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Cellular Localization and Role of Prohormone Convertases in the Processing of Pro-melanin Concentrating Hormone in Mammals*

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Melanin concentrating hormone (MCH) and neuropeptide EI (NEI) are two peptides produced from the same precursor in mammals, by cleavage at the Arg^{145}-Arg^{146} site and the Lys^{129}-Arg^{130} site, respectively. We performed co-localization studies to reveal simultaneously the expression of MCH mRNA and proconvertases (PCs) such as PC1/3 or PC2. In the rat hypothalamus, PC2 was present in all MCH neurons, and PC1/3 was present in about 15–20% of these cells. PC1/3 or PC2 was not found in MCH-positive cells in the spleen. In GH_4C_1 cells co-infected with vaccinia virus (VV):pro-MCH along with VV:furin, PACE4, PC1/3, PC2, PC5/6A, PC5/6B, or PC7, we observed only efficient cleavage at the Arg^{145}-Arg^{146} site to generate mature MCH. Co-expression of pro-MCH together with PC2 and 7B2 resulted in very weak processing to NEI. Comparison of pro-MCH processing patterns in PC1/3- or PC2-transfected PC12 cells showed that PC2 but not PC1/3 generated NEI. Finally, we analyzed the pattern of pro-MCH processing in PC2 null mice. In the brain of homozygotic mutants, the production of mature NEI was dramatically reduced. In contrast, MCH content was increased in the hypothalamus of PC2 null mice. In the spleen, a single large MCH-containing peptide was identified in both wild type and PC2 null mice. Together, our data suggest that pro-MCH is processed differently in the brain and in peripheral organs of mammals. PC2 is the key enzyme that produces NEI, whereas several PCs may cleave at the Arg^{145}-Arg^{146} site to generate MCH in neuronal cell types.

Prohormones give rise to multiple peptides through cleavage at specific sites, usually basic residues, by endoproteases (reviewed in Refs. 1 and 2). Seven prohormone convertases (PCs)[1] have been identified so far in different mammalian cellular models, and all participate in selective cleavage of precursors on the C-terminal side of pairs of basic residues (reviewed in Refs. 3–5). Based on their tissue distribution and intracellular localization, the mammalian subtilisin/kexin-like serine proteinases can be subdivided into four classes: (i) furin (6) and the recently discovered PC7 (7) process precursors that reach the cell surface via the constitutive secretory pathway; (ii) PC1/3 and PC2 (reviewed in Refs. 5 and 8) process precursors whose products are stored in neuroendocrine secretory granules; (iii) PACE4 (9), the isoforms PC5/6-A (10–12), and PC5/6-B (13), which are expressed in both endocrine and nonendocrine cells, conceivably process precursors in both the constitutive and regulated secretory pathways; (iv) PC4, which is predominantly synthesized in testicular germ cells (14, 15), appears important for fertilization (16). Defining the cellular co-localization of each PC with its cognate substrate(s) is necessary in order to ascribe a role for these enzymes in particular precursor processing events. In addition, dramatic developmental changes in the tissue expression of the PCs have been reported (17, 18). These PCs could therefore participate in the differential processing of neuroendocrine precursors, such as those encoding multiple bioactive peptides. Indeed, PC1/3 and PC2 cleave pro-opiomelanocortin at distinct pairs of basic residues in established cell lines (19) and in a temporal order in the corticotrophs (20). Specific patterns of cleavage by either PC1/3 or PC2 have also been demonstrated for proglucagon (21–23), proneurotensin (24), pro-thyrotropin-releasing hormone (25), proenkephalin (26, 27), and procholecystokinin (28, 29). The functional relevance of the proconvertases in a whole animal model was recently established by disrupting the corresponding genes such as these encoding PC2 (30) and PC4 (16).

Melanin-concentrating hormone (MCH) and neuropeptide EI (NEI) are two distinct peptides encoded in the same precursor and co-expressed and secreted by neurons in the brain (31–34) and different cell types (macrophages, Sertoli cells, spermatogonia) in the peripheral tissues of mammals (35–37) (Fig. 1). Both peptides are synthesized in the perikarya of neurons in the lateral hypothalamic area (LHA) and zona incerta and serve as neurotransmitters/neuromodulators in a number of

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[1] The abbreviations used are: PC, proconvertase; MCH, melanin-concentrating hormone; NEI, neuropeptide EI; LHA, lateral hypothalamic area; NGF, nerve growth factor; rMCH, rat MCH; WT, wild type; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; RIA, radioimmunoassay; HPLC, high pressure liquid chromatography; RP-HPLC, reverse phase HPLC; VV, vaccinia virus.

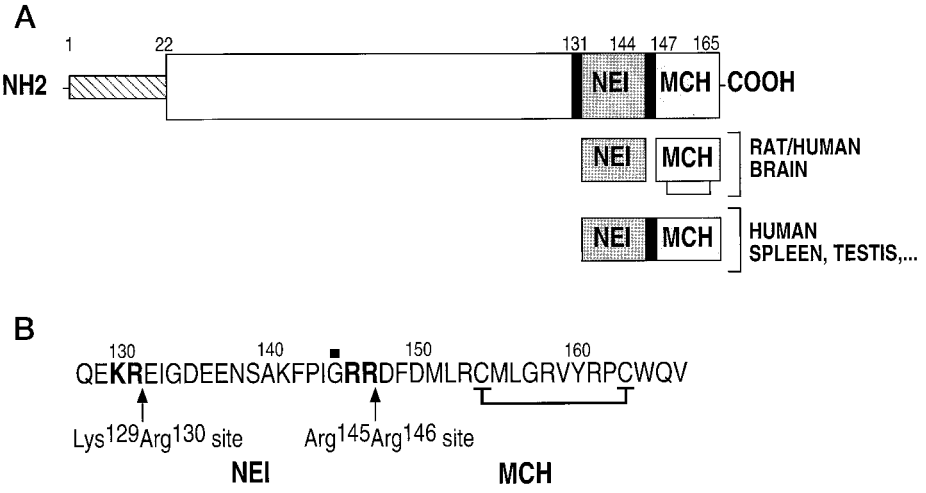

FIG. 1. *A*, schematic representation of the mammalian pro-MCH and of its peptide products detected in rodents and humans. *B*, sequence of the C-terminal domain of mammalian pro-MCH. The positions of the two pairs of basic residues are shown in *boldface type*. The disulfide bridge in MCH is indicated. ■, amidation site.

neural functions (reviewed in Refs. 38 and 39). Recently, several laboratories have suggested an important role for MCH in the regulation of satiety/appetite and associated pathologies such as obesity (40–43).

In the rat and human brain, exclusively mature peptides, *i.e.* cyclic MCH and amidated NEI, were identified (44, 45). Larger forms were found in the human colon, thymus, or adipose tissues (45) (Fig. 1, *A* and *B*). In the rat gut (35), testis (37), and spleen[2] most of the MCH immunoreactivity also did not correspond to the mature peptides, but the structure of these pro-MCH derivatives is not yet established. These tissue-specific differences in MCH-derived peptides probably result from differential processing of pro-MCH. In the current study, we examined (i) the cellular co-expression of different convertases in the rat brain and selected peripheral tissues (spleen, testis); (ii) the processing of mammalian pro-MCH in GH_4C_1 cells expressing various convertases using vaccinia expression systems; (iii) the processing of rat pro-MCH after stimulation by 50 ng/ml nerve growth factor (NGF) and 20 mM lithium of PC12 cells engineered to express either PC1/3 or PC2; (iv) the effects of disruption of the PC2 gene on the processing of pro-MCH in the brain, spleen, and testis of transgenic mice. Our main finding is that active PC2 is necessary for the formation of mature NEI in the brain and in cellular models. In contrast, many convertases (including PC1/3 and PC2) have the capacity to generate MCH from its precursor both in virally infected GH_4C_1 cells and in an animal model.

MATERIALS AND METHODS

Co-localization Studies: in Situ Hybridization/Immunohistochemistry—Frozen sections (12 μm-thick) of brain, spleen, or testis from adult Wistar rats (180 g weight) were cut on a cryostat at −20 °C and thaw-mounted on silane-coated slides. Tissue sections were fixed in 4% paraformaldehyde, 1× PBS for 15 min at room temperature, washed in 2× PBS for 5 min two times, and prehybridized for at least 1 h. An adequate volume of hybridization mixture containing ^{33}P-labeled RMCH2 oligoprobe (35) was spotted on each slide, sealed under coverslip, placed in a humidified chamber, and incubated at 25 °C below the temperature of fusion (T_m). The slides were rinsed three times for 20 min in 1× SSC at 42 °C under agitation and dehydrated in serial 70–100% ethanol baths. The labeling was controlled by overnight exposure using a phosphor imager (bioimaging analyzer, BAS-1500, Fujifilm). MCH-positive slides were then incubated for 24 h at 4 °C with primary antiserum (PC1/3 and PC2 antisera were provided by one of us) at a dilution of 1:200 in phosphate-buffered saline buffer containing 0.3% Triton X-100, 0.02% normal goat serum. Sections were washed in 1 × PBS solution, and peroxidase immunohistochemistry was done with an Immuno Pure ABC staining kit (Pierce) following the manufacturer's recommendations. Finally, the sections were dipped in LM1 nuclear emulsion (Amersham Pharmacia Biotech), exposed for about 1 week, and developed. About 50 neurons/section (at three different levels in the rostral-to-caudal orientation) were examined for co-localization of MCH mRNA and PC(s) in the hypothalamus. Cells labeled with MCH oligoprobe were taken as positive where at least 10 grains or strong hybridization signal confined to the cytoplasm were detected. Cells were considered PC-positive where they presented a strong brown color.

Vaccinia Virus (VV) Constructs and Infections—Purified recombinant VV using the full-length cDNA inserts of the rat MCH (VV:rMCH), the mouse PC1/3 (VV:PC1/3), PC2 (VV:PC2), PC5/6 A or B (VV:PC5), PC7 (VV:PC7), human furin (VV:hfurin), human PACE4 (VV:hPACE4), and mouse pro-7B2 (VV:27-kDa 7B2) and active 7B2 (VV:21-kDa 7B2) were constructed as reported previously (19, 22, 46). The VV:recombinant convertases were found to be expressed at high levels in GH_4C_1 cells (Refs. 19, 22, 25, and 26 and data not shown). Conditions for cellular infections were as described previously (19, 22). Briefly, GH_4C_1 cells at P15 (50×10^6 cells/plate) were infected with recombinant VV for 1 h and plated for 18 h, and cell extracts and 18-h media were collected. In some cases after 18-h infection, cells were incubated with serum-free medium for 4 h, and the media were collected. Proteins or peptides were extracted as described below. Two to four separate sets of experiments were performed with plates in duplicate for each VV:recombinant convertase preparation (mean values are shown).

PC1/3 or PC2-PC12 Cell Transfectants—Wild type (WT) PC12 cells, PC1-expressing PC12 cell lines (L1.2 and E1.2), and PC2-expressing PC12 cell lines (L2.2 and E2.15) obtained from Dr. P. Kitabgi's laboratory (Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France) were cultured as described previously (24). The PC12 cell lines were stimulated for 24 h (T_{24}) or not (control; *C*) with optimum concentrations of NGF (50 ng/ml) and LiCl (20 mM) to induce MCH mRNA synthesis (47). Expression of MCH mRNA and pro-MCH-derived peptides (MCH, NEI) was determined by RT-PCR (47) and by RIA coupled to RP-HPLC as described below. MCH mRNA was detected only in NGF/lithium-treated PC12 cells, whereas PC1/3 or PC2 was expressed in resting and lithium/NGF-treated cells (Ref. 24 and data not shown).

PC2 Null Mutant Mice Model—PC2 null mutant mice were generated after homologous recombination in introducing the neomycin gene into the third exon of the mouse PC2 gene (30). This resulted in the production of an inactive truncated PC2 protein (30). Tissues (brain, gut, testis) from 12 WT and 12 PC2 null mice (PC2 KO) were dissected and immediately frozen at −70 °C. Tissues were used either for RNA or peptide extraction as described below.

RNA Extraction and Semiquantitative RT-PCR Analysis—Hypothalami from three wild type and PC2 null mice were dissected following partial thawing, and total RNA was extracted as described previously (35). The quality of the RNA was monitored by ethidium bromide staining of 0.8% agarose gel after electrophoresis.

Semiquantitative RT-PCR analysis was performed using competition between reverse-transcribed RNA from tissues and a short template (MCHΔ) (48) amplified with RMCH2 (5′-CCAACAgggTCggTAgACTCgTCCCAgCAT-3′) and RMCH60 (5′-gAgCggCTTCATgAAggATgAC-3′) primers. Detailed protocols of the reverse transcription and amplification methods were published previously (45, 49). Briefly, hypothalamic RNAs (1 μg) were reverse-transcribed in 20 μl of reaction mixture according to a standard protocol (49). PCR was performed in a

[2] G. Hervieu and J.-L. Nahon, unpublished data.

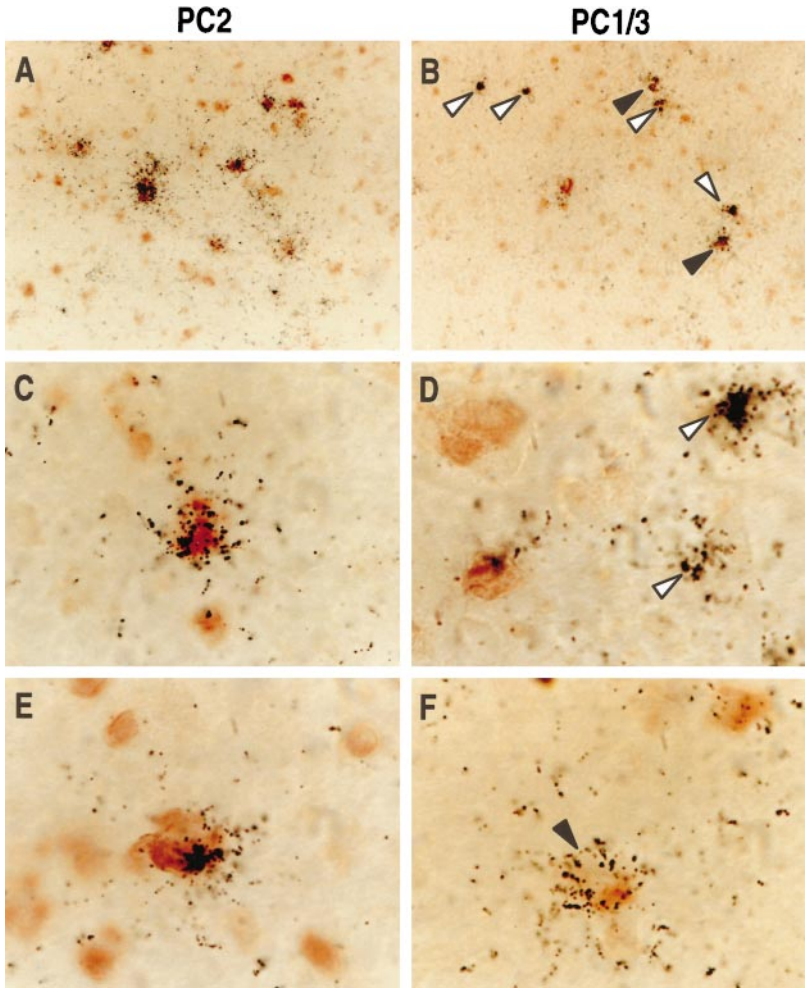

FIG. 2. **Co-localization of MCH mRNA and PC2 or PC1/3 proteins in the rat hypothalamus.** Rat brain sections were hybridized with ^{33}P-labeled RMCH2 oligonucleotide, and PC1/3 or PC2 protein was detected using specific antisera as described under "Materials and Methods." PC2 was found in all MCH mRNA-expressing neurons (*A*, *C*, and *E*). PC1/3 co-localized with MCH in some neurons (*solid arrows*), whereas other MCH mRNA-expressing neurons lacked PC1/3 immunostaining (*open arrows*) (*B*, *D*, and *F*). Magnification: × 40 (*A* and *B*); × 250 (*C*–*F*).

reaction solution (30 μl) containing 1× PCR buffer (Appligene, France), 200 μM deoxy-NTPs, 10 μCi of [^{32}P]dCTP (Amersham, Buckinghamshire, United Kingdom), 1 μM each of RMCH2 and RMCH60 primers, 0.2 units of *Taq* polymerase (Appligene, France), 2 μl of 10-fold dilution of the reverse-transcription mixture, and various quantities of MCHΔ (0.01–10 ng/μl). The PCR profile involved heating at 95 °C for 3 min with dNTP, primers and reverse-transcribed RNA only, and then PCR solution and *Taq* polymerase were added to perform 25 cycles of amplification as follows: 50 s at 94 °C (denaturation), 50 s at 50 °C (annealing), 1 min 30 s at 72 °C (extension). The amplification products (10 μl of the PCR mixture) were separated by electrophoresis on a 2.3% metaphore-agarose (FMC Bioproducts, Rockland, ME) gel, fixed for 5 min with 7% trichloroacetic acid, and then dried and finally exposed to x-ray films. Quantitative densitometric analysis was carried out using a computerized image analysis system (bioimaging analyzer, BAS-1500, Fujifilm).

Preliminary experiments established that primers amplify target and internal MCHΔ sequences with similar efficiencies, and appropriate ratios of unknown and competitive templates were also initially determined (data not shown). As a negative control, only MCHΔ amplification was found in the absence of reverse-transcribed RNA (not shown).

Peptide Extraction—Virus-infected GH_4C_1 cells were sonicated in 1 ml of 0.06 N HCl, 0.1% (v/v) trifluoroacetic acid solution. Extracts were passed through a C18 silica cartridge (C18 Sep Pak, Waters Associates, Milford, MA), and absorbed peptides were eluted with 55% (v/v) CH_3CN and lyophilized.

Peptides were extracted from PC-transfected PC12 cells with 0.1 N HCl. Extracts were centrifuged 10 min at 3,500 × *g*. Supernatants were kept at 95 °C for 10 min and stored at −20 °C until use.

After homogenization by a Polytron homogenizer, tissues were boiled for 10 min in 0.1 M HCl and centrifuged twice at 10000 × *g* at room temperature for 20 min. The supernatants were neutralized with NaOH, and the peptide solutions were lyophilized. The dried peptide extracts were resuspended in water and used either for a direct RIA or an RP-HPLC purification.

RP-HPLC Condition—Reverse-phase HPLC (RP-HPLC) was performed with a C18 Merck Lithosorb column 100-RP (5 μm) or a C18 PLRP-S column 300A (8 μm) (Polymer Laboratories, Amherst, MA). The mobile phase was 0.1% trifluoroacetic acid solution. The peptides were eluted with an acetonitrile/trifluoroacetic acid 0.1% gradient from 20 to 60% in 40 min with a 1-ml/min flow rate after an isocratic step at 20% for 10 min. The retention times of the synthetic rat/human MCH and NEI were, respectively, 31 or 33 min and 8–9 or 13 min, depending on the column used. Synthetic MCH and NEI were gifts from Drs. Carl M. Hoeger and Jean Rivier (Salk Institute, La Jolla, CA). Synthetic NEI-GRR (43) was custom-synthesized by Neosystem (Strasbourg, France).

MCH and NEI Radioimmunoassays—RIAs were performed as described (36, 45). Briefly, 50 μl of rat MCH or NEI antiserum (final dilution 1:300,000), 50 μl of the standard peptide or biological samples, 50 μl of the iodinated peptide (10^5 cpm/ml), and 100 μl of phosphate buffer were mixed. After 24 or 48 h of incubation at 4 °C, bound radioactivity was precipitated by adding 50 μl of bovine serum, 50 μl of 0.6% Tween 20, and 350 μl of 25% polyethylene glycol 6000 for 10–20 min. Tubes were then centrifuged for 10 min at 4000 rpm at 4 °C, and the supernatant was discarded. The pellets were counted in a Crystal Packard for automatic calculation of the bound/total ratio. Blank values (*i.e.* radioactivity precipitated without antibodies) were always <6%. The routine sensitivity of the assays was 250 and 50 fmol/sample for the MCH and NEI RIA, respectively. The NEI antiserum recognized equally well the amidated NEI peptide and NEI-GR, but NEI-GRR cross-reacted <0.1% with this antiserum (43, 45).

RESULTS

Co-localization of MCH mRNA and Proconvertases in the LHA—Using ^{33}P-labeled RMCH2 oligoprobe on the one hand and different proconvertase antisera on the other, we simultaneously examined the expression of MCH mRNA and the

TABLE I
Processing of pro-MCH in GH_4C_1 cells
A comparison of cellular and secreted contents after furin, PC1/3, or PC2:vaccinia virus infections is shown.

VV infection	MCH-IR			NEI-IR		
	Total[a]	Peptide[b]	Cleavage[c] Arg^{145}-Arg^{146}	Total[a]	Peptide[b]	Cleavage Lys^{129}-Arg^{130}
	pmol / 10^6 cells	*pmol / 10^6 cells*	*%*	*pmol / 10^6 cells*	*pmol / 10^6 cells*	*%*
Human furin						
Cells	4.6	2.1	45	0.3	<0.001	ND[d]
18-h media	10.1	4.1	40	0.2	<0.001	ND
4-h media	1.8	0.5	30	<0.001	<0.001	ND
PC1/3						
Cells	3.3	1.1	35	0.2	<0.001	ND
18-h media	8.7	3.2	37	0.1	<0.001	ND
4-h media	1.7	0.5	30	<0.001	<0.001	ND
PC2						
Cells	2.6	0.8	33	0.1	<0.001	ND
18-h media	6.9	2.8	40	<0.001	<0.001	ND
4-h media	1.7	0.6	35	<0.001	<0.001	ND
MCH						
Cells	3.0	<0.001	ND	<0.001	<0.001	ND
18-h media	11.0	<0.001	ND	0.2	<0.001	ND
4-h media	1.3	<0.001	ND	<0.001	<0.001	ND

[a] Cellular content in total MCH-IR and NEI-IR (pmol/10^6 cells).
[b] Cellular content in mature MCH or NEI determined after RP-HPLC analysis (pmol/10^6 cells).
[c] Percentage of cleavage at the basic sites as described under "Materials and Methods." The values are the means for triplicate determinations with two cell plates.
[d] ND, not done.

amounts of PC1/3 and PC2 protein in the rat LHA. As shown in Fig. 2, *A* and *B*, both PC1/3 and PC2 immunoreactivities were found within or close to MCH mRNA-expressing neurons. At higher magnification, strict co-localization was demonstrated for PC2 and MCH mRNA in all perikarya, whereas only part of the MCH mRNA-expressing neurons co-localized with PC1/3 (Fig. 2, *C* and *E*, and Fig. 2, *D* and *F*, respectively). We estimated that about 15–20% of MCH neurons contained both PC1 and PC2 (27 ± 7 of 150 individual neurons examined per rat; $n = 2$ rats).

Pro-MCH Processing in GH_4C_1 Cells—Pro-MCH processing was determined in GH_4C_1 cells after co-infection of recombinant vaccinia virus engineered to express rat pro-MCH or either of the prohormone convertases listed in Tables I and II. Media were collected during an 18-h period of infection (18-h media) and following a further 4 h of infection in a fresh serum-free media (4-h media). Cell extracts were isolated after the 22 h of infection. Immunoreactive peptide content was measured with specific RIAs in total cellular extracts or collected media and in fractions following RP-HPLC analysis. Both MCH and NEI RIA have previously been shown to be highly specific for their respective peptides (36, 45). However, it is worth noting that MCH antisera recognized equally well the mature peptide, processing intermediates and the full-length precursor (unpublished data),[2] whereas the NEI antisera can identify amidated NEI, NEI-G, and NEI-GR forms but cross-react poorly with other pro-MCH derivatives and the precursor itself (36, 45). Therefore, the MCH-IR concentrations in whole cell extracts or collected media reflected the total amounts of intracellular pro-MCH derivatives (processed plus unprocessed forms), and the NEI-IR concentrations indicated the amounts of processed NEI peptide.

No MCH-IR or NEI-IR was detected in WT GH_4C_1 cells, whereas infection with VV:MCH resulted solely in the production of pro-MCH (Tables I and II). We assessed first the processing capability of one ubiquitous PC (furin) and the two PCs (PC1/3 and PC2) found co-localized with MCH and NEI in pro-MCH-expressing perikarya by coinfecting GH_4C_1 cells with VV:MCH and either of the PC. As shown in Table I, MCH-IR was found in both media and cell extracts after furin, PC1/3, or PC2:VV infection, whereas NEI-IR was absent. The ratio of MCH peptide was very similar in cell extracts, 18-h media, and 4-h media in all of the PC:VV co-infection, consistent with the constitutive secretion of prohormone derivatives in the GH_4C_1 cells. The cleavage efficiency at the Arg^{145}-Arg^{146} site was quite similar between PC1/3 and PC2 and tends to be higher in cell extracts after the furin:VV infection.

We extended this analysis to the other PCs and determined the intracellular content of pro-MCH peptides in GH_4C_1 cells infected with either of PC:VV. As shown in Table II and Fig. 3*A*, all tested prohormone convertases were able to cleave the pro-MCH at the pair of basic residues Arg^{145}-Arg^{146} to yield mature MCH with an order of efficiency that was PC7 > furin > PACE4 > PC1/3 ≥ PC2 = PC5/6-B ≫ PC5/6-A. In contrast, no detectable processing of pro-MCH at the pair of basic residues Lys^{129}-Arg^{130} was observed with any of the prohormone convertases other than PC7, albeit this enzyme displayed very low cleavage efficiency (Table II and Fig. 3*A*). Similar patterns of pro-MCH processing by each of the PC(s) were found when peptides were determined in the media collected during the 18-h infection period as found for furin, PC1/3, and PC2:VV infection (Table I and data not shown).

The PC2-specific binding protein 7B2 has been shown to be essential for the expression of active PC2 (50–52). VV:PC2 plus 27-kDa 7B2 produced a large amount of mature MCH (63% cleavage efficiency; not shown). In contrast, simultaneous infection of GH_4C_1 cells with VV:MCH, the 7B2 precursor (VV:27-kDa 7B2), or the processed 7B2 (VV:21-kDa 7B2) and VV:PC2 resulted in a weak cleavage at the Lys^{129}-Arg^{130} site (0.6–0.4% cleavage efficiency) as further demonstrated by RP-HPLC analysis (Fig. 3*B*). In addition, only mature NEI was revealed (Fig. 3*B*), and putative processing intermediates such as the pro-MCH-(130–165) (44-min peak, Fig. 7) were lacking (data not shown).

These results indicated that in the vaccinia expression system, any of the prohormone convertases may potentially liberate MCH without being able to produce an equivalent amount of NEI. Coinfection of GH_4C_1 cells with VV:PC7 or VV:PC2 plus 7B2 (precursor or active form) resulted in weak processing to mature NEI.

PC2 but Not PC1/3 May Generate NEI in Stably PC-transfected PC12 Cells—We hypothesized that PC1/3 and PC2 may be responsible for cleavage at pairs of basic residues found in pro-MCH to yield mature NEI and MCH but only under re-

TABLE II
Processing of pro-MCH in GH_4C_1 cells infected with VV:PC recombinants

VV infection	MCH-IR			NEI-IR		
	Total[a]	Peptide[b]	Cleavage[c] Arg^{145}-Arg^{146}	Total[a]	Peptide[b]	Cleavage[c] Lys^{129}-Arg^{130}
	pmol/10^6 cells	*pmol/10^6 cells*	*%*	*pmol/10^6 cells*	*pmol/10^6 cells*	*%*
Human furin	5.6	3.3	59	0.7	<0.001	ND[d]
Human PACE4	6.4	3.1	49	<0.001	<0.001	ND
PC1/3	6.4/2.8	3.2/0.9	40	0.2/0.07	<0.001	ND
PC2	4.1/3.0	1.4/1.1	35	0.1/0.02	<0.001	ND
PC5/6A	5.4	1.2	23	<0.001	<0.001	ND
PC5/6B	6.0	2.5	42	<0.001	<0.001	ND
PC7	1.8	1.6	93	0.09	0.04	2
MCH	4.0	<0.001	ND	<0.001	<0.001	ND
WT	<0.001	<0.001	ND	<0.001	<0.001	ND

[a] Cellular content in total MCH-IR and NEI-IR (pmol/10^6 cells).
[b] Cellular content in mature MCH or NEI determined after RP-HPLC analysis (pmol/10^6 cells).
[c] Percentage of cleavage at the basic sites as described under "Materials and Methods." The values are the means for triplicate determinations with two cell plates. Two separate sets of viral infection were shown for PC1/3 and PC2 as indicated by the slashes. The data corresponding to human furin, PC1/3, and PC2:VV infection are additional to those summarized in Table I.
[d] ND, not done.

stricted physiological conditions not reached in the vaccinia expression system. Accordingly, PC1/3- or PC2-stably transfected PC12 cells from P. Kitabgi's laboratory (Valbonne, France) were used in an additional study. It has been demonstrated that wild type PC12 cells lack both of these PCs and that the stably transfected cell lines express large amounts of active enzyme capable of processing endogenous prohormones such as proneurotensin (24). Furthermore, we have previously shown that MCH mRNA is absent in resting PC12 cells but can be induced by the addition of NGF and lithium for 24 h, resulting in MCH mRNA production (47). The levels of MCH-IR and NEI-IR were determined using RIA on RP-HPLC fractions of cellular extracts from control (Fig. 4, *C*) or NGF/lithium-treated (Fig. 4, *T24*) PC12 cells. The MCH RIA revealed a broad peak of immunoreactivity in all PC12 cell lines tested under resting or stimulated conditions (data not shown). Further Western blot analysis demonstrated cross-reactivity of MCH antiserum to several protein species in PC12 cellular extracts (not shown). Therefore, it was not possible to ascertain whether PC1/3 or PC2 cleave at the Arg^{145}-Arg^{146} site in this cellular model. However, a single peak, corresponding to mature NEI, was identified only in the PC2-transfected cells (L2.2) treated with NGF and lithium (Fig. 4). The level of NEI was estimated to about 0.3 pmol/10^6 cells, *i.e.* at least 10-fold the amount of peptide found in VV:MCH/PC2 plus 7B2-infected GH_4C_1 cells (see Table II and Fig. 3*B*). No NEI immunoreactivity was found in WT and PC1-transfected cells or in unstimulated L2.2 cells (Fig. 4). These results then support the hypothesis of preferential cleavage by PC2 at the Lys^{129}-Arg^{130} site in neuroendocrine cells. The same pattern of pro-MCH processing was observed in another PC2-transfected PC12 cell line (E2.15; Ref. 24) (data not shown).

PC2 Is Necessary for the Formation of NEI but Not MCH in the Hypothalamus—To test directly whether PC2 was actually involved in producing MCH and NEI, we determined whether pro-MCH processing could be affected by the lack of active PC2 in a transgenic mice model (30). Peptides were extracted from the whole brain, hypothalamus, brain without hypothalamus, spleen, and testis of WT or KO mice, and MCH-IR and NEI-IR contents were measured by RIA in crude extracts and after RP-HPLC separation.

The results of representative experiments concerning MCH and NEI assays in the brain, spleen, and testis of WT and KO mice are summarized in Table III. As expected, MCH-IR and NEI-IR contents were quite similar in WT mice. Surprisingly, the KO mice showed higher amounts of MCH-immunoreactive materials by comparison with the WT mice, solely in the hypothalamus. On the other hand, the NEI-IR content was drastically diminished in both whole brain and hypothalamus of KO mice compared with WT (≥16-fold reduction; Table III). Hypothalamic cell extracts were purified on RP-HPLC, and similar amounts of mature MCH and NEI were found in WT mice, whereas MCH concentrations were 2–3-fold higher in KO (Fig. 5). In agreement with previous data using crude extracts (Table III), no mature NEI or other processed form was observed in the hypothalami of KO mice. These findings strongly suggest that cleavage at the pair of basic residues Lys^{129}-Arg^{130} of pro-MCH in the brain is dependent upon PC2 activity, whereas cleavage at the Arg^{145}-Arg^{146} site is not affected by the lack of active PC2.

The apparent increase in MCH-IR in the hypothalamus of PC2 null mice could result either from enhanced synthesis, possibly reflected at the level of MCH mRNA, or regulation at post-translational levels, such as an increase in storage of MCH-containing secretory granules. The first possibility was examined by using a semiquantitative RT-PCR assay (48). As shown in Fig. 6, the levels of MCH mRNA were similar in WT and KO mice, indicating that changes in MCH-IR contents were not accompanied by modifications in MCH gene expression. Further attempts to reveal differences in the pattern of pro-MCH derivatives using either Western blot (not shown) or RP-HPLC analysis (Fig. 5, *left panels*) with MCH antiserum demonstrated the presence of a single peak corresponding to mature MCH in both WT and KO mice. These results are therefore consistent with the hypothesis of an increase of storage of MCH in the soma located within the LHA of the KO mice.

A Large Form of MCH Peptide Is Produced in Peripheral Tissues of Mice—In peripheral organs such as spleen and testis, RIA of cell extracts for MCH-IR indicated no significant differences between the WT and KO mice, suggesting that PC2 was not involved in the processing of pro-MCH in these tissues (Table III). No NEI-IR could be detected in spleen or testis of both types of mice (Table III). Further RP-HPLC analysis with spleen extracts revealed a single peak of MCH-IR at 44 min, and no MCH-IR or NEI-IR was found co-eluting at the position of the corresponding synthetic peptide (Fig. 7). The material corresponding to the 44-min peak displayed the characteristics of the intermediate cleavage form, pro-MCH-(130–165), already identified in several human peripheral tissues (45). The apparent increase in MCH-IR material in KO mice *versus* WT

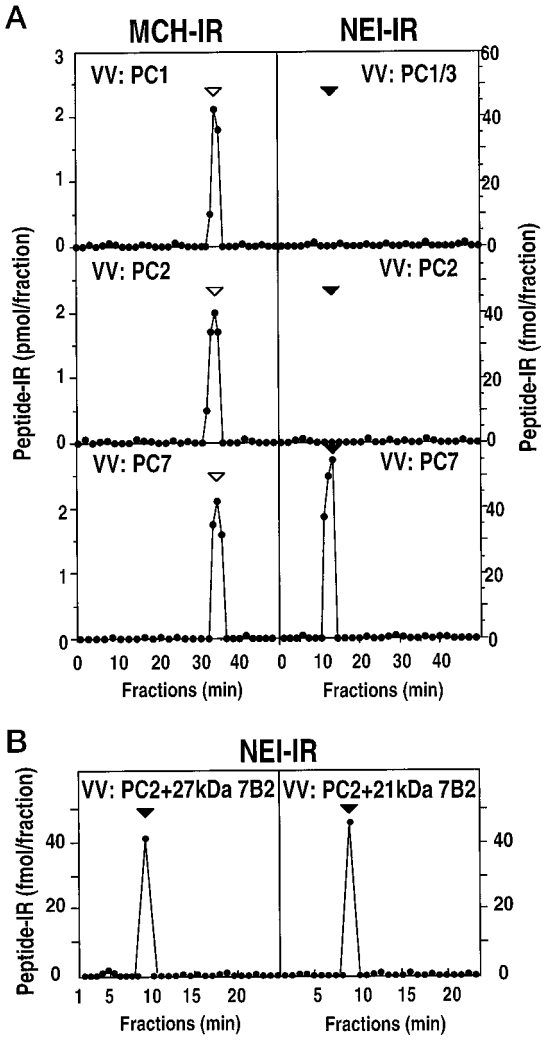

FIG. 3. **RP-HPLC analysis of MCH-IR and NEI-IR in GH_4C_1 cells.** *A*, peptides extracted from VV:PC1-infected cells, VV:PC2-infected cells, or VV:PC7-infected cells together with VV:rMCH were applied to a C18 Merck Lithosorb column, and all the fractions were assayed for their MCH-IR (*left panels*) and NEI-IR (*right panels*) contents. The *open* and *black arrowheads* indicate, respectively, the elution position of the synthetic rat/human MCH (33 min) and NEI (13 min) standards. *B*, NEI-IR content in GH_4C_1 cells infected with VV:PC2 and VV:7B2. GH_4C_1 cells (50 or 55 $\times$ 10^6 cells/plate in duplicate) were coinfected with VV:rMCH (1 plaque-forming unit), VV:PC2 (1 plaque-forming unit), and either VV:27-kDa 7B2 (1 plaque-forming unit) or VV:21-kDa 7B2 (1 plaque-forming unit). The same amounts of extracts were chromatographed using a C18 PLRP column, and NEI-IR was determined by RIA. The elution position (9 min) of synthetic amidated NEI is indicated by a *black arrowhead*.

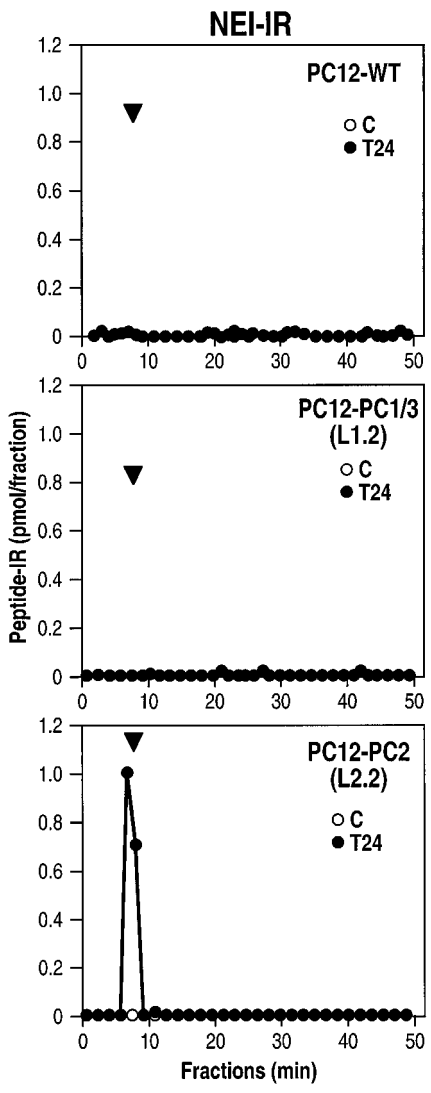

FIG. 4. **RP-HPLC analysis of NEI-IR in WT, PC1/3-transfected (L1.2), and PC2-transfected (L2.2) PC12 cell lines.** Aliquots (100 μl) of cell extracts (from a subconfluent 10-cm dish) were fractionated by RP-HPLC on a C18 PLRP column, and all the fractions were assayed for their NEI-IR as described under "Materials and Methods." The elution position of synthetic NEI (8 min, *black arrowhead*) is indicated.

mice noted in Fig. 7 was not reproducibly found with other spleen peptide samples (not shown).

DISCUSSION

It is now well established that PC1/3 and PC2 are the major enzymes involved in the processing of precursors directed to the regulatory secretion pathway within endocrine and neuroendocrine cells. On the other hand, furin, PACE4, PC5/6, and PC7 exhibit a widespread distribution and are capable of processing proproteins within constitutively secreting cells (1–5, 7, 11, 53). Information regarding the role of these enzymes in the processing of the precursor encoding MCH and NEI was so far lacking. We suggest here that PC2 is necessary for the generation of mature NEI in neuronal cells, whereas all of the known PCs are potentially capable of producing MCH from its precursor in mammals.

The first criterion to define the physiological importance of endoproteases involved in processing of a specific precursor is obviously to find one or several of these enzymes in the cells expressing the prohormone of interest. In this study, we demonstrate strict co-localization of PC2 with MCH mRNA in neurons of the LHA, the main site of synthesis of pro-MCH in the rat brain (32). These results support and extend previous *in situ* hybridization data showing that most of MCH mRNA-expressing magnocellular cells of the LHA contained also PC2 mRNA (54). These results indicate that PC2 represents a strong candidate for the processing of pro-MCH in these neurons. In addition, PC1/3 was also found in some but not all MCH mRNA-expressing perikarya. In a semiquantitative analysis, we estimated that about 15–20% of MCH-positive cells in the LHA co-express also PC1/3. On the other hand, MCH-expressing cells in the gut (macrophages) or testis (spermatogonia, Sertoli cells) lacked PC1/3 or PC2 (data not shown).

The cleavage susceptibility of pro-MCH to various prohormone convertases was analyzed first by vaccinia expression systems that generated high levels of pro-MCH and PCs, thereby facilitating the processing analysis. GH_4C_1 cells were co-infected with recombinant vaccinia virus engineered to ex-

TABLE III
Processing of pro-MCH in the brain and peripheral tissues of wild-type and PC2 null mice

MCH-IR and NEI-IR (fmol/mg of tissue) were assayed as described under "Materials and Methods." The values for individual WT mouse are the mean ± S.E. from triplicate determinations; similar levels of MCH-IR/NEI-IR were found in separate experiments.

Genotype	Total MCH-IR	Total NEI-IR	NEI/MCH
	fmol/mg tissue	*fmol/mg tissue*	
Whole brain			
WT (n = 4)	9.5 ± 1.5	9.6 ± 2.4	1.00
KO (n = 2)	ND[a]	<0.01	ND
Hypothalamus			
WT (n = 1)	24.8 ± 2.2	27 ± 0.5	1.09
KO (n = 3)	65.2 ± 7[b]	<0.01	ND
Brain without hypothalamus			
WT (n = 1)	11.3 ± 1.0	11.9 ± 2.5	1.00
KO (n = 3)	11.0 ± 0.7	0.7 ± 0.06[b]	0.06
Spleen			
WT (n = 3)	31.4 ± 9	<0.01	ND
KO (n = 4)	32.9 ± 10	<0.01	ND
Testis			
WT (n = 3)	1 ± 0.2	<0.01	ND
KO (n = 2)	2 ± 0.8	<0.01	ND

[a] ND, not done.
[b] $p < 0.05$.

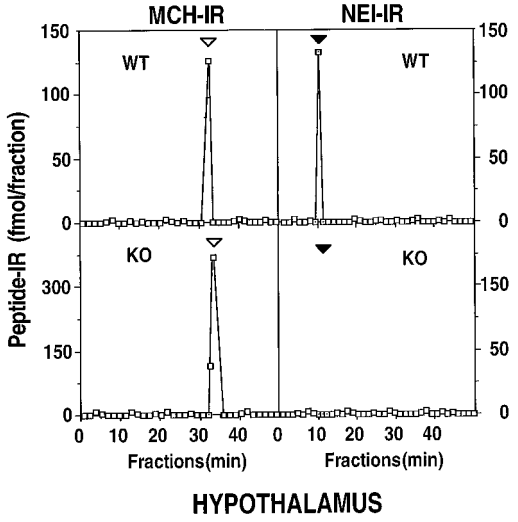

FIG. 5. **RP-HPLC analysis of MCH-IR and NEI-IR in the hypothalamus of one WT and one KO mouse.** Peptide extracts of at least two wild type and PC2 null mice were subjected to RP-HPLC analysis as described under "Materials and Methods" and gave similar results. Elution positions of synthetic MCH at 33 min (*open arrowhead*) and synthetic NEI at 10 min (*black arrowhead*) are shown.

press pro-MCH and different prohormone convertases, namely furin, PACE4, PC1/3, PC2, PC2 with various forms of 7B2, PC5/6-A, PC5/6-B, and PC7. Thereafter, peptides produced and MCH precursor were identified by RIA and RP-HPLC analysis. All seven processing enzymes are capable of cleaving on the C-terminal side of the Arg^{145}-Arg^{146} site to produce high amounts of MCH, the most efficient being PC7 and PC2 in the presence of 7B2 (see Tables I and II and Fig. 3). In great contrast, large amounts of NEI-IR were not found with any of the above combinations of pro-MCH and PCs, although minor cleavage at the Lys^{129}-Arg^{130} site was observed to produce mature NEI with PC7 or PC2 expressed with either 27-kDa 7B2 or 21-kDa 7B2 (see Fig. 3, *A* and *B*). Several interpretations may be proposed to explain these data: (i) none of the tested enzymes may actually cleave at this site of pro-MCH; (ii) all or some of these PCs process pro-MCH to produce an NEI form undetectable by our RIA, for instance NEI-GRR (45); (iii) the enzyme(s) involved in cleavage at this site required defined conditions that may not be reproduced in this vaccinia expression model. We cannot rule out, on one hand, the likelihood that a novel uncharacterized convertase is required for this cleavage or, on the other hand, that trimming by carboxypeptidases was reduced in these cells, preventing exposure of epitopes recognized by NEI antiserum (43, 45). However, we favor another possibility that is discussed below.

Because PC1/3 and PC2 co-localized with MCH in the LHA, it was of obvious interest to investigate the role of these processing enzymes in cellular models that expressed PC(s) and pro-MCH in the appropriate intracellular compartment. We have examined therefore the processing of pro-MCH in PC12 cell lines stably transfected with PC1/3 or PC2 (24). It has been shown that the same transfectants expressed active PC1/3 or PC2, resulting in different processing patterns of proneurotensin close to those found in the gut (PC1/3 transfectants) or the brain (PC2 transfectants) (24). We had previously revealed that MCH mRNA production may be induced in PC12 cells by adding NGF and lithium salt to the media for 24 h (47). Interpretation of results using our MCH RIA was impeded by the cross-reactivity of the MCH antiserum with unknown proteins of high molecular weights expressed in large amounts in all of the PC12 cell lines tested so far (data not shown). However, the data obtained with the NEI-RIA support the conclusion that PC2 but not PC1/3 may cleave efficiently at the Lys^{129}-Arg^{130} site to generate NEI when PC12 cells are committed to differentiate into neuron-like cells in the presence of NGF.

To further assess the physiological role of PC2 in the pro-MCH processing *in vivo*, we studied the cleavage of this precursor in a strain of mice lacking functional PC2 (30). Using this model, we showed here that cleavage at the Lys^{129}-Arg^{130} site was dramatically impaired, whereas cleavage at Arg^{145}-Arg^{146} was not altered in the hypothalamus. As an internal control, no difference in processing of pro-MCH was observed in cells expressing MCH-related peptides but lacking PC2, such as immune cells located in the spleen (Table III). PC2 corresponds therefore to the key endoprotease responsible for mature NEI production by virtue of cleavage at the Lys^{129}-Arg^{130} site in neuronal cells, whereas this enzyme may also be involved but is not the only enzyme that can cleave pro-MCH at the Arg^{145}-Arg^{146} site to release MCH (Fig. 8).

Strikingly, as regards NEI production, the results obtained using the stably transfected PC12 cell lines or PC2-KO mice do not agree with the vaccinia expression data mentioned above. Similar discrepancies have been noted previously between data obtained from vaccinia virus-infected models and other cellular models, such as cells stably transfected with PC-encoding vector, when secretogranin II (55), proglucagon (21, 22), and proneurotensin[3] processing was analyzed. These differences could be due to a possible alteration in the formation and storage of fully functional secretory granules in the vaccinia virus-infected cells. More specifically, it is well established that optimal PC2 enzyme activity requires acidic pH and high Ca^{2+} levels (56, 57). Furthermore, PC2 cleavage of precursors operates late along the biosynthetic pathway after the dissociation of PC2 from the C-terminal CT peptide of 7B2. These conditions may not be ideally achieved in the secretory pathway of vaccinia virus-infected cells, leading to conflicting results when compared with other cellular or animal models (55).

Based on the *in vitro* data, numerous prohormone convertases may be candidates for processing at the Arg^{145}-Arg^{146} site to produce mature MCH (see Table II and Fig. 8). PC2 and PC1/3 have been found co-expressed with MCH in the neurons of LHA, and they represent therefore the strongest candidates.

[3] P. Kitabgi, personal communication.

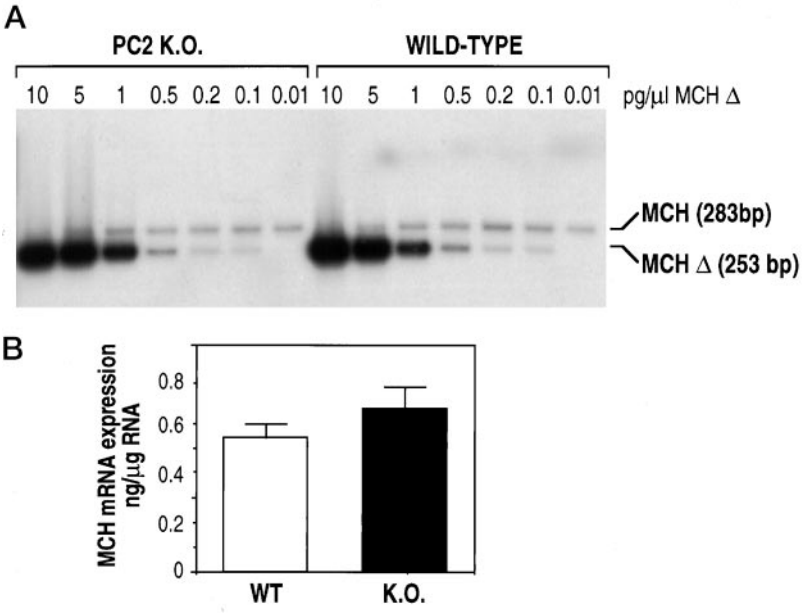

FIG. 6. **MCH mRNA expression in wild type and PC2 null mice.** *A*, 1 μg of whole cell RNA was reverse transcribed, and competitive PCR amplification was performed with various amounts (ng/μl) of a short template (MCHΔ) as described under "Materials and Methods." The PCR products corresponding to endogenous MCH mRNA and standard RNA are labeled *MCH* and *MCH*Δ, respectively. *B*, bar graph shows densitometric data analysis of RT-PCR for three animals per group of WT and KO mice.

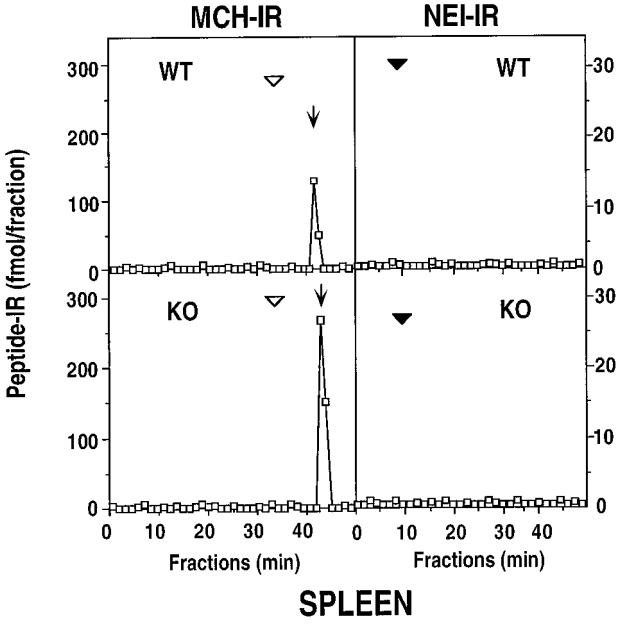

FIG. 7. **RP-HPLC analysis of MCH-IR and NEI-IR in the spleen of one WT mouse and one KO mouse.** Identical results were obtained for NEI-IR measurement in two other animals. MCH-IR contents were lowest in two other PC2 null mice. The *black* and *open arrowheads* are as in Fig. 4. The *arrow* indicates the position of the putative pro-MCH (131–165) product (44 min).

However, PC2 is not required for efficient processing of pro-MCH to produce mature MCH in the PC2 null mice model, and PC1/3 was found only in 15–20% of MCH-expressing neurons in the rat brain. Therefore, other yet uncharacterized proconvertase enzymes may possibly be involved in this cleavage. Among them, the PC5/6-A exhibits some overlapping of expression with MCH in the LHA[4] and may potentially cleave at the Arg^{145}-Arg^{146} site to liberate MCH (see Table II). PC7 is widely expressed in the hypothalamus, and it could also be present in MCH neurons, but co-localization studies are presently lacking.

An intriguing result concerns the 2–3-fold increase in mature MCH content observed selectively in the hypothalamus of PC2 null mice in comparison with controls (see Table III). The elevated MCH concentrations were not paralleled by enhanced gene transcription and/or MCH mRNA stability, as illustrated by our semiquantitative RT-PCR data. One could envisage first that the targeting of MCH-containing granules to axonal projections is somehow altered. Transport of secretory vesicles involves sequential steps and numerous protein factors (58). Impaired processing of one of these regulatory factors in the PC2 null mice may contribute to a partial decrease in granular transit. Alternatively, chronic elevation of hypothalamic MCH in PC2 null mice may be directly dependent upon the lack of NEI production or other peptidic secretagogues involved in the regulation of pro-MCH synthesis and release. In this context, it is worth noting that similar increase in MCH content was observed in *fat*/*fat* mice, which produced the NEI-GRR form but very low amounts of fully processed amidated NEI (43). The physiological significance of MCH content enhancement in PC2 null mice remains at this stage a matter for speculation.

Production of pro-MCH derivatives was not modified in spleen and testis of mutant PC2 mice, in agreement with co-localization studies that demonstrated that macrophage-like cells in the spleen and Sertoli cells or spermatogonia in the testis do not express detectable levels of PC2. The levels of MCH-IR contents in mice were close to those found in rat (35) or human (45) peripheral tissues. Interestingly, no peak corresponding to either mature MCH or NEI was found in mouse spleen. The MCH immunoreactivity was exclusively associated with a peptidic form corresponding to a 44-min peak. The same product was previously identified as an intermediate cleavage form of pro-MCH, bearing NEI and MCH, in human peripheral tissues (45). This large MCH-IR form may represent a novel peptide with hitherto unknown biological activity. Further experiments are required to establish the structure of this material in mice, but it is attractive to suggest that a similar kind of pro-MCH processing operates in peripheral tissues of human and mouse and leads to the release of an unprocessed NEI-MCH product. Interestingly, fully processed MCH and NEI peptides, albeit in low amounts, were identified in rat gut and testis (35, 37), raising the possibility of species-specific regulation of MCH precursor processing in peripheral organs. As illustrated in Fig. 8, the enzymes that participate in the cleavage at the Lys^{129}-Arg^{130} pair of pro-MCH in peripheral tissues remain unknown. Based on the cell-specific expression of PC7

[4] P. Villeneuve and A. Beaudet, personal communication.

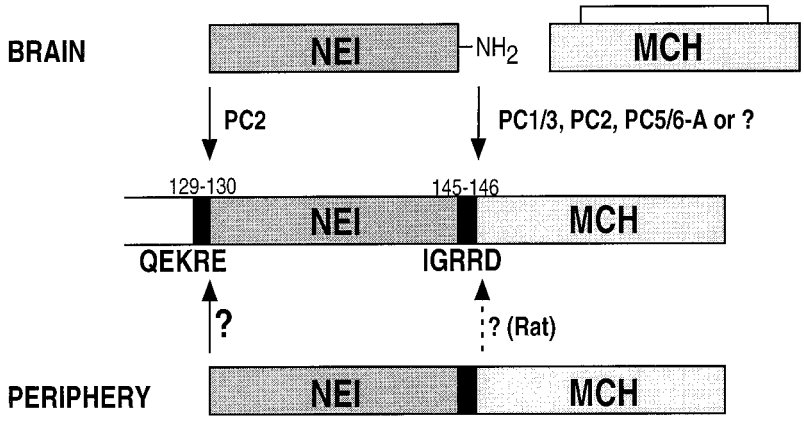

FIG. 8. **Schematic representation of the roles of PC2 and other PCs in mammalian pro-MCH processing in the brain and in the peripheral tissues.** Positions and sequence of cleavage sites are indicated. The *arrows* indicate the point of cleavage by specific PCs. *Question marks* signify potential involvement of other PCs in cleavage at the indicated site; *(Rat)* indicates partial cleavage at this site found only in peripheral tissues of the rat (36, 37).

in testis and spleen (7) and data from the vaccinia expression systems, we proposed that PC7 could be a putative candidate to cleave at this site. However, the ubiquitous enzyme furin or PACE4 may also be involved in the processing of MCH precursor in cells lacking the regulated secretory pathway.

In conclusion, this study reveals the preeminent role of PC2 in the production of NEI and provides evidence that PC2, PC1/3, and/or P5/6A are potential candidates involved in the release of MCH from its precursor in neurons of LHA. In addition, PC7 or other ubiquitous convertases of the constitutive secretory pathway may possibly cleave at the Lys^{129}-Arg^{130} site to generate a large product, encompassing the NEI and MCH domains of the precursor, found predominantly in peripheral tissues of mice and humans. As for the other prohormones studied so far, the differential processing of pro-MCH reflects the tissue-specific expression of the various prohormone convertases potentially involved in the cleavage at basic sites (reviewed in Refs. 3 and 4). The structural basis for the Lys^{129}-Arg^{130} site preference of PC2 in the brain or of the putative ubiquitous processing enzyme in peripheral tissues remains to be elucidated. The temporal as well as the tissue-specific regulation of pro-MCH processing should be further examined in cells (other than PC12 cell lines) expressing stably both pro-MCH and the proconvertases of interest and in the future PC1/3 or PC5/6A null mice models.

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