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Approaches to anorexia in rodents: focus on the *anx*/*anx* mouse

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Abstract

Eating disorders constitute major medical health problems in the western world. Even though little is known about the mechanisms behind abnormal eating behavior, it has become clear that the central nervous system (CNS), particularly the hypothalamus, plays a significant role. The anorexic *anx*/*anx* mouse is a unique model for studying food intake and energy expenditure. The *anx* mutation is linked to marked alterations in hypothalamic distributions of signal substances known to have potent regulatory roles in the control of food intake. We have identified a mutation in *anx*/*anx* mice that is likely to cause the anorectic phenotype. Using RNA profiling, we have found 29 genes with differential expression in the *anx*/*anx* mouse brain. The *anx* gene, its protein product or molecules in the *anx* pathway may thus be interesting targets for development of new pharmaceuticals for the treatment of eating disorders. Based on the histochemical alterations found in the *anx*/*anx* mouse, we hypothesised and showed that many sera from anorectic/bulimic patients contain antibodies that bind specifically to the hypothalamic food intake regulatory system in rat. This finding represents a novel research avenue that may lead to a better understanding of eating disorders. It also suggests that targeted immunological approaches may be used in therapy.

Keywords: Anorexia; Hypothalamus; Mouse model; Genetic; Positional cloning; Neuropeptide Y; AgRP (agouti-related protein); POMC (pro-opiomelanocortin); Autoantibody

1. Introduction

Eating disorders such as obesity, anorexia and bulimia are complex disorders displaying a variety of symptoms apart from an abnormal eating behaviour. Like many other motivated behaviours, feeding requires the integration of internal and external signals, and it is not clear if the physiological correlates observed in these disorders are causes or effects of the altered eating behaviour. There is considerable uncertainty regarding the biological basis of eating disorders in humans and a good understanding of the physiology underlying feeding behaviour is therefore essential. Even though animal models may not mimic all of the aspects of human eating disorders, they can provide useful information on underlying causes and how to treat at least some of the symptoms. In fact, several animal models have been used as hypothesis generators, and in some cases they have been most helpful in revealing the nature of specific human conditions of eating disorders. Furthermore, animal models may help us predict the effects of small molecule drugs and/or therapeutic proteins. The identification of genes behind mouse phenotypes that resemble the desired clinical effects of a drug, or the clinical state one wish to cure, may aid substantially in predicting new drug targets.

2. The anorexia (*anx/anx)* mouse

The anorexia mouse (*anx*/*anx*) is characterised by poor appetite, and its stomach content is reduced compared to normal littermates from around postnatal day 5 and continue so until death (Maltais et al., 1984). Interestingly, the daily changes in stomach content in *anx*/*anx* mice are very similar to those observed in normal littermates from birth to 20 days of age (Maltais et al., 1984). These data indicate that *anx*/*anx* mice fail to properly regulate the amount of food consumed rather than failing to eat for other reasons.

Anorexic mice can be recognised at about 5–8 days of age by a thinning of the neck and tail. Later on they are easily recognised by their growth failure and emaciated appearance,

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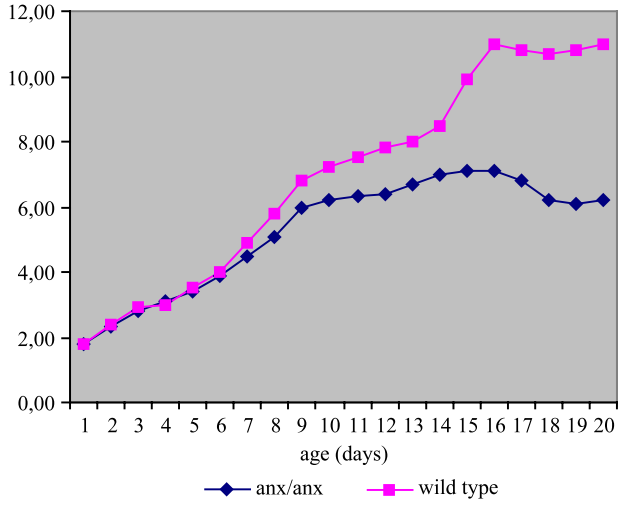

Fig. 1. Daily average body weights observed for *anx*/*anx* and wild type littermates. Figure adapted from Maltais et al. (1984).

as well as their abnormal behaviour including body tremors, headweaving, hyperactivity and uncoordinated gait. A significant difference in body weight between *anx*/*anx* and normal sib controls occurs at postnatal day 9 (Fig. 1) (Maltais et al., 1984). The animals die at the age of 3–5 weeks depending on the genetic background. Histological analysis show no abnormalities in the gastrointestinal system, and blood parameters such as total red blood cells, hematocrit, haemoglobin and mean cell volume are within normal range (Maltais et al., 1984). No abnormalities have been found in sections of organs stained with histochemical methods.

3. Genetics of the *anx/anx* mouse

The autosomal recessive *anx* mutation arose at the Jackson laboratory (Bar Harbor, ME, USA) in 1976 in the F2 generation of a cross between DW/J and an inbred strain derived from a cross between *M. m. poschiavinus* and an inbred Swiss stock (Maltais et al., 1984). The male *anx* carrier was crossed to a B6C3H-*a*/*a* F1 female, and the mutation has been maintained on this hybrid background at the Jackson laboratory. Linkage was found with the non-agouti locus, *a*, on Chromosome 2 (Maltais et al., 1984).

Using a positional cloning approach, we have mapped the *anx* gene to a 0.2-cM interval corresponding to 1.28 Mbp on mouse chromosome 2. This interval has been sequenced and analyzed using bioinformatic tools, and 54 coding genes and 63 possible noncoding RNA genes have been identified. All genes have undergone a mutation screening by exon- and cDNA-sequencing and a very likely *anx* candidate gene has been identified. This gene harbours a sequence alteration leading to an amino acid exchange in the signal peptide of the candidate protein in *anx*/*anx* mice, possibly resulting in an altered subcellular location of the protein. This mutation predicts a substitution of the charged polar amino acid arginine (R) by the hydrophobic nonpolar amino acid tryptophan (W) within the signal peptide. This mutation has not been found in any of the mouse strains C57B6/J, C3HeJ, BALB/cByJ or CAST/Ei nor has it been found in any sequence in the public databases. As this amino acid exchange is likely to change the polarity within the signal peptide domain, we investigated whether it would interfere with the probability of the protein sequence to function as a signal peptide. The first 31 amino acids of the wild type and mutant sequences were submitted to the SignalP V2.0 signal peptide prediction package http://www.cbs.dtu.dk/services/SignalP) (Nielsen et al., 1997; Nielsen and Krogh, 1998). According to the hidden Markov model, there was an increase in the probability of the mutated sequence acting as a signal peptide (0.867 for the mutant sequence; 0.345 for the wild type sequence). Furthermore, the predicted peptide cleavage site was altered from between positions 24 and 25 in the wild type sequence to between positions 23 and 24 in the mutant sequence. These results suggest an altered efficacy/function of the signal peptide in *anx*/*anx* mice.

Evidence for the existence of *anx* modifying genes was provided in a cross where B6C3-*a*/*a*-*a* +/+ *anx* was crossed with CAST/Ei. CAST/Ei is derived from the subspecies *M. m. castaneus*, and as such it is genetically very different from most other inbred strains that originate from the subspecies *M. m. musculus* and *M. m. domesticus* (Silver, 1995). These subspecies diverged evolutionary approximately 1 million years ago (Silver, 1995). Interestingly, *anx*/*anx* mice in the F2 generation of this cross do not show symptoms, including lower body weight, until around postnatal day 20. They also survive longer, approximately 4 to 5 weeks as compared to 3 weeks on the B6C3H background. The differences in phenotype between *anx*/*anx* mice from these two crosses suggest that *anx* modifying gene(s) exist. CAST/Ei could thus be introducing allele(s) of gene(s) with modifying effects on the *anx* phenotype. Mapping of the modifying gene(s) may further contribute to our understanding of the fine tuned control of appetite.

4. Profiling of mRNA expression in *anx/anx* brain

An mRNA differential display analysis was performed on newborn and 3-week-old *anx*/*anx* and +/+ mice, to identify genes involved directly and indirectly in the *anx* phenotype. As differential display experiments often result in false positive results, we performed duplicate analyses on three animals in each group. In total, 102 amplified gene fragments differing in expression levels between *anx*/*anx* and +/+ mice were identified. Fifty-three of these fragments had a different expression pattern in newborn *anx*/*anx* mice and *anx*/*anx* mice of both ages. The remaining 49 fragments were differently expressed only in 3-week-old *anx*/*anx* mice. These are likely to reflect secondary responses to the starvation. All fragments were excised from the gel and

sequenced. Seventy of the fragments have been identified as genes or ESTs. These genes were subjected to microarray analysis, and 29 have been confirmed to show altered expression pattern in *anx*/*anx* mouse brain. Further analyses with regard to their role in regulation of food intake and energy expenditure is ongoing.

This is, to our knowledge, the first expression profile on long-term starvation and it will hopefully contribute to elucidating the molecular pathways involved in the response to the deficit imposed by the *anx* mutation and starvation.

5. Alterations in hypothalamic peptide distributions in *anx/anx* mice

The hypothalamus has long been known as an important site for the regulation of food intake. Histochemical studies on the *anx*/*anx* mouse have demonstrated abnormalities in the hypothalamic arcuate nucleus summarised in Table 1 (Broberger et al., 1997a, 1998a, 1999; Johansen et al., 2000). This nucleus houses several neurochemically defined cell populations (Everitt et al., 1986), among them two populations that seem to play antagonistic roles in energy balance control. One population expresses neuropeptide Y (Allen et al., 1983; Chronwall et al., 1985; DeQuidt and Emson, 1986) and agouti-related protein (AgRP) (Broberger et al., 1998b; Hahn et al., 1998; Shutter et al., 1997), both stimulators of food intake. The other population expresses two inhibitors of food intake, pro-opiomelanocortin (POMC) (Bloch et al., 1978; Bloom et al., 1978; Watson et al., 1978) and cocaine- and amphetamine-regulated transcript (CART) (Elias et al., 1998; Kristensen et al., 1998). Both these cell populations receive peripheral signalling from fat tissue via the peptide hormone leptin (Elmquist et al., 1999) as well as other hormones such as insulin and ghrelin.

Table 1
Histochemical alterations in the hypothalamus of *anx*/*anx* mice

	In situ hybridization	Immunohistochemistry
ACTH	See POMC	Cell number: ↓
AgRP	mRNA in arc: 0	Cell bodies in arc: ↑↑ Terminals: ↓
α-MSH	See POMC	Terminals: ↓
CART	mRNA in arc: ↓↓ Number of cells in DMH: ↓	Cell bodies in arc: ↓ Number of cells in DMH: 0 Terminals: ↓
CCK	mRNA: 0	Terminals: 0
Galanin	n.d.	Terminals: 0
Neuropeptide Y	mRNA in arc: 0	Cell bodies in arc: ↑↑ Terminals: ↓
Neuropeptide Y Y_1 receptor	mRNA in arc: ↓	Cell body number: ↓ Dendrites: ↓
Neuropeptide Y Y_2 receptor	mRNA in arc: ↓	n.d.
Neuropeptide Y Y_5 receptor	mRNA in arc: ↓	n.d.
POMC	mRNA in arc: ↓	See ACTH and α-MSH

Summary of the histochemical alterations observed in the hypothalamus of 3-week-old *anx*/*anx* mice compared to +/+ littermates. Increases and decreases are indicated with arrows. 0 indicates no alteration detected; not determined is denoted n.d. ACTH, adrenocorticotropic hormone; AgRP, agouti-related protein; α-MSH, α-melanocyte-stimulating hormone; arc, arcuate nucleus; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; DMH, dorsomedial hypothalamic nucleus; POMC, pro-opiomelanocortin (for details, see Broberger et al., 1997a, 1998a, 1999; Johansen et al., 2000).

The stimulatory effect of neuropeptide Y on food intake is well characterised and has been shown to act primarily on carbohydrate consumption (Stanley and Leibowitz, 1985). Neuropeptide Y is expressed in arcuate cells that innervate the rest of the hypothalamus including the paraventricular nucleus, an important target area for regulation of food intake. When administered centrally into the paraventricular nucleus even low doses of neuropeptide Y have a powerful stimulatory effect on food intake (Clark et al., 1984; Stanley and Leibowitz, 1985). AgRP is an endogenous antagonist of the anorexigenic melanocortin peptides (Fan et al., 1997; Ollmann et al., 1997). It is expressed by 95% of the neuropeptide Y neurones in the arcuate nucleus (Broberger et al., 1998b).

POMC is the precursor protein for seven mature melanocortin peptide hormones. Among these are the adrenocorticotropic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH). Fasting decreases POMC expression levels (Brady et al., 1990) and melanocortin treatment induces anorexia (Fan et al., 1997; Grill et al., 1998). CART encodes putative peptides that have potent appetite-suppressing activity (Kristensen et al., 1998; Lambert et al., 1998). It even blocks neuropeptide Y-induced feeding responses in rats, when injected intracerebroventricularly (Kristensen et al., 1998; Lambert et al., 1998). There is evidence that the CART peptide may act partly through the feeding-regulating circuitry operated by neuropeptide Y, melanocortins and leptin (Elias et al., 1998; Kristensen et al., 1998; Lambert et al., 1998).

There is much anatomical and pharmacological evidence that the balance between neuropeptide Y and POMC transmission may be correlated to the level of food intake. The projections of the arcuate POMC/CART neurones have been shown to be linked and parallel to the arcuate neuropeptide Y system (Broberger et al., 1997b, 1998a; Csiffáry et al., 1990; Fuxe et al., 1997; Zhang et al., 1994). Furthermore, POMC neurones express the neuropeptide Y receptors Y_1 and Y_5 (Broberger et al., 1997b; Fuxe et al., 1997) and activation of these two receptors stimulates food intake (Gerald et al., 1996; Stanley et al., 1992), possibly involving inhibition of POMC signalling.

5.1. Neuropeptide Y and agouti-related protein

In *anx*/*anx* mice, the neuropeptide Y/AgRP neurones are characterised by increased neuropeptide Y- and AgRP-like immunoreactivities in the cell body and decreased staining

in terminals as compared to wild type littermates (Broberger et al., 1997a, 1998a). However, the mRNA levels of both neuropeptide Y and AgRP were reported to be similar in the arcuate nucleus of *anx*/*anx* and wild type mice (Broberger et al., 1997a, 1998a). This suggests that increased peptide levels in cell bodies are not due to increased mRNA synthesis, but that neuropeptide Y and AgRP peptides are accumulated in the cell bodies, possibly as a result of a deficiency in axonal transport mechanisms.

5.2. Pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript

anx/*anx* mice display decreased levels of POMC, neuropeptide Y Y_1 and Y_5 receptors and CART mRNA in the arcuate nucleus (Broberger et al., 1999; Johansen et al., 2000). Furthermore, *anx*/*anx* mice have a decreased number of cell bodies with detectable POMC/CART peptides, as determined by staining with ACTH, α-MSH and CART antiserum (Broberger et al., 1999). They also show a dramatic reduction of neuropeptide Y Y_1 receptor-positive dendritic extensions of the POMC cells (Broberger et al., 1999). It may be speculated that neuropeptide Y and AgRP cannot be properly released in the arcuate nucleus and that the POMC neurones need some molecule(s) secreted from the neuropeptide Y/AgRP cells to remain functional.

In addition to the arcuate nucleus, CART is also expressed in the dorsomedial hypothalamic nucleus and lateral hypothalamic area as well as in other parts of the brain and periphery (Douglass et al., 1995; Koylu et al., 1997). The intensity of CART signal in cells in the dorsomedial hypothalamic nucleus and lateral hypothalamic area of *anx*/*anx* mice does not appear to differ from healthy littermates, but the number of labelled cells seems decreased (Johansen et al., 2000). A possible interpretation of this finding is that at least two distinct populations of CART expressing cells may exist in the dorsomedial hypothalamic nucleus and lateral hypothalamic area, differing in their response to the regulatory mechanisms affected in the *anx*/*anx* mouse. Further studies dissociating the functions of different hypothalamic CART-expressing populations will be needed to address the functional relevance of these findings.

6. Experimental approaches in the rat using human antisera

Autoimmune disease affects 3% of the world population, accounting for diseases such as rheumatoid arthritis and autoimmune diabetes. Implication of autoimmunity in neurological diseases is also a known phenomenon, where either cell-mediated or antibody-mediated autoimmune mechanisms may predominate in a given disease, such as multiple sclerosis or myasthenia gravis, Lambert–Eaton myasthenic syndrome, neuromyotonia and Ramussen encephalitis, respectively (for review, see Whitney and McNamara, 1999).

Based on the immunohistochemical alterations, we detected in the *anx*/*anx* mouse, we hypothesised that in some cases anorexia may develop as a result of production of autoantibodies targeting hypothalamic peptidergic neurotransmitter systems controlling food intake and body weight. Hence, we tested whether serum from anorectic/bulimic patients may contain antibodies that would bind specifically to neuropeptides or their receptors or enzymes present in the hypothalamic food intake regulatory system. In a preliminary study, sera from 57 anorexia and/or bulimia nervosa patients were screened by immunohistochemistry of γ-globulin (IgG) on rat brain and pituitary sections (Fetissov et al., 2002). Eight sera displayed similar peptidergic-like staining of different intensity in neuronal cell bodies of the arcuate nucleus and processes characteristic for α-MSH distribution and 42 sera displayed binding to pituitary melanotrophes. Positive cell bodies and processes were also found after incubation with same sera in the lateral hypothalamic area with a distribution similar to that of melanin concentrating hormone. By double immunohistochemistry, we identified that labelled neurones and terminals originated in the arcuate nucleus, expressed POMC-derived peptide α-MSH (Fig. 2) and preadsorption of sera with α-MSH reduced this labelling. It is important to note that in 13 sera from control individuals without known anorexia we found two which also showed the above described peptidergic immunostaining pattern in the rat hypothalamus (Fetissov et al., 2002). We are therefore now analysing a larger number of patients with anorexia/bulimia together with more controls, to establish to what extent this type of autoantibodies is present also in an apparently normal population of individuals.

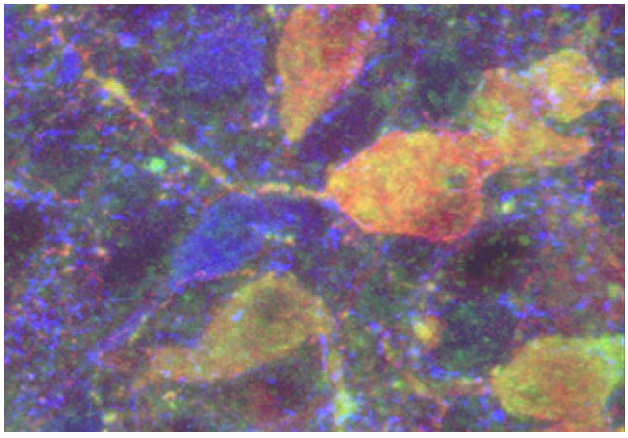

Fig. 2. Photomicrograph of part of the arcuate nucleus in the rat. Immunohistochemical detection of neuropeptide Y (blue staining), α-MSH (red staining) and IgG serum from an anorexia patient (green staining). Note yellow staining over the α-MSH staining cell indicating staining with both the α-MSH and the anorexia antisera.

7. Discussion

Over the past 10 years, there has been a tremendous increase in the understanding of the genetic regulation of food intake and energy expenditure, partly based on the use of monogenic rodent models of obesity. Several genes have been cloned that, when mutated, cause obesity in the mouse and rat (Barsh et al., 2000; Johansen and Schalling, 2002). These genes and their products have unravelled biochemical pathways involved in obesity. Some of these genes, such as leptin and its receptor, have been shown to be important for the regulation of food intake and/or metabolism also in humans.

There has been less of a focus on genetic models of anorexia. One reason may be that there are more genetic animal models that shift the regulation of food intake, satiety or metabolic turnover towards obesity than anorexia. A possible explanation for this could be that even a relatively mild anorectic phenotype could lead to malnutrition and/or death by starvation early enough in life to affect the number and viability of the offspring.

The *anx* gene, its protein product and molecules active in the *anx* pathway constitute attractive targets for development of anti-obesity drugs. In the case of the recessive *anx*/*anx* mouse, the phenotype resembles the desired human clinical effect of such drugs. Since it appears easier to produce small molecule compounds with antagonistic than agonistic effects, one could argue that studying animal models with a phenotype resembling the desirable effect of the drug could lead to better targets and increase the success rate for the pharmaceutical industry. This makes the anorexia mouse an attractive model for providing new targets for the development of anti-obesity drugs.

If our hypothesis that anorexia/bulimia nervosa and obesity also have an autoimmune component proves to be correct, the mechanism(s) of autoantibody production need to be established. In addition, since some healthy individuals display anti-α-MSH IgG, presence of these antipeptide antibodies may not be sufficient for development of anorexia or bulimia and an additional pathophysiological mechanism(s) should be required. For instance, these mechanisms might be dependent on access of these antibodies to central peptidergic neurones and therefore permeability of the blood–brain barrier can play a critical role in this process, since it may be affected by changes in hormone secretion, disease or dieting. If this hypothesis is correct immunological approaches may possibly be developed for therapy of eating disorders.

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