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Impaired Processing of Brain Proneurotensin and Promelanin-Concentrating Hormone in Obese *fat/fat* Mice*

CAROLE ROVERE, AGNÈS VIALE†, JEAN-LOUIS NAHON, AND PATRICK KITABGI

Institut de Pharmacologie Moléculaire et Cellulaire du CNRS, Université de Nice-Sophia Antipolis, Sophia Antipolis, 06560 Valbonne, France

ABSTRACT

Mice homozygous for the *fat* mutation exhibit marked hyperproinsulinemia and develop late onset obesity. The *fat* mutation was recently mapped to the gene encoding carboxypeptidase E (CpE), a processing enzyme involved in trimming C-terminal paired basic residues from prohormone-derived peptides. The mutation resulted in a loss of CpE activity that correlated with aberrant proinsulin processing. Neurotensin (NT) and melanin-concentrating hormone (MCH) are two neuropeptides that, among other central effects, inhibit food intake. Here, using RIA techniques coupled to reverse phase HPLC, we analyzed the processing products derived from the NT and MCH precursors in the brain of + /*fat* and *fat*/*fat* mice. Compared to control hypothalamic and brain extracts, *fat*/*fat* extracts had markedly reduced levels (>80%) of NT and neuromedin N (NN), another active pro-NT-derived peptide. In contrast, they exhibited high concentrations of biologically inactive NT-KR and NN-KR (NT and NN with a C-terminal Lys-Arg extension), two peptides that were undetectable in control extracts. MCH, which is located at the C-terminus of its precursor, was present in 2- to 3-fold higher amounts in *fat*/*fat* than in + /*fat* hypothalamus. Neuropeptide-Glu-Ile, another pro-MCH-derived neuropeptide separated from MCH by an Arg-Arg sequence, was present in amounts similar to those of MCH in control extracts. In contrast, neuropeptide-Glu-Ile was more than 10 times less abundant than MCH in extracts from obese mice. Our data are consistent with a deficit in CpE activity affecting the maturation of both pro-NT and pro-MCH. This suggests that abnormal neuropeptide and hormone precursor processing is a general phenomenon in *fat*/*fat* mice and supports the idea that defects in the production of neuropeptide involved in the control of feeding might lead to the development of obesity in these animals. (*Endocrinology* **137:** 2954–2958, 1996)

MICE THAT ARE homozygous for the *fat* mutation are characterized by marked hyperproinsulinemia and develop a late-onset obesity. It has recently been shown that the *fat* mutation maps to the gene encoding carboxypeptidase E (CpE) (1), a processing enzyme involved in the trimming of paired basic residues remaining at the C-terminus of prohormone-derived peptides (2). A single $Ser^{202}Pro$ mutation occurs in the mutant CpE allele and abolishes enzyme activity. This results in abnormal processing of proinsulin, leading to the production of poorly active diarginyl insulins (1). The deficit in biologically active insulin is thought to put increased demand on proinsulin biosynthesis and β-cell secretion; hence, the marked hyperproinsulinemia observed in *fat/fat* mice. However, several lines of evidence suggest that the hyperproinsulinemia might not be related to the late-onset obesity that develops in mutant mice (1). Instead, it was proposed that since CpE is expressed in all neuroendocrine tissues including brain, defects in the processing of neuropeptides involved in the control of feeding behavior and energy balance might cause the animals to become obese (1). However, to our knowledge, the processing of neuropeptide/hormone precursors other than proinsulin has not been examined to date in *fat/fat* mice.

A number of neuropeptides are known to regulate feeding behavior (see Ref. 3 for review). Among these peptides, central neurotensin (NT) and melanin-concentrating hormone (MCH) have been shown to inhibit food intake (4-7). The structures of rat pro-NT (8) and mouse pro-MCH (9) are schematized in Fig. 1. Both are multifunctional precursors containing several biologically active peptides that are in most cases flanked by sequences of paired basic residues. The processing of these precursors is thought to occur in the regulated secretory pathway where it is initiated by cleavage at the C-terminal side of dibasic sites mediated by prohormone convertases (PCs) such as PC1 and PC2, followed by CpE-catalyzed removal of C-terminal basic residues (Fig. 1) (reviewed in Refs. 10-12). Concerning pro-NT, processing in most brain regions has been shown to occur at the three most C-terminal Lys-Arg doublets to yield the biologically active peptides NT and neuromedin N (NN) (13). In the case of pro-MCH, which is chiefly synthesized in the hypothalamus, the two dibasic sites that flank neuropeptide-Glu-Ile (NEI) are processed to produce comparable amounts of both NEI and MCH (14). Figure 1 shows the peptides that are expected from the sequential actions of the PCs and CpE on the two precursors. A lack of carboxypeptidase activity would result in the production of intermediate peptides with C-terminal dibasic extensions. Note that MCH being located at the C-terminus of its precursor does not require CpE activity to be produced in a mature form.

In the present study, we analyzed the processing products derived from the NT and MCH precursors in the brain of

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Address all correspondence and requests for reprints to: Dr. Patrick Kitabgi, Institut de Pharmacologie Moléculaire et Cellulaire du CNRS, Université de Nice-Sophia Antipolis, Sophia Antipolis, 660 route des Lucioles, 06560 Valbonne, France. E-mail: kitabgi@unice.fr.
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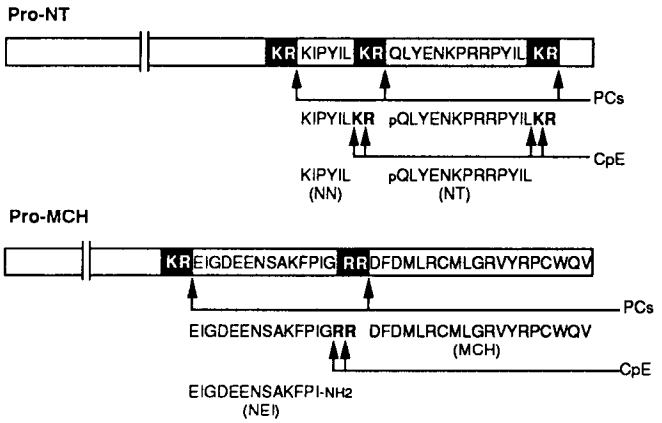

FIG. 1. Diagrammatic representation of pro-NT and pro-MCH and of the sequential enzymatic events thought to lead to the production of mature peptides. PCs first cleave at the C-terminal side of dibasic KR or RR sequences. C-Terminal dibasic extensions are then removed by CpE.

+/*fat* and *fat/fat* mice. Our data are consistent with a deficit in CpE activity affecting the maturation of both precursors. This suggests that abnormal neuropeptide and hormone precursor processing is a general phenomenon in *fat/fat* mice and supports the idea that defects in the production of neuropeptides involved in the control of feeding might lead to the development of obesity in these animals.

Materials and Methods

Synthetic peptides

NT and NN were purchased from Neosystem (Strasbourg, France). The peptides pQLYENKPRRPYILKR (NT-KR) and EIGDEENSAKFPI-GRR (NEI-GRR) were custom synthesized by Neosystem. The peptide KIPYILKR (NN-KR) was provided by Solange Lavielle (Université Paris VII, Paris, France). MCH and NEI were gifts from Carl M. Hoeger and Jean Rivier (The Salk Institute, La Jolla, CA).

Tissue extraction

Whole frozen brains from three BKS-+/*fat* and three BKS-*fat/fat* mice were kindly provided by Yves Rouillé and Donald F. Steiner (University of Chicago, Chicago, IL). The brains were allowed to thaw on ice, the hypothalami were dissected out, and the tissues were weighed. The brains (minus hypothalamus) were homogenized in 10 vol (vol/wt) ice-cold 0.1 N HCl with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The hypothalami were similarly homogenized in 500 μl of 0.1 N HCl. The extracts were then centrifuged at 20,000 × *g* for 30 min at 4 C, and the supernatants were heated for 10 min in a boiling water bath. The extracts were assayed for their protein content using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA) and kept frozen until use.

Antisera and RIAs

The characterization of the antisera directed against the C-terminus of NT (29G), the N-terminus of NT (28H; gifts from Jean-Claude Cuber, Lyon, France), and the N-terminus of NN (NN-Ah) and the RIA conditions employing these antisera have been previously described (15, 16). NT-KR cross-reactivity was 100% and less than 0.01% with antisera 28H and 29G, respectively. NN-KR cross-reacted 100% with antiserum NN-Ah.

Anti-MCH and anti-NEI antisera were kindly provided by Joan. M. Vaughan and Wylie W. Vale (The Salk Institute). The characteristics of these antisera have been previously described (17, 18). RIA conditions for MCH and NEI have been previously reported (19). NEI-GRR cross-reacted less than 0.1% with the NEI antiserum.

Citraconylation

Portions of brain and hypothalamic acid extracts were citraconylated, submitted to Arg-directed tryptic digestion (13, 16), and assayed for N-terminal immunoreactive NN (iNN). The value of CTiNN thus obtained provides an index of the total amount of pro-NT (either processed or unprocessed) that was synthesized and stored in the tissues at the time of extraction.

Reverse phase HPLC

Brain (10 mg tissue equivalent) and hypothalamic (4 and 2 mg tissue equivalent for control and mutant mice, respectively) extracts were injected onto a 4 × 250-mm Lichrosorb RP 18 (7 μm) column. Elution of pro-NT-derived products was carried out in 0.1% trifluoroacetic acid and 0.05% triethylamine-acetonitrile at a flow rate of 1 ml/min. The column was equilibrated in 10% acetonitrile, and 10 min after sample injection, a linear gradient was run from 10-40% acetonitrile in 42 min and then from 40-50% acetonitrile in 30 min. Fractions of 1 ml were collected, lyophilized, and reconstituted in 300 μl 0.1% trifluoroacetic acid containing 200 μg/ml BSA. The fractions were then assayed for their immunoreactive NT (iNT) and iNN content. Synthetic NT, NT-KR, NN, and NN-KR were used to calibrate the column and eluted with retention times of 41, 34, 43, and 34 min, respectively. Elution of pro-MCH-derived products was carried out in 0.1% trifluoroacetic acid-acetonitrile at a flow rate of 1 ml/min. The column was equilibrated in 20% acetonitrile, and 10 min after sample injection, a linear gradient was run from 20-60% acetonitrile in 40 min. The fractions were assayed for their immunoreactive MCH (iMCH) and NEI (iNEI) content. The retention times of synthetic MCH, NEI, and NEI-GRR were 34, 14, and 7 min, respectively.

Results

Pro-NT

Direct assays of C-terminal iNT, N-terminal iNT, N-terminal iNN, and CTiNN in brain and hypothalamic extracts from +/*fat* and *fat/fat* mice gave the results shown in Table 1. CTiNN, N-terminal iNT, and iNN contents were similar in the brain and hypothalamus of control and mutant animals. C- and N-terminal iNT levels were almost identical and were comparable to iNN concentrations in control mice. In marked contrast, C-terminal iNT levels were approximately 10-fold lower than the corresponding N-terminal iNT concentrations in cerebral tissues from mutant mice.

Figure 2 compares the typical HPLC profiles obtained with hypothalamic extracts from one control and one obese mouse. The results obtained with all control and mutant tissue samples are summarized in Table 2. All HPLC profiles of extracts from control mice showed single peaks of iNT and iNN that coeluted with synthetic NT and NN, respectively. In addition, the C- and N-terminal iNT peaks were superimposable. In extracts from obese mice, most of the N-terminal iNT and iNN materials coeluted with synthetic NT-KR and NN-KR, respectively. Small peaks of iNN and superimposed C- and N-terminal iNT that coeluted with NN and NT, respectively, were also present. Comparison of the pre- and post-HPLC data (Tables 1 and 2) indicates that the recovery of immunoreactive materials after HPLC was close to 90%.

Pro-MCH

As shown in Table 3, crude hypothalamic extracts from control and mutant mice contained large amounts of

TABLE 1. Levels of iNT, iNN, and CTiNN in brain and hypothalamic extracts from +/*fat* and *fat/fat* mice

	iNT (pmol/g)		iNN (pmol/g)	CTiNN (pmol/g)
	C-Terminal	N-Terminal		
+/*fat* brain	10.7 ± 1.3	10.4 ± 0.2	11.9 ± 2.3	25.2 ± 2.1
fat/fat brain	1.0 ± 0.1	9.6 ± 2.3	6.3 ± 1.3	27.1 ± 3.3
+/*fat* hypothalamus	51.2 ± 5.9	48.3 ± 6.7	56.3 ± 7.9	72.7 ± 6.5
fat/fat hypothalamus	6.3 ± 0.6	63.4 ± 2.3	47.6 ± 9.1	77.2 ± 23.3

C- and N-terminal iNT, and N-terminal iNN and CTiNN (an index of the total amount of pro-NT) were assayed as described in *Materials and Methods*. The values are the mean and SEM from three control and three mutant mice.

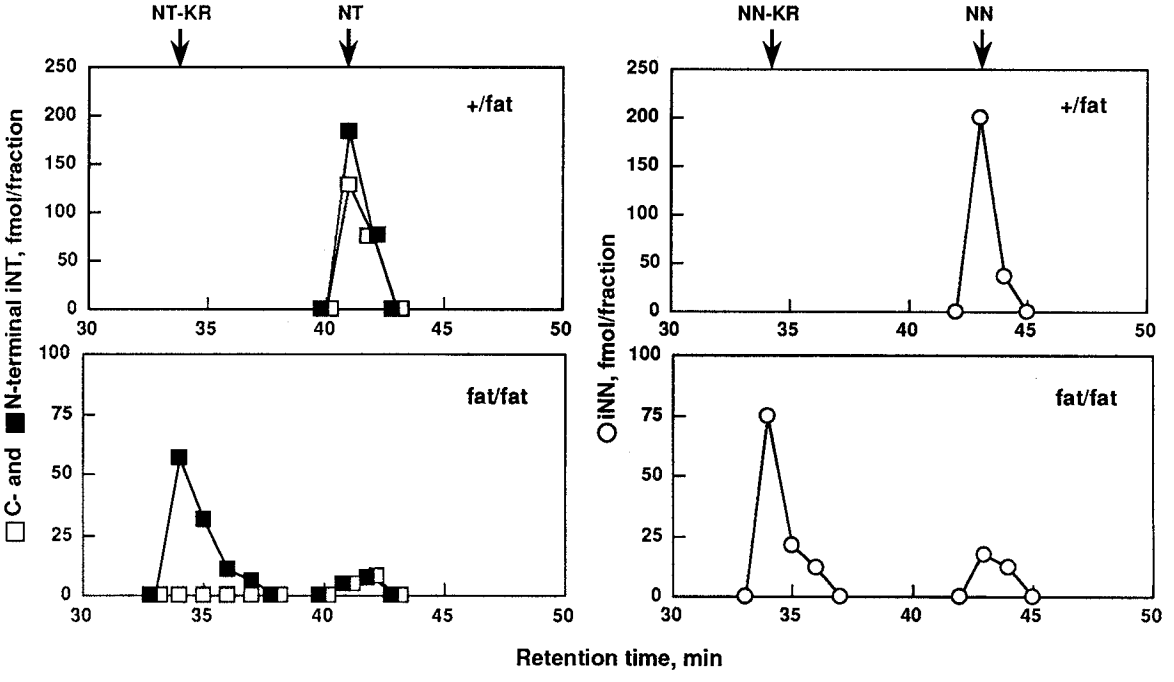

FIG. 2. Amounts of C- and N-terminal iNT (*left panels*) and N-terminal iNN (*right panels*) in HPLC-fractionated hypothalamic extracts from one control (*top panels*) and one *fat/fat* mouse (*bottom panels*). Four and 2 mg tissue equivalents from control and *fat/fat* extracts, respectively, were injected onto the HPLC column. HPLC conditions are given in *Materials and Methods*. All fractions were assayed for the various immunoreactive components, but only those fractions that reacted positively in the RIAs are represented. The elution positions of synthetic NT, NT-KR, NN, and NN-KR are indicated by *arrows*.

iMCH. Interestingly, a 2- to 3-fold increase in iMCH was noted in *fat/fat* mice compared to the controls. The amounts of iNEI were comparable to those of iMCH in control mice. In contrast, iNEI levels were 10-fold lower than iMCH concentrations in mutant mice. HPLC analyses of hypothalamic extracts from one control and one mutant animal are shown in Fig. 3. Similar data were obtained with the other animals and are summarized in Table 3. Single peaks of iMCH and iNEI coeluted with synthetic MCH and NEI, respectively, in control and mutant animals. The iNEI peak was similar in size to the iMCH peak in +/*fat* mice, whereas it was markedly smaller in *fat/fat* mice. No iNEI material with the retention time of NEI-GRR could be detected in mutant mice. The recovery of immunoreactive materials after HPLC was 50-60% for iMCH and 20-40% for iNEI (Table 3).

Discussion

The major finding of the present study is that the processing of two neuropeptide precursors, pro-NT and pro-MCH, was markedly impaired in the brain of *fat/fat* mice. Several pieces of evidence suggest that this impairment most likely resulted from the defect in CpE activity recently described in these animals (1). Thus, all of the precursor-derived products that need C-terminal trimming of dibasic sequences to achieve their mature form, *i.e.* NT, NN, and NEI were massively reduced (80-90%) in the brain of mutant compared to control mice. In addition, it could be demonstrated in the case of pro-NT that the major processing products detected in the mutants, *i.e.* NT-KR and NN-KR, were indeed bearing C-terminal dibasic extensions. NEI-GRR could not be detected in hypothalamic extracts from obese mice. This does not mean, however, that this material was not present, but, rather, reflects the fact that it cross-reacts poorly (<0.1%) with the anti-NEI antiserum used here. The fact that mature NT, NN, and NEI, although markedly decreased, were nonetheless detectable in the mutant brain could be explained by either a residual activity of the mutated CpE or the existence of other peptidases with CpE-like activity. The first hypothesis appears unlikely in view of the data reported by Naggert *et al.* (1). The second hypothesis would imply that the CpE-like enzyme, if present in all neuroendocrine cells, is not very efficient or, alternatively, that it is active but is localized in

TABLE 2. Amounts of NT, NT-KR, NN, and NN-KR in brain and hypothalamic extracts from +/*fat* and *fat/fat* mice

	NT (pmol/g)	NT-KR (pmol/g)	NN (pmol/g)	NN-KR (pmol/g)
+/*fat* brain	8.8 ± 1.6	ND	8.4 ± 1.5	ND
fat/fat brain	1.1 ± 0.3	6.7 ± 1.7	0.9 ± 0.2	6.1 ± 1.2
+/*fat* hypothalamus	46.2 ± 7.7	ND	52.4 ± 7.1	ND
fat/fat hypothalamus	4.9 ± 1.5	46.4 ± 4.5	11.7 ± 9.1	46.3 ± 4.4

Tissue extracts were fractionated on reverse phase HPLC, and the eluting peptides were identified by RIA and comigration with synthetic standards as described in Fig. 2. The NT values shown here were determined with the C-terminally directed antiserum. Similar values were obtained with the N-terminally directed antiserum (not shown). The values are the mean and SEM from three control and three mutant mice. ND, Not detectable.

TABLE 3. Levels of iMCH, MCH, iNEI, and NEI in crude and HPLC-fractionated hypothalamic extracts from +/*fat* and *fat/fat* mice

	iMCH (pmol/g) crude extract	MCH (pmol/g) post-HPLC	iNEI (pmol/g) crude extract	NEI (pmol/g) post-HPLC
+/*fat* hypothalamus	69.1 ± 2.9	37.7 ± 3.2	43.5 ± 11.0	17.2 ± 7.3
fat/fat hypothalamus	156 ± 30	91.7 ± 6.6	16.1 ± 6.3	3.5 ± 0.9

iMCH and iNEI were assayed in crude extracts as described in *Materials and Methods*. Tissue extracts were subjected to reverse phase HPLC, and the eluting peptides were identified by RIA and comigration with synthetic standards as described in Fig. 3. The values are the mean and SEM from three control and three mutant mice.

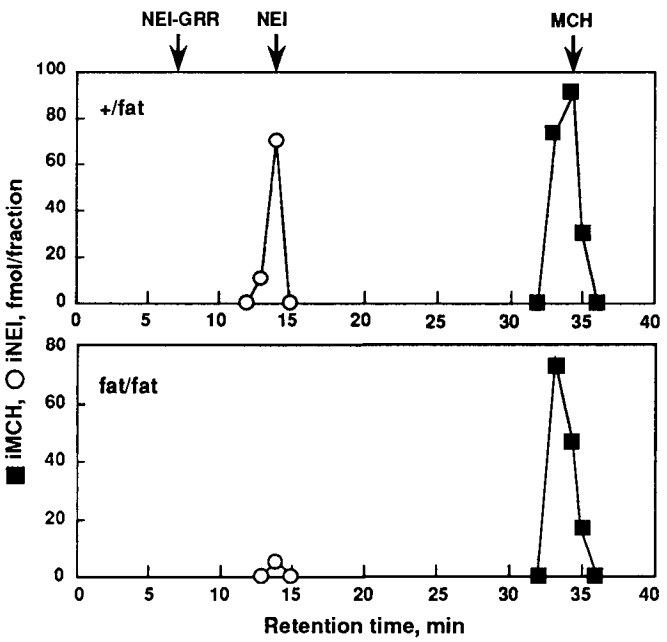

FIG. 3. Amounts of iMCH and iNEI in HPLC-fractionated hypothalamic extracts from one control (*top panel*) and one *fat/fat* mouse (*bottom panel*). Four and 2 mg tissue equivalents from control and *fat/fat* extracts, respectively, were injected onto the HPLC column. HPLC conditions are given in *Materials and Methods*. All fractions were assayed for the various immunoreactive components, but only those fractions that reacted positively in the RIAs are represented. The elution positions of synthetic MCH, NEI, and NEI-GRR are indicated by *arrows*.

only 10-20% of the neurons that produce pro-NT and pro-MCH. Further work will be required to clarify this issue.

The production of MCH was not impaired in the brain of *fat/fat* mice. This can be explained by the fact that this peptide being located at the C-terminus of its precursor does not require CpE activity to be produced in mature form. It further indicates that the PC(s) required for cleaving the dibasic that precedes MCH is fully active in the brain of *fat/fat* mice. It was also observed that the amounts of authentic and C-terminally extended NT and NN in mutant mice were comparable to the amounts of NT and NN, respectively, in control animals. This again means that the PC(s) performing the cleavages of the dibasic sites that flank these peptides is present and active in NT-producing neurons in *fat/fat* brain. This conclusion is in agreement with that reached by Naggert *et al.* (1) concerning PC1 and PC2 activities in the β-granules of *fat/fat* pancreatic β-cells.

The consequences of a defect in CpE activity for the biological activity of neuropeptides will be diverse, depending on the pharmacology and precursor location of these peptides. 1) Peptides that are internal to their precursor and whose C-terminal integrity is required for biological activity will be produced in inactive form. This is the case for NT and NN, which have been shown to interact with the same receptor through their common C-terminal pharmacophore and to produce similar biological effects (20). Structure-activity studies have demonstrated that C-terminal amidation or extension leads to a virtual loss of biological activity of NT and NT-related peptides (21). The present observation that *fat/fat* brain produces mostly NT-KR and NN-KR makes it likely that neurotensinergic transmission will be markedly impaired in these animals. This may also be the case for NEI, which requires an amidated C-terminus to be active (Nahon, J. L., unpublished observations). 2) Peptides that are internal to their precursor and whose C-terminal integrity is not required for biological activity, such as enkephalins, will be produced in active form. 3) Finally, neuropeptides that are located at the C-terminus of their precursor, such as MCH, will be produced intact.

A number of studies have suggested that NT may act as an inhibitor of food intake in the central nervous system (3-6). The markedly decreased production of active NT in the brain of *fat/fat* mice might, therefore, contribute to the development of obesity in these animals. The situation with pro-MCH is less clear. The effect, if any, of NEI on feeding behavior has not been investigated. It is, therefore, difficult to link the decreased NEI production seen in *fat/fat* hypothalamus to the obese state of these animals. With regard to MCH, it has been recently reported that the peptide potently decreases food intake when injected into the brain (7). The present study shows that au-

thentic MCH content is increased in the hypothalamus of obese compared to lean mice, which should tend to oppose the development of obesity. Interestingly, there is some evidence that MCH activates the hypothalamo-corticotrope axis via stimulation of hypothalamic CRF-containing neurons (22). CRF itself is a most potent anorectic agent (3, 23), and it may be speculated that it could mediate at least in part the inhibitory effect of MCH on food intake. Examination of the organization of pro-CRF and the structure-activity relationships of the peptide (23) indicates that a CpE-like activity is required for the production of biologically active CRF. From the present data showing impaired processing of two neuropeptide precursors, it is possible that CRF may not be correctly processed in *fat/fat* mice. This would further contribute to the obese state of these animals and render MCH inoperant as an anorectic agent. This might also provide a tentative explanation for the elevated MCH concentrations seen in *fat/fat* hypothalamus. Thus, one could speculate that a lack of active CRF synthesis will put an increased demand on CRF-producing cells and on the mediators (such as MCH) that stimulate these cells. A similar type of explanation has been put forward to account for proinsulin hyperproduction in pancreatic β-cells of *fat/fat* mice (1).

The above discussion is necessarily speculative and illustrates only some of the complex perturbations that may occur in peptidergic regulatory systems consequent to a defect in peptide processing such as that observed in *fat/fat* mice. It does not intend to suggest that the present data, limited as they are to two peptide precursors, account for the obese state of the mutant mice. Many peripheral and central neuropeptides (such as cholecystokinin, galanin, and neuropeptide Y) have been reported to exert positive or negative effects on feeding behavior (3), and the status of these peptides in *fat/fat* mice needs to be examined. We can only say that our data are consistent with a deficit in CpE activity affecting the maturation of pro-NT and pro-MCH. Together with the original observation of aberrant proinsulin processing (1), this suggests that abnormal neuropeptide and hormone maturation may be a general phenomenon in *fat/fat* mice and supports the idea that defects in the production of neuropeptides involved in the control of feeding behavior might lead to the development of obesity in these animals. *fat/fat* mice should provide an interesting model to further investigate alterations in neuropeptidergic transmission that may result from the impaired CpE activity that characterizes these animals.

Acknowledgment

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Note Added in Proof

Qu *et al.* (Nature 380:243–247, 1996) reported that icv injected MCH exerted an orexigenic effect in Long Evans rats. This is at variance with the anorexigenic effect of MCH described by Presse *et al.* in Wistar rats (7). Apart from the obvious rat strain difference, another major difference between the two studies stands in MCH doses used by Qu *et al.*, which were at least 30 times higher than those administered in the study by Presse *et al.* (7). This raises the possibility that the elevated MCH levels observed here in the hypothalamus of *fat/fat* mice might contribute directly to the obese state of these animals.

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