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Neuropeptide Y mRNA and serotonin innervation in the arcuate nucleus of *anorexia* mutant mice

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Abstract

The *anorexia* (*anx*) mutation causes reduced food intake in preweanling mice, resulting in death from starvation within 3–4 weeks. In wild-type rodents, starvation induces increased neuropeptide Y (NPY) mRNA levels in the arcuate nucleus that promotes compensatory hyperphagia. Despite severely decreased body weight and food intake at 3-weeks age, *anx/anx* mice do not show elevated NPY mRNA levels in the hypothalamic arcuate nucleus compared to wild-type/heterozygous littermates. The NPY mRNA levels can be upregulated in normal mice at this chronological age, because 24-h food deprivation increased arcuate NPY mRNA in wild-type littermates. The unresponsiveness of NPY expression in the arcuate of *anx/anx* mice was paralleled by serotonergic hyperinnervation of the arcuate nucleus, comparable to the serotonergic hyperinnervation previously reported in the rest of the *anx/anx* brain. This result is consistent with the hypothesis that wasting disorders are accompanied by disregulation of NPY mRNA expression in the arcuate nucleus, and suggests that reduced food intake, the primary behavioral phenotype of the *anx/anx* mouse, may be the result of altered hypothalamic mechanisms that normally regulate feeding. © 1998 Elsevier Science B.V.

Keywords: Wasting disorder; Failure to thrive; Feeding; Food deprivation; Ontogeny; Gene expression; Hypothalamus; 5-Hydroxytryptamine

1. Introduction

The *anorexia* (*anx*) mutation results in a wasting disorder in preweanling mice [15]. By the second postnatal week, *anx/anx* mice begin to have decreased food intake and body weights relative to wild-type littermates. Death due to starvation usually occurs in week 3 or 4, at which time the body weight of *anx/anx* mice is less than half the weight of wild-type littermates. Among the neurological deficits of *anx/anx* mice are hyperactivity, body tremors, and head weaving. The molecular nature of the mutation is unknown, nor have the altered peripheral or brain mechanisms been identified to cause the decreased food intake and weight gain. Increased levels of serotonin (5-HT) have been implicated: the serotonin antagonist 5,7-dihydroxytryptamine partially alleviates the *anx* symptoms, while increasing serotonin brain levels in wild-type littermates by administering the serotonin precursor 5-hydroxy-DL-tryptophan replicates some of the *anx* phenotype [15]. Consistent with these results, our laboratory has found a large increase in the number and density of serotonergic fibers in the forebrain of *anx/anx* mice compared to wild-type littermates [25]. The link between increased brain serotonin innervation and the disruption of normal feeding in *anx/anx* mice is unknown.

The normal response to decreased food intake and body weight is a compensatory hyperphagia. This response appears to be absent in *anx/anx* mice. In many disease states, such as cancer and AIDS, there is also a progressive weight loss without a compensatory increase in food intake. It has recently been proposed that the normal hypothalamic mechanisms of food intake are disrupted or downregulated in human diseases and in animal models of wasting disorders, thus blocking compensatory hyperphagia [21]. Because the *anx/anx* mouse appears to suffer from decreased food intake and wasting comparable to human infant 'failure to thrive' syndrome [6], it is possible that the normal hypothalamic mechanisms controlling food intake have been disrupted by the *anx* mutation.

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One of the best characterized hypothalamic mechanisms that regulates food intake after starvation is upregulation of neuropeptide Y (NPY) mRNA levels in the cell bodies of the arcuate nucleus [2,23,28] and the subsequent accumulation and release of NPY protein in the paraventricular nucleus (PVN) [14,20]. Disregulation of NPY synthesis in the arcuate is correlated with behavioral feeding disorders. Thus, in genetically obese rodents that overeat (e.g., *ob / ob* mice, *db / db* mice, and *fa / fa* rats), NPY mRNA in the arcuate is overexpressed compared to non-obese controls [22,29]. Conversely, in rats made anorexic by implantation of a Yoshida sarcoma tumor, NPY protein levels in the PVN were lower than in normal controls, while NPY protein levels in the PVN of food-restricted controls were elevated [17].

The *anx / anx* mouse provides another test of the arcuate NPY hypothesis. If the arcuate NPY system is normally regulated in the *anx / anx* mouse, then the weight loss in the second and third postnatal week should induce an increase in NPY mRNA in the arcuate nucleus compared to non-deprived wild-type littermates. In this case, the lack of normal food intake might be due to disruption of factors subsequent to or parallel to the synthesis of NPY in the arcuate. If, however, the mechanisms regulating NPY synthesis in the arcuate are disrupted, then the starving *anx / anx* mouse should show normal or reduced levels of NPY mRNA compared to wild-type controls. To test these predictions, we used in situ hybridization to quantify the level of NPY mRNA expression in the arcuate nucleus of *anx / anx* mice compared to wild-type littermates in the third postnatal week. As a positive control, wild-type littermates were 24-h food-deprived. Others have shown that acute food deprivation and chronic food restriction (as experienced by *anx / anx* mice) produce comparable increases in arcuate NPY mRNA [2].

Because serotonergic hyperinnervation has been previously described in other brain regions of the *anx / anx* mouse [25] but not in the hypothalamus, we also quantified serotonin innervation of the arcuate nucleus by immunohistochemistry. Increased serotonin may be partially responsible for the lack of eating in *anx / anx* mice. Serotonin agonists and re-uptake inhibitors cause decreased food intake in rodents, via the 5-HT 1A/1B and 5-HT 2A/2C receptor subtypes [24]. Serotonin can also indirectly affect feeding because hypothalamic serotonin can regulate arcuate NPY levels [8–10].

2. Materials and methods

Homozygous anorexia mice were produced from heterozygous breeder pairs (B6C3Fe-a/a-*anx* A/+ a) obtained from the Jackson Laboratory [25]. Weanling mice (postnatal days 21–24) group housed with parents were separated into three groups: *anx / anx*, control wild type, and food-deprived wild type. *Anx / anx* mice were identified by their reduced body weight, body tremor, and mild hyperactivity. Because homozygous and heterozygous mice cannot be phenotypically distinguished, wild-type groups probably contained both genotypes. *Anx / anx* and control mice were allowed ad lib access to the mother, food, and water in the home cage prior to perfusion. Food-deprived wild-type mice were removed from their home cage and parents, and housed in pairs in a clean cage with access to water but not food for 24 h prior to perfusion. [1] Two mice in each group from each litter were processed in parallel with littermates. A total of 12 *anx / anx*, 12 control, and 8 food-deprived mice from 6 litters were processed for NPY in situ hybridization. Seven mice in each group from 3 additional litters were processed for serotonin immunoreactivity. Mice were matched for sex whenever possible; post-hoc analysis revealed no effect of sex on body weight or NPY mRNA levels. Therefore, data from male and female mice were pooled.

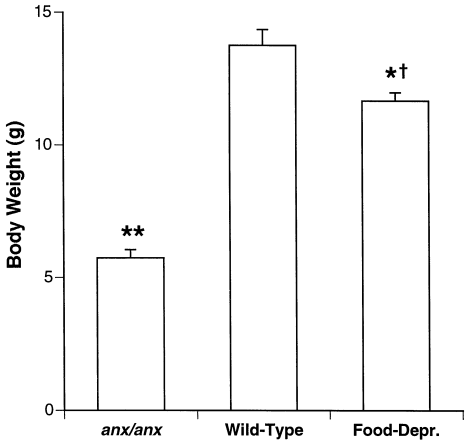

Fig. 1. Mean b.wt ± S.E.M. of *anx / anx* ($n = 12$), control wild type ($n = 12$), and 24-h food-deprived wild-type mice ($n = 8$) immediately prior to perfusion. $* p < 0.01$, $** p < 0.0001$ vs. wild type; $^{\dagger} p < 0.0001$ vs. *anx / anx*.

Mice were weighed, overdosed with sodium pentobarbital and, when completely unresponsive, transcardially perfused with heparinized isotonic saline containing 0.5% $NaNO_2$, then with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB). The brains were dissected, blocked, post-fixed for 24 h, and transferred into 30% sucrose for cryoprotection. Forty-micron coronal sections were cut on a freezing, sliding microtome through the rostral–caudal extent of the arcuate nucleus. Between 5 and 14 sections (8 sections on average) were collected from each mouse.

2.1. Immunohistochemistry

Free-floating tissue sections were washed twice for 15 min in 0.1 M sodium phosphate buffered saline (PBS),

[1] There was no food-deprived *anx / anx* group, because in preliminary trials revealed that *anx / anx* mice cannot tolerate longer than 6 h deprivation at postnatal day 21.

then permeabilized in 0.2% Triton, 1% bovine serum albumin (BSA) in PBS for 30 min. After washing twice in PBS–BSA, sections were incubated overnight with rabbit anti-serotonin antiserum at a dilution of 1:25,000 (Eugene Tech., Allendale, NJ). Sections were washed in PBS–BSA twice and incubated for 1 h with biotinylated anti-rabbit goat antibody (Vector Laboratories); bound secondary antibody was then amplified with the Vector Elite ABC kit. Antibody complexes were visualized by a 5-min 0.05% diaminobenzadine reaction.

Serotonin immunoreactivity within the arcuate nucleus was quantified by digitizing dark-field images (720 μm $\times$ 540 μm) of the immunostained arcuate. An average of 6.5 ± 0.6 sections through the intermediate arcuate were quantified per mouse. 5-HT-positive pixels were defined as light-gray and white pixels brighter than background as determined with the thresholding function of NIH Image software (W. Rasband). The number of 5-HT-positive pixels within the arcuate was counted bilaterally using a 100-pixel (110 μm) diameter circular punch of the arcuate just dorsal to the median eminence and lateral to the third ventricle (see Fig. 2 for example), and averaged within each mouse. The number was converted to a percent of 5HT-positive pixels quantified in control wild-type mice

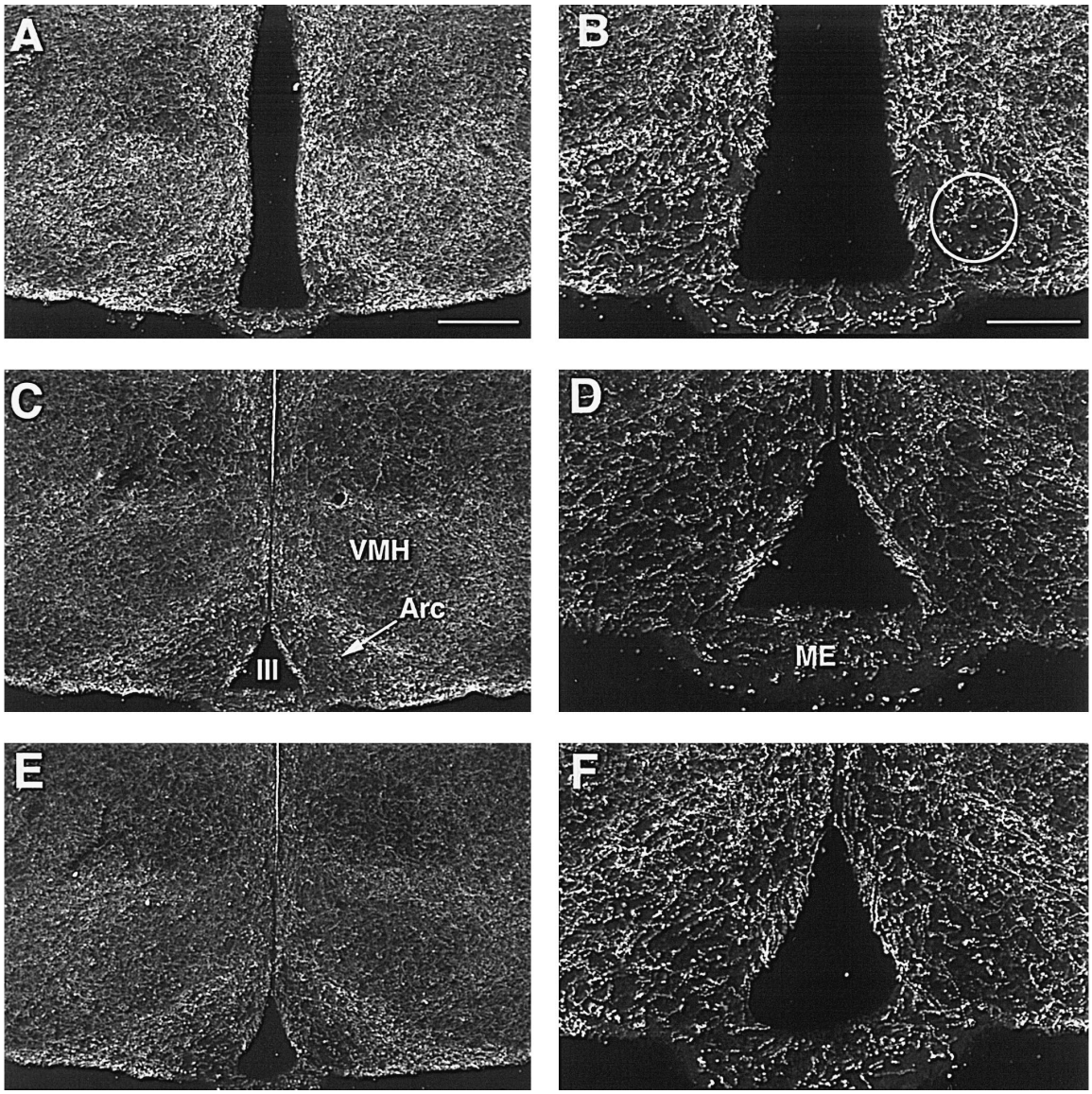

Fig. 2. Darkfield photomicrographs of serotonin immunoreactivity in the hypothalamus (low magnification in A, C, E) and arcuate nucleus (higher magnification in B, D, F) of *anx/anx* (A, B), control wild type (C, D) and 24-h food-deprived wild-type mice (E, F). Increased serotonin immunoreactivity was seen throughout the hypothalamus and arcuate nucleus of *anx/anx* mice compared to wild type. White circle in B represents 100-pixel (110 μm) diameter punch used to quantify 5HT-positive pixels in the arcuate. Scale bar in A, 250 μm. Scale bar in B, 100 μm. ARC, arcuate nucleus; ME, median eminence; VMH, ventromedial hypothalamus; III, third ventricle.

processed in parallel with *anx / anx* and food-deprived littermates.

2.2. In situ hybridization

Free-floating tissue sections were collected into 20 ml glass scintillation vials containing ice-cold 2 × SSC (0.3 M NaCl, 0.03 M Na Citrate) for in situ hybridization. The SSC was pipetted off, and sections were suspended in 1 ml of prehybridization buffer (50% formamide, 10% dextran sulfate, 2 × SSC, 1 × Denhardt's solution, 50 mM DTT, and 0.5 mg/ml denatured salmon sperm DNA). After 2 h prehybridization at 48°C, labeled denatured NPY cDNA probe (1×10^7 CPM) was added to the vials, and hybridized overnight at 48°C. The NPY cDNA probe was a 0.5-kb *Eco*RI restriction fragment comprising most of the cDNA of rat preproNPY [13], labeled with S^{35}-dATP by the random-priming method (Boehringer Mannheim).

Following hybridization, the sections were washed at 15-min intervals in decreasing concentrations of SSC (2 ×, 2 ×, 1 ×, 0.5 ×, 0.25 ×, 0.125 ×, 0.125 ×) at 48°C. The tissue sections were then mounted on gelatin-subbed slides, air-dried, and apposed to Amersham Hyperfilm autoradiographic film at 4°C. Exposure times varied from 6 to 24 h to obtain autoradiographic images within a linear range of optical density. Slides were then dipped in undiluted Kodak NTB-2 photoemulsion, and stored in light-tight boxes at 4°C for 1–4 weeks. After development in Kodak D-19, the slides were counterstained with Cresyl violet and cover-slipped. Tissue sections from one mouse from each treatment condition were hybridized within the same vial, and apposed to film together on the same microscope slide. Sections from different mice were identified by punctures or nicks made in the brain during sectioning. Thus, in situ hybridization was carried out on representative members of each experimental group at the same time under identical conditions, allowing direct comparison of mRNA expression.

Images of the arcuate nucleus (1600 μm × 1200 μm) were digitized from autoradiographic films through a Zeiss Stemi-2000 stereoscope attached to a Dage-MTI CCD 72 camera and MCID image analysis system. NPY mRNA expression levels were determined by quantifying the number and mean relative O.D. of pixels with densities of at least 2 standard deviations above the mean density of the image background ('NPY pixels'). The mean background value was subtracted from the mean NPY pixel values. The NPY pixel values were averaged across all sections from each individual mouse, and the average NPY value for each mouse then averaged across all mice within the experimental groups. The data is presented as percent of control wild-type NPY pixel value.

Significant differences in body weight, immunoreactivity and in situ hybridization were detected with one-way ANOVA and Fisher's PLSD post-hoc test using Statview software (Abacus, Berkeley, CA).

3. Results

Body weight was significantly affected by food deprivation and the *anx* mutation [$F(2,28) = 89.8$, $p < 0.0001$] (Fig. 1). As previously reported [15,25], *anx / anx* mice had body weights less than 50% that of control wild-type mice. Twenty-four-hour food deprivation significantly reduced wild-type body weight compared to non-deprived

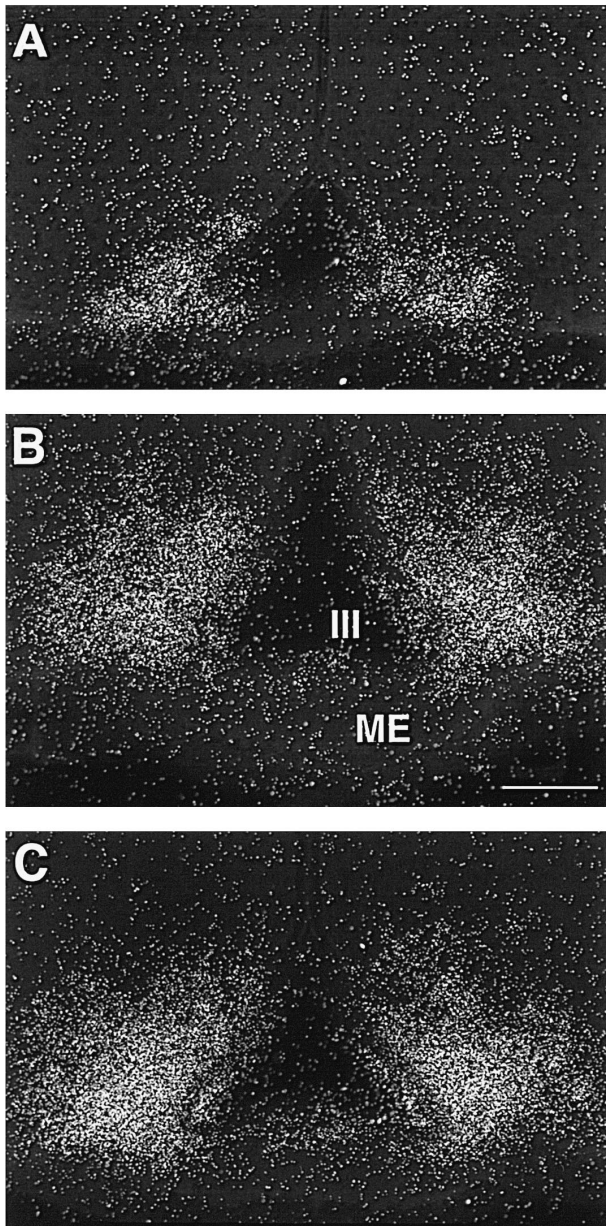

Fig. 3. Darkfield photomicrographs of NPY in situ hybridization in the arcuate nucleus from *anx / anx* (A), control wild type (B) and 24-h food-deprived wild-type mice (C) that were processed in parallel. Food deprivation, but not the *Anx* mutation, increased the intensity of NPY cDNA hybridization within the arcuate nucleus. Although there was a trend for the area of NPY in situ hybridization to be smaller in *anx / anx* mice than wild-type mice (as seen in A), no significant difference was found overall. Scale bar in A, 100 μm. ME, median eminence; III, third ventricle.

wild-type mice, but food-deprived wild-type mice still weighed significantly more than *anx / anx* mice.

Fibers stained with serotonin immunoreactivity were seen throughout the hypothalamus and forebrain of wild-type and *anx / anx* mice (Fig. 2), but the density of serotonin-immunoreactive fibers was greatly elevated in the *anx / anx* mice. In all control and food-deprived wild-type mice, serotonin-immunoreactive fibers were less dense in the center of the arcuate nucleus and ventromedial hypothalamus, but formed densities lateral and dorsal to the arcuate. Increased serotonin-immunoreactive fibers were seen throughout the arcuate nucleus in all 7 *anx / anx* mice examined. Quantification of 5-HT-positive pixels within a 110-μm-diameter punch of digitized arcuate sections revealed a significant difference in serotoninergic innervation ($F(2,18) = 4.6$, $p = 0.02$; Figs. 2 and 4). The absolute number 5-HT-positive pixels within the arcuate punch was small (e.g., a mean of 520 of 7860 pixels per arcuate section in wild-type mice). The arcuate of *anx / anx* mice, however, contained significantly higher numbers of 5-HT-positive pixels than control and food-deprived wild type; no significant difference in 5-HT innervation was found between control and food-deprived wild type. Consistent with the previous report, increased serotonin immunoreactivity was also observed in the cortex and forebrain of *anx / anx* mice compared to wild-type controls [25].

In situ hybridization of NPY cDNA revealed no significant difference between the three groups in the area of NPY pixels within the arcuate nucleus despite a trend towards a smaller area in anorexic mice ($F(2,28) = 3.0$, $p = 0.07$). Significant differences were found in mean NPY pixel intensity, however ($F(2,28) = 6.0$, $p = 0.007$; Figs. 3 and 4). The mean pixel intensity of NPY pixels was not significantly different between *anx / anx* and control wild-type mice. Twenty-four-hour food deprivation significantly increased arcuate NPY pixel intensity compared to non-deprived wild-type and *anx / anx* mice.

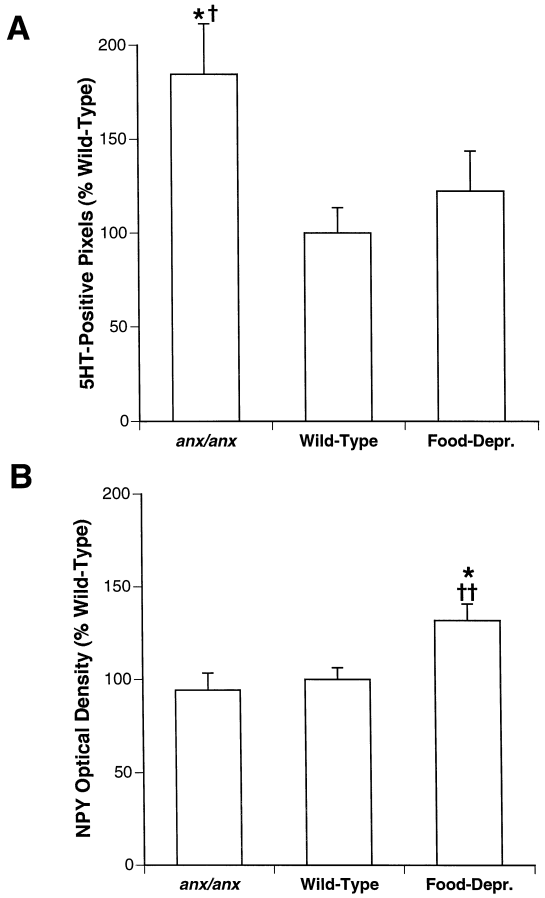

Fig. 4. (A) Mean number ± S.E.M. of 5-HT-positive pixels within bilateral, circular punches of 5-HT-immunostained arcuate nuclei from *anx / anx*, control wild type, and 24-h food-deprived wild-type mice ($n = 7$ for all groups), expressed as percent of control wild type staining. $* p < 0.01$ vs. control wild type; $^{\dagger} p < 0.05$ vs. food-deprived wild type. (B) Mean relative O.D. ± S.E.M. of autoradiographic images of arcuate NPY in situ hybridization from *anx / anx* ($n = 12$), control wild type ($n = 12$), and 24-h food-deprived wild-type mice ($n = 8$), expressed as percent of control wild-type density. $* p < 0.01$, $** p < 0.0001$ vs. control wild type; $^{\dagger} p < 0.0001$ vs. *anx / anx*.

4. Discussion

Despite severely decreased body weight and food intake at 3-week age, *anx / anx* mice do not show elevated NPY mRNA levels in the hypothalamic arcuate nucleus compared to wild-type littermates. In wild-type mice of the same chronological age, the arcuate nucleus was able to respond to food-deprivation because 24-h food deprivation increased arcuate NPY mRNA in wild-type littermates. The unresponsiveness of NPY expression in the arcuate of the *anx / anx* mice was correlated with serotonergic hyperinnervation of the arcuate nucleus, comparable to the serotonergic hyperinnervation previously reported in the rest of the *anx / anx* brain [25]. Food deprivation did not alter the pattern of serotonin innervation within the arcuate of wild-type mice.

This result suggests that reduced food intake, the primary behavioral phenotype of the *anx / anx* mouse, may be the result of altered hypothalamic mechanisms that normally regulate feeding. We hypothesize that there is a causal link between serotonin and NPY expression in the arcuate of *anx* mice, because: (1) serotonin hyperinnervation is the primary neurochemical phenotype of the *anx* mouse; (2) a lack of feeding is the primary behavioral phenotype of the *anx* mouse; and (3) NPY and serotonin are among the best-characterized central neurochemicals that regulate feeding behavior. Currently, we can only speculate on potential mechanisms by which serotonin hyperinnervation might compromise NPY mRNA expression. A variety of pharmacological, dietary, and metabolic manipulations that alter central serotonin levels will be

required to demonstrate causal effects on NPY expression and feeding behavior in the *anx* mice and wild-type littermates.

Serotonin regulates feeding behavior at multiple receptor sites [24]. The serotonin reuptake inhibitor and releaser fenfluramine decreases meal size and increases the intermeal interval [4], leading to weight loss in lean and obese rodents [11]. Stimulation of both 5-HT 1B and 5-HT 2C receptors with a high dose of the serotonin agonist mCPP replicates the effects of serotonin reuptake inhibitors [5]. Eliminating the 5-HT2C receptor in transgenic mice causes overeating and obesity [27]. Antagonist studies suggest that 5-HT 1B receptors mediate serotonin's effects on the size of meals, while 5-HT 2C receptors affect the rate of eating [24].

There is also evidence that serotonergic innervation of the arcuate can inhibit the deprivation-induced increase in NPY mRNA seen in wild-type mice. Serotonin fibers make direct contact with NPY cells of the rat arcuate [12]. Systemic administration of the serotonin re-uptake inhibitor fluoxetine blocks deprivation-induced increases in rat arcuate NPY mRNA [8]; conversely, chronic administration of the serotonin antagonist methysergide elevates rat arcuate NPY mRNA [10] and protein levels [9]. Thus, endogenous serotonin serves as a negative regulator of NPY synthesis in the rodent arcuate.

The increased density of serotonin fibers in the mutant arcuate may directly block an increase in arcuate NPY mRNA in the *anx / anx* mouse, thus preventing compensatory hyperphagia that would ameliorate starvation. However, although we observed increased serotonin immunoreactivity, we do not know whether this represents a net increase in serotonin or an increase in serotonin fibers without a net increase in serotonin. Furthermore, we have not directly established increased synaptic serotonin or serotonin release within the arcuate of *anx / anx* mice. Despite these caveats, the hypothesis that elevated serotonergic innervation of the arcuate compromises NPY regulation in the *anx / anx* mouse is plausible and testable.

The cause of serotonergic hyperinnervation in *anx / anx* mice is unknown. The hyperinnervation could be directly and mechanistically linked to the *anx* mutation. For example, the *anx* mutation might disrupt trophic signals (e.g., brain-derived neurotrophic factor [16,18]) that control serotonergic fiber sprouting and pruning during postnatal development [7]. Conversely, serotonergic hyperinnervation may be secondary to the chronic caloric and protein deprivation experienced by *anx / anx* mice [19,26], although the effects of chronic food deprivation specifically on serotonergic innervation of the hypothalamus have not been examined.

The *anorexia* mutation may alter other neuronal and humoral components of the hypothalamic feeding network. For example, NPY mRNA expression in the arcuate nucleus is enhanced by glucocorticoids and suppressed by corticotropin-releasing hormone, insulin, and leptin [1,21]. The presence of these factors and the arcuate's sensitivity to them have not yet been assessed in *anx / anx* mice.

In addition to the effects of serotonin or other extrinsic factors on the arcuate, an intrinsic deficit of NPY regulation in arcuate cells cannot be ruled out. The *anx* mutation (located on chromosome 2) is not within the NPY gene itself (located on chromosome 6; Mouse Genome Database, The Jackson Laboratory, Bar Harbor, ME). It has recently been reported, however, that NPY immunoreactivity is localized within the soma, rather than fibers, of arcuate cells in *anx / anx* mice [3]; this suggests that the *anx* mutation may also compromise NPY protein synthesis, processing, or transport.

5. Conclusion

Arcuate NPY mRNA levels are not elevated in *anx / anx* mice even as caloric restriction and starvation progresses. The apparent block of deprivation-induced increase in arcuate NPY mRNA seen in wild-type mice is paralleled by serotonergic hyperinnervation of the arcuate nucleus. Thus, the *anorexia* mutation is an inversion of the abnormal hypothalamic regulation seen in obese rodent models, in which arcuate NPY mRNA is continuously elevated [22,29]. This result is consistent with the hypothesis that wasting disorders are accompanied by disregulation of NPY mRNA expression in the arcuate nucleus, thus blocking the hyperphagia that normally compensates for food deprivation [21].

The description of NPY mRNA and serotonergic fibers in the arcuate nucleus contributes to the neurochemical phenotyping of *anx / anx* mice. The causal links between the underlying mutation, serotonergic hyperinnervation, and neonatal anorexia remain to be discovered. The *anx / anx* mouse will be a useful model of neonatal malnutrition and failure to thrive, particularly for exploring the role of NPY, serotonin, and other hypothalamic mechanisms regulating food intake.

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