a large component of the orexigenic response to tail pinch may involve activation of Y1 signaling not previously described.

We recently found that sst2 activation in the brain also induced a rapid onset phasic effect that is mediated by brain NPY1-opioid pathways in freely fed rodents (54, 57). However, the selective peptide sst2 antagonist failed to alter the 5-min tail pinch-induced eating when injected intracerebroventricularly under similar dosing conditions blocking the robust 2-h eating response to intracerebroventricular pan-somatostatin agonist ODT8-SST icv (14, 54). These data ruled out a role of orexigenic sst2 signaling upstream of NPY1 pathways. In addition, under our experimental conditions, the blockade of opioid receptors did not reproduce the magnitude of suppression of the feeding response observed with the intracerebroventricular injection of the NPY1 receptor antagonist. Naloxone (5 mg/kg, ip) pretreatment resulted in a nonsignificant trend to decrease the 5-min feeding response to tail pinch while completely suppressing the eating induced by intracerebroventricular ODT8-SST under otherwise similar conditions (54). The ability of naloxone to modulate tail pinch-induced feeding seems to vary between reports since naloxone or naltrexone (4 mg/kg, peripheral) was found to have either no effect (3, 27), partially reduce (34), or block (37, 46) acute tail pinch-induced eating in rats. These conflicting findings may be related to different modalities of tail-pinch procedures recruiting different pathways (30 s vs. 2–5 min and/or different clips). However, naloxone or a μ-selective opioid antagonist microinjected directly into the substantia nigra reduced the 4-min tail pinch-induced food intake by 80% without influencing pain sensitivity (20). This provides insight to potential local μ-opioid mechanisms involving the nigrostriatal dopaminergic system well established to play a role in the orexigenic response in this model (4, 5, 20, 33, 46).

Among physiological mechanisms of NPY activation, acyl ghrelin, which is the only peripherally produced orexigenic peptide (for review see Ref. 56), stimulates feeding in the fed state by activating NPY-containing neurons in the arcuate nucleus (23, 66). In the tail-pinch model, the rapid onset of eating response (within seconds) and the nonsignificant changes in circulating ghrelin at the end of the 5-min tail pinch, do not support a primary role of circulating ghrelin. However, under conditions of two tail pinches, we observed the activation of arcuate neurons as shown by the robust Fos induction at 100 min after the first tail pinch. Although no double labeling was performed, the localization of Fos immunoreactivity within the arcuate nucleus is not confined to the NPY-expressing neurons localized in the ventromedial part (66) but may also encompass the anorexigenic pro-opiomelanocortin-containing neurons reported to be located more dorsally (68). Retrograde transsynaptic transport of pseudorabies virus studies have revealed that the lateral hypothalamus relayed projections from lateral arcuate pro-opiomelanocortin neurons to the insular and anterior cortex and thalamic nuclei along with NPY/Agoouti-related peptide neurons to the accumbens shell (25). Based on these retrograde tracing studies, the lateral hypothalamus emerged as part of one integrative center of energy status and pathways directed to reward and the cognitive aspect of food intake (25). Of relevance, we also observed an increase in Fos expression in the lateral hypothalamus.

Fig. 6. Daily 5-min tail pinch for 14 days results in a blunted orexigenic response. When rats were pinched daily for 14 days, the 5-min food intake was highest during the days 1–5 and lowest during days 10–14 (A, B). Data are means ± SE of 8 rats/group. *P < 0.05 and ***P < 0.001 vs. days 1–5.

Fig. 7. Daily 5-min tail pinch for 14 days did not influence the cumulative food intake (A) while reducing body weight gain (B, C) due to decreased lean and fat mass (C). Data are means ± SE of 8 rats/group. *P < 0.05 vs. control.
following tail pinch as well as within the insular cortex and cingulate cortex, recently identified as the primary taste cortex (16, 25). Finally, the perifornical area within the lateral hypothalamus receives prominent NPY-ergic input from the arcuate nucleus (13), and the strongest feeding response to NPY can be elicited by local injection into the perifornical area (52). Collectively, present data and previous functional studies point to several brain regions and transmitters that may be involved in the mediation and/or neuronal modulation of the rapid onset and robust orexigenic response following acute tail pinch.

Whether circulating ghrelin plays a role in Fos expression in the arcuate nucleus under these conditions as demonstrated previously with peripheral injection of ghrelin (66) is worth further investigation. Indeed plasma acyl ghrelin showed a trend to increase (41%) within 5 min of exposure to tail pinch. Consistent reports also indicate that exposure to a novel environment induces a rise in circulating ghrelin levels monitored 1 h later (60). In addition, repeated tail pinches in mice increase gastric ghrelin gene expression (6).

Besides the induction of Fos in hypothalamic feeding regulatory centers such as the arcuate nucleus and lateral hypothalamus, we also observed a robust expression in pontine and medullary catecholamine-containing neurons including the locus coeruleus and ventrolateral medulla. This is indicative of sympathetic nervous system activation (1). In particular excitation of neurons in the ventrolateral medulla leads to hyperglycemia in rats (64). This may provide neuroanatomical substrata for the rise in blood glucose occurring at 5 min after tail pinch as reported previously after a 2-min tail-pinch exposure (32).

Increased activity of catecholaminergic neurons is well known to activate limbic and hypothalamic CRF neurons (11, 70). Using neuroanatomical and pharmacological approaches, we observed evidence that activation of hypothalamic CRF signaling modulates food intake induced by tail pinch. Two repetitive tail-pinch stimuli induce Fos expression in CRF-containing neurons of the pPVN, unlike the central amygdaloid nucleus, indicative of selective activation of the hypothalamic CRF signaling system. At the end of the 5-min tail pinch, plasma levels of ACTH started to increase (+37%) without reaching statistical significance. This may be due to the early timing of the measurement as ACTH is known to reach a peak in the circulation at 20 min following CRF injection in rats or an immunological stressor such as cytokine injection (63). Moreover, the CRF antagonist astressin-B injected intracerebroventricularly significantly increased the tail-pinch induced feeding response by 48%. Previous studies showed that intracerebroventricular pretreatment with the CRF antagonist α-helical CRF(9–41) enhanced the duration of eating during the 5-min tail pinch and the food intake response to the intracerebroventricular injection of NPY (21). Moreover, the PVN has been identified to be the selective site of CRF-induced curtailing of the feeding response to NPY (22). Collectively, these data would be consistent with CRF neurons being activated in the PVN by tail pinch and dampening the NPY-mediated feeding response.

In the chronic study, repeated tail pinch for 5 min/day reproducibly increased the 5-min food intake at each daily trial during the 14-day experimental period. However, the magnitude of eating response was blunted over time as shown by the significant 50% reduction during days 12–14 compared with the similar food ingestion occurring during the first 5 days. In addition, the repeated 5-min daily tail pinch reduces body weight gain after 8 days compared with undisturbed rats. Likewise, in an earlier report, the use of a more frequent tail-pinch paradigm (5-min tail pinch, 12 trials/day for 10 days) resulted in a 50% reduction of food intake throughout the tail-pinch period and body weight gain in rats (32). Therefore, daily tail pinch for 5 min either once or repeatedly, while inducing hyperphagia during the trial, impairs the body weight gain thereafter. Magnetic resonance imaging analysis indicates that the decreased body weight is related to the inhibition of fat gain (−99%) and reduction of lean mass (−25%) compared with undisturbed rats.

Interestingly, the fecal pellet output during tail pinch showed an opposite pattern: it was lowest during the first 5 days and higher during days 10–14 (+58%), resulting in a negative correlation of 5-min food intake and fecal pellet output. The activation of brain CRF signaling pathways has been involved in various acute stressors-induced stimulation of defecation (61), inhibition of food intake (29, 48, 50) and body weight gain (10, 50). Taken together these data will be consistent with a more prominent involvement of the CRF pathway under conditions of consecutive 5-min daily tail pinch to dampen the orexigenic response. Likewise, other chronic stress models did not result in the induction of obesity. For example, repeated daily exposure to short-term restraint stress over a 5-wk period led to body weight reduction in Wistar rats (30). Social stress resulting from dominant-subordinate relationships in male rats is associated with body weight loss and altered body composition in the subordinate animal (62). In another study
C57BL/6 mice that were subjected to chronic social/overcrowding stress with access to high-fat diet for 6 wk displayed an inhibition of increase in weight gain and caloric intake (15).

**Perspectives and Significance**

The present study unraveled additional brain signaling systems recruited by acute mild tail pinch, besides the reproducibly established nigrostriatal and mesocorticolimbic dopamine systems (4, 5, 20, 33, 46). In particular, it may be speculated that tail pinch activates circuitries specific for the appetite urge namely the hypothalamic arcuate-PVN NPY1 pathways stimulating feeding (24) and concomitantly the arcuate-lateral hypothalamic-pro-opiomelanocortin and PVN/CRF pathways that modulate the magnitude of eating response.

Humoral signals such as circulating ghrelin do not seem to be involved in the immediate acute response; however, the contribution of this hormone in the enhanced eating response to a second tail-pinch trial (32) deserves consideration in view of its release by stressors (60) and its trend to increase plasma ghrelin at the end of the 5-min tail pain observed in the present study. Additionally, the CRF signaling system activated in the PVN by acute tail pinch may predominate after repeated daily exposure, which could explain the 50% dampening of the food intake response, increased defection, and decreased body weight gain prominently observed during the last 5 days of the 14 days experimental period. Therefore, acute tail pinch is a suited model to study underlying multiple mechanisms that sensitize behavioral responsiveness to food while daily exposure to tail pinch leads to decreased fat and lean mass gain similar to other repeated stressors. However, of interest, one report indicated that when six tail pinches/day lasting 10–15 min each for a period of 5 days were applied in presence of sweetened milk, this led to body weight gain in rats (44). In the context of present data and previous reports (32, 44), it can be speculated that high caloric intake is a determinant component for repeated tail-pinch stress to induce obesity. Indeed palatable diet is associated with a reduction in the endocrine response to chronic stress and the increase in comfort food in turn increases body weight (69). Whether repeated tail pinch will alter food preference warrants further investigations.

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