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Research report

Differential expression of monoamine oxidase A, serotonin transporter, tyrosine hydroxylase and norepinephrine transporter mRNA by anorexia mutation and food deprivation

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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. We have found serotonin (5HT) hyperinnervation in the anx brain; altered noradrenergic (NE) innervation may also mediate eating disorders. We examined the expression of synthetic or catabolic monoamine enzyme genes in brainstem nuclei: serotonin transporter (5HTT) and monoamine oxidase A (MAOA) in the raphe nuclei (RN), and MAOA, norepinephrine transporter (NET), and tyrosine hydroxylase (TH) in the locus ceruleus (LC). We compared 3-week old anx with control and 24-h food-deprived wildtype littermates using in situ hybridization to measure mRNA levels by quantitative autoradiography. The anx mutation was correlated with decreased MAOA mRNA in the LC (but not RN), decreased 5HTT mRNA in the RN, and a trend towards lower NET mRNA in the LC. Food deprivation decreased MAOA mRNA in the LC (but not RN), increased TH mRNA in the LC, and did not alter NET or 5HTT mRNA levels. Thus, the effect of the anx mutation on MAOA expression in the LC paralleled the effect of food-deprivation, but the anx mutation and food-deprivation had differential effects on the expression of TH, NET, and 5HTT genes. Decreased 5HTT expression in the anx RN is consistent with upregulation of serotonergic neurotransmission that may accompany 5HT hyperinnervation. Central NE levels or innervation may be altered in anx mice by decreased expression of NET and MAOA and a lack of TH upregulation induced by food deprivation as in wild-type mice. © 1998 Elsevier Science B.V.

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1. Introduction

Anorexia (anx) mutant mice are a model system of a neonatal wasting disorder characterized by decreased food intake after postnatal day 10, neurological symptoms of motor dysfunction, and eventual death from starvation in the fourth postnatal week [12]. The underlying genomic mutation and the mechanism by which anx compromises neonatal ingestive behavior are unknown.

Several reports have begun to explore the neurochemical changes caused by the anx mutation that may contribute to the behavioral and physiological phenotypes [2,7,12,18]. The most dramatic alteration is a general up-regulation of central serotonergic innervation. Treatment with the serotonin antagonist 5,7-dihydroxytryptamine alleviates the neurological symptoms of anx mice [12]. The cerebral cortex, cerebellum, hippocampus, and hypothalamus, among other regions, contain far more serotonin-immunoreactive fibers in anx mice compared to wildtype littermates [7,18].

To continue the neurochemical phenotyping of the anx mouse, we examined the synthetic and catabolic pathways of monoamines in the anx mice by measuring gene expression in the raphe nuclei (RN) and the locus ceruleus (LC) by in situ hybridization. The RN contain the cell bodies that project serotonergic fibers throughout the brain; thus the serotonin hyperinnervation of anx mice may be associated with altered gene expression in the raphe. The expression of two genes were visualized in the raphe: the serotonin transporter (5HTT), which mediates serotonin reup-

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take after synaptic release, and monoamine oxidase A, which degrades 5HT intracellularly after reuptake. The expression of tryptophan hydroxylase (TPH), the rate-limiting enzyme of serotonin synthesis, was not examined because in the mouse RN, unlike the rat, TPH mRNA is not observable by cDNA in situ hybridization. This may be due to a very low copy number of TPH mRNA in the mouse RN [14].

The LC is the source of most brain norepinephrine. Anx mice do not show any gross abnormality in catecholamine innervation as visualized by tyrosine hydroxylase (TH) immunoreactivity [18]. Nonetheless, norepinephrine is a critical modulator of ingestive behavior, and alterations in noradrenergic innervation can induce serotonin hyperinnervation in neonatal rodents [20]. Therefore, we examined gene expression in the LC of TH, the rate-limiting enzyme in norepinephrine synthesis, the norepinephrine transporter (NET), which mediates norepinephrine reuptake, and MAOA, which degrades norepinephrine.

Anx mice were examined at 3 weeks of age, when the anx phenotype is well-developed. Two control groups were also examined: wildtype littermates, and 24-h food-deprived wildtype littermates. The comparison with food-deprived littermates is a useful positive control for the effects of starvation. Physiological alterations after starvation may represent either (1) the consequences of the metabolic and nutritional deficit, or (2) part of the compensatory response to conserve energy and induce hyperphagia to reverse the deficit. Analysis of neurochemical changes in anx mice is complicated because many alterations are likely to reflect either or both of these categories. Although acute food deprivation is not a perfect control for the ingestive effects of the anx mutation (i.e., the 3-week old anx mice do exhibit some suckling, but do not ingest enough to prevent death from starvation), it is a useful one. Ultimately, pair-fed littermates of equal body weight (either starved littermates or repleted anx mice) will be required to disentangle the direct effects of the mutation and the consequences of anorexia. Nonetheless, comparable changes in gene expression can be seen after acute total food deprivation and chronic food restriction [8].

2. Materials and methods

Homozygous anorexia mice were produced from heterozygous breeder pairs (B6C3Fe-a/a-anx A/+a) obtained from the Jackson Laboratory [18]. Weanling mice (postnatal days 21–24) group housed with parents were separated into three groups: anx/anx (anx), control wildtype, and food-deprived wildtype. Anx mice were identified by their reduced body weight, body tremor, and mild hyperactivity. Because homozygous and heterozygous mice cannot be phenotypically distinguished, wildtype groups probably contained both genotypes. Anx and control mice were allowed ad lib access to the mother, food, and water in the home cage prior to perfusion. Food-deprived wildtype 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. Mice in each group from each litter were processed in parallel with litter mates. Mice were matched for sex whenever possible, and data from male and female mice were pooled.

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 locus ceruleus and raphe nuclei. All sections were examined, with alternate sections divided between two probes for each hybridization run. Between four and ten sections (six sections on average) for the locus ceruleus, between three and nine sections (six sections on average) for the raphe nuclei were collected from each mouse for each of the two probes.

2.1. 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 sodium 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, ^{35}S-ATP labeled cDNA probes (1×10^7 CPM) were added to the vials, and hybridized overnight at 48°C.

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.

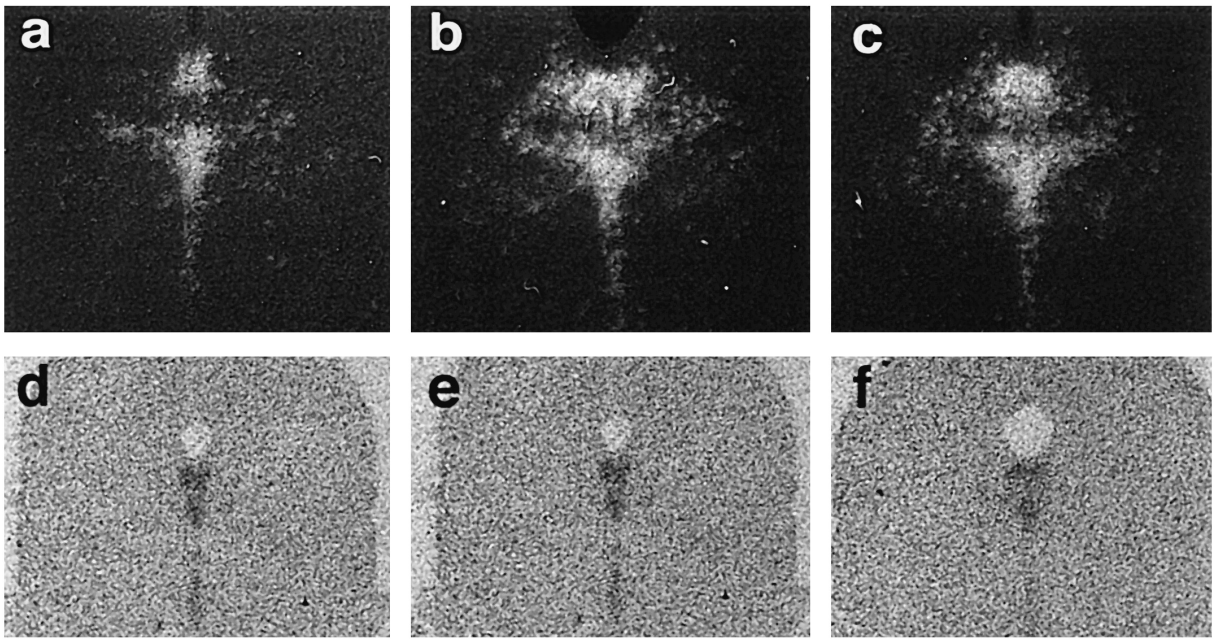

Fig. 1. Examples of in situ hybridization in the raphe nuclei of anx (a,d), wildtype (b,e) and food-deprived wildtype mice (c,f). (a)–(c) Darkfield photomicrographs of 5HTT cDNA hybridization from emulsion-dipped slides. 5HTT expression was significantly reduced in anx mice. (d)–(f) Bright field photomicrographs from autoradiographic films of MAOA cDNA hybridization.

Four cDNA probes were labeled with S^{35}-dATP by the random-priming method (Boehringer Mannheim). Sections containing the raphe nuclei were hybridized with (1) a 3.5-kb *Eco*R1 restriction fragment of a rat MAOA cDNA [8] and (2) a 0.8-kb *Eco*R1 restriction fragment of a rat 5HTT cDNA [21]. Sections containing the locus ceruleus were hybridized with: (1) a 1.6-kb *Pst*1 restriction fragment of a rat TH cDNA [9]; (2) a 0.5-kb *Eco*R1 restriction fragment of a rat NET cDNA [4]; and (3) the rat MAOA cDNA.

The total sets of mice (anx, control, and food-deprived littermates) examined with each probe were seven for MAOA, five for TH, five for NET, and eight for 5HTT.

Images of the RN and LC (2500 μm $\times$ 1875 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. Messenger RNA expression levels for each probe were determined by quantifying the number and mean relative optical density of pixels with densities of at least 2 S.D. above the mean density of the image background ('mRNA pixels'). The mean background value was subtracted from the mean mRNA pixel values. The mRNA pixel values were averaged across all sections from each individual mouse, and the average mRNA value for each mouse then averaged across all mice within the experimental groups. The data is presented as percent of control wildtype mRNA pixel value.

Significant differences in body weight and in situ hybridization were detected by paired *t*-tests of control vs. anx and control vs. food-deprived values. (Statview software, Abacus, Berkeley, CA).

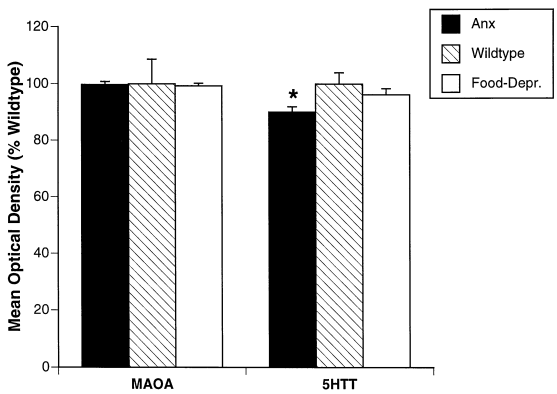

Fig. 2. Quantification of in situ hybridization in the raphe nuclei. Mean optical density of MAOA and 5HTT expression in anx, wildtype, and 24-h food-deprived wildtype mice. $* p < 0.05$ vs. wildtype.

3. Results

The body weights of both anx mice (5.4 ± 0.2 g) and 24 h food-deprived mice (10.7 ± 0.4 g) were significantly lower than non-deprived wildtype littermates (13.1 ± 0.4 g; $p < 0.0001$, $p < 0.005$, respectively).

In the RN, MAOA mRNA levels and area were not different between anx, wildtype, and food-deprived mice (Figs. 1 and 2). The anx mutation was associated with significantly lower levels of 5HTT mRNA ($p < 0.005$) and significantly smaller area (94 ± 1% of wildtype, $p < 0.005$). Food-deprivation did not alter 5HTT mRNA relative to non-deprived controls (Figs. 1 and 2).

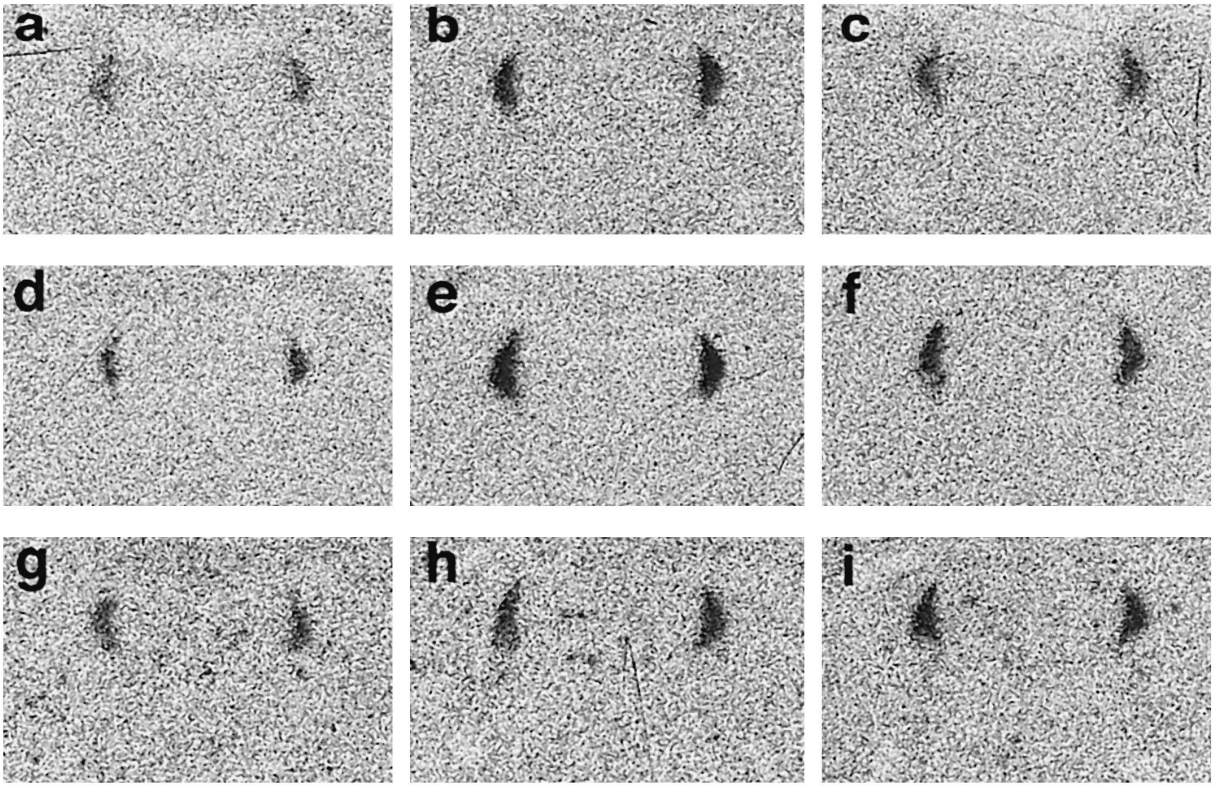

Fig. 3. Bright field photomicrographs from autoradiographic films of in situ hybridization in the locus ceruleus of anx (a,d,g), wildtype (b,e,h) and food-deprived wildtype mice (c,f,i). (a)–(c) MAOA cDNA hybridization. MAOA expression was decreased in anx and food-deprived mice relative to wildtype. (d)–(f) NET cDNA hybridization. There was a trend for decreased NET expression in the LC of anx mice. (g)–(i) TH cDNA hybridization. TH expression was increased in food-deprived mice relative to anx and wildtype mice.

MAOA, TH, and NET mRNA were strongly expressed in the LC of anx, wildtype littermates, and food-deprived wildtype littermates (Fig. 3). Quantification of relative optical densities revealed that MAOA expression in the LC was significantly lower in anx mice than in wildtype littermates (Fig. 4, $p < 0.005$). Although the anx mutation did not significantly change TH or NET mRNA levels compared to wildtype control littermates (Fig. 4), all anx mice had lower average NET mRNA levels than paired wildtype controls, and there was a trend towards significance (Fig. 4, $p < 0.06$).

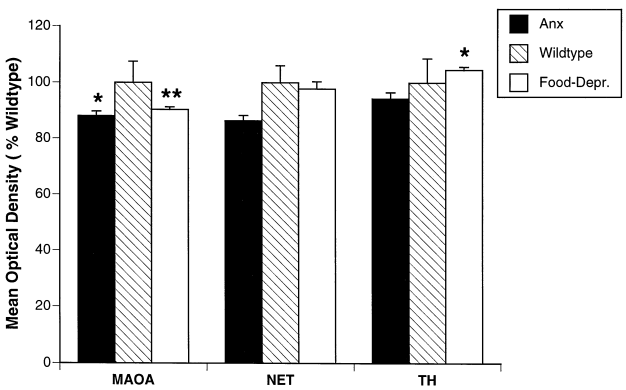

Fig. 4. Quantification of in situ hybridization in the locus ceruleus. Mean optical density of MAOA and NET, and TH expression in anx, wildtype, and 24-h food-deprived wildtype mice. $* p < 0.05$, $** p < 0.001$ vs. wildtype.

The area of MAOA expression (number of MAOA mRNA pixels) was slightly but significantly greater in anx mice than wildtype mice ($103 \pm 1\%$ of wildtype, $p < 0.05$). No other differences were found in other comparisons of mRNA area.

Food deprivation also significantly decreased MAOA expression in the LC compared to non-deprived wildtype littermates (Fig. 4, $p < 0.001$). In contrast to anx mice, food deprivation significantly elevated TH mRNA levels ($p < 0.05$) but did not alter NET mRNA levels (Fig. 4).

4. Discussion

These results expand the neurochemical phenotype of the anx mutation. Previously, we and others have characterized two central markers of the anx mutation: (1) hyperinnervation by serotonergic fibers throughout the brain [18], and (2) in the hypothalamic arcuate nucleus, NPY expression that is not upregulated despite starvation [7], and NPY peptide that is not properly transported from the cell body [2]. This study adds that MAOA mRNA levels in the LC and 5HTT mRNA levels in the RN are abnormally

low in anx mice (and suggest that larger studies may reveal a significant decrease in NET mRNA). These small but significant changes in mRNA levels may have a large cumulative effect on protein synthesis. To determine the functional significance of these in situ hybridization results, quantitative measures of monoamine levels and release (e.g., by HPLC), transporter protein (e.g., by 5HT- and NE-binding and transport), and MAOA activity (e.g., by enzyme assay) will be required. We can speculate, however, on the significance of altered gene expression in the RN and LC, and on possible links between NE and 5HT.

4.1. Altered RN gene expression

Serotonin may be involved in mediating the anorexia and movement disorders of anx mutants, because of the pronounced serotonergic hyperinnervation in anx brains [18], the reversal of some anx symptoms by serotonin antagonists [12], and the induction of anx-like symptoms in wildtype littermates by serotonin agonists [12]. Serotonin acting at $5HT_{1B}$, $5HT_{2C}$, and $5HT_{2A}$ receptors can decrease feeding directly [17], and serotonin can block fasting-induced increases in hypothalamic arcuate NPY mRNA and NPY secretion [5]; we have shown serotonin hyperinnervation in the arcuate nucleus and normal levels of NPY mRNA despite starvation in anx mice [7]. The current finding that 5HTT mRNA is downregulated may be consistent with increased 5HT neurotransmission, because decreased 5HTT function (e.g., with fluoxetine) decreases the rate of serotonin clearance from synapses [11]. Decreased 5HTT mRNA in neonatal anx mice suffering from chronic starvation is also consistent with a recent report that long-term starvation decreases $^{3}H-$ paroxetine binding in the cortex of 10-week old rats [6].

Paradoxically, the size of the raphe appears smaller in anx mutants (because we observed a smaller area of MAOA and 5HTT in in situ hybridization). And it was previously found that TPH activity was decreased in the raphe, despite hyperinnervation of brain regions by 5HT fibers [18]. It is not clear whether these changes represent causal factors or compensatory responses to 5HT hyperinnervation, or steady-state levels of gene expression after prolonged hyperinnervation or chronic starvation.

4.2. Altered LC gene expression

Because changes in gene expression were found in the LC of anx mice, NE neurotransmission may also be implicated in the anx syndrome. Decreased MAOA and decreased NET in NE neurons may contribute to increased synaptic efficacy of endogenous NE. For example, MAOA knockout mice exhibit increased NE levels beginning in postnatal week 3 [3] and pharmacological blockade of NE reuptake (e.g., with desipramine) increases synaptic NE levels [11]. Although two major catabolic regulators of NE were decreased, however, the expression of the rate-limiting enzyme in NE synthesis, TH, was not increased. Quantitative measures of NE levels and NE innervation (e.g., in the hypothalamic feeding areas) will be required to determine if the changes in LC gene expression are causal or compensatory for altered NE innervation.

We also found that 24 h food-deprivation increases TH expression while decreasing MAOA expression in the LC of wildtype mice. The LC provides a major NE and NPY input to the hypothalamic paraventricular nucleus (PVN) [16], and local injections of NE or NPY into the PVN stimulate food intake [10]. Thus, upregulation of LC NE may represent a response to food-deprivation, and activity of LC efferents to the PVN may promote compensatory food-intake after fasting, similar to the role of arcuate-PVN efferents. However, the ability of starved anx mice to upregulate TH expression in the LC appears compromised relative to food-deprived wildtype littermates. The lack of response of LC TH to starvation in anx mice parallels their lack of response of arcuate NPY gene expression [7], and may be significant in the context of the anx mutation as a wasting disorder.

4.3. A possible link between NE and 5HT

This study suggests that reuptake of two important monoamines, 5HT and NE, may be altered by the anx mutation. Other studies have demonstrated a link between sprouting of serotonin fibers in neonatal rodents and decreased catecholamines [20]. For example, 6-hydroxydopamine lesions of dopamine fibers in the striatum [19] or NE fibers in the cortex [1] of neonatal rats induces sprouting of serotonin fibers. In another model, the brindled mottled mutant mouse displays serotonin hyperinnervation throughout the cerebral cortex (but not other brain regions) [13]. The direct effect of the brindled mottled mutation is to alter copper metabolism, but because copper is a cofactor for dopamine-β-hydroxylase, NE and epinephrine levels are severely decreased, which induces 5HT fiber sprouting. These models provide precedents of catecholamines altering 5HT innervation that supports investigation of NE innervation in anx mice. These models also demonstrate, however, that the phenotype of 5HT hyperinnervation may be secondary to an apparently unrelated mutation (e.g., in copper metabolism).

Although further neurochemical analysis of the anx brain is ongoing, we and others are just beginning the task of interpreting these neurochemical changes as direct and indirect results of the anx mutation. The neurochemical alterations must then be integrated with the physiological, neurological, and behavioral symptoms of the postweanling anx mouse. We are undertaking a combination of molecular genetics (e.g., differential display of neuronal gene products from anx and wildtype [15]) and neurochemical phenotyping to identify the mutation and its neurobiological consequences. The anx mouse should continue to prove a useful model of monoamine disregulation with molecular and behavioral consequences in neonates.

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