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Tissue distribution and processing of proSAAS by proprotein convertases

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Abstract

The conversion of inactive precursor proteins into bioactive neuropeptides and peptide hormones involves regulated secretory proteins such as prohormone convertases PC1 and PC2. The neuroendocrine protein 7B2 represents a specific binding protein for PC2, and the protein proSAAS, which interacts with PC1, exhibits certain structural and functional homologies with 7B2. With the intention of better understanding the physiological role of proSAAS and its derived peptides, we investigated its tissue localization using a new radioimmunoassay (RIA) to a C-terminal proSAAS-derived peptide. Immunoreactivity corresponding to this SAAS-derived peptide is mostly localized to the brain and gut. Analysis of the brain distribution of the proSAAS-derived peptides indicates that the hypothalamus and pituitary are the two richest areas, consistent with the previously described high expression of PC1 in these two areas. In order to investigate the cleavage of proSAAS by prohormone convertases, we incubated recombinant His-tagged proSAAS with recombinant mouse proPC2 or furin, separated the cleavage products using high-pressure gel permeation chromatography and analyzed the products by RIA. Our results indicate that either PC2 or furin can accomplish *in vitro* rapid removal and efficient internal processing of the C-terminal peptide, exposing the inhibitory hexapeptide to possible further digestion by carboxypeptidases. Finally, we also studied proSAAS processing in the brains of wild-type and PC2 null mice and found that proSAAS is efficiently processed *in vivo*. Whereas the C-terminal peptide is mostly internally cleaved in wild-type mouse brain, it is not processed as efficiently in the brain of PC2 null mice, suggesting that PC2 is partially responsible for this cleavage *in vivo*.

Keywords: convertase, furin post-translational processing neuropeptide, proSAAS, PC1, PC2.

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The prohormone convertases (PCs), such as PC1 (also known as PC3) and PC2, are involved in the generation of bioactive neuropeptides or peptide hormones that are first synthesized as much larger precursors. These enzymes belong to a distinct subfamily of subtilisin-related proteinases and are involved in the processing of a great number of important peptide hormones, such as proinsulin, proglucagon and proopiomelanocortin (for review, see Rouillé *et al*. 1995; Seidah and Chrétien 1999). Until last year, only one endogenous inhibitor of a member of this family had been identified: the protein 7B2. This protein appears to control the production of activatable proPC2 through its N-terminus (residues 1–151) and the enzymatic activity of this convertase in the secretory pathway through its C-terminal portion (residues 155–186) (for review, see Muller and Lindberg 1999).

Recently, a second convertase inhibitor that exhibits some structural and functional homologies with 7B2 was discovered: the proSAAS protein, which, like 7B2, contains a proline-rich region within the first half of the molecule. ProSAAS is a granin-related protein which contains a C-terminal peptide inhibiting PC1 with nanomolar potency (Cameron *et al*. 2000b; Fricker *et al*. 2000; Qian *et al*. 2000). The cell biology of the interaction of 7B2 with PC2

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Abbreviations used: ACN, acetonitrile; BCIP, 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CPB, carboxypeptidase B; HPGPC, high-pressure gel permeation chromatography; ir, immunoreactive; NBT, *p*-nitroblue tetrazolium chloride; PC1, prohormone convertase 1; PC2, prohormone convertase 2; PCR, polymerase chain reaction; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TFA, trifluoroacetic acid.

has been studied but is not completely understood (for review, see Muller and Lindberg 1999); nothing is known to date concerning the cell biology of the proSAAS/PC1 interaction. Endogenous inhibitors/binding proteins of PCs may play important roles in several hormonal disorders: for example, mice null for 7B2 die at an early age from a lethal endocrine disorder consistent with a Cushing's disease-like syndrome, which arises from enhanced secretion of ACTH by the pituitary (Westphal *et al*. 1999). We speculate that proSAAS may also play a role in the regulation of secreted hormone levels through action on PC1.

In order to target certain post-translational processing steps during the maturation of neuropeptides and peptide hormones, a better understanding of the interaction of PC1 with proSAAS is required. The distribution of proSAAS mRNA has been reported previously (Fricker *et al*. 2000), but nothing is known about the distribution of the protein and its derived peptides. In this study, we aim to better characterize the molecule proSAAS, first by determining the localization of derived peptides in various mouse tissues as well as determining their brain distribution. We also investigated the *in vitro* processing of proSAAS by two different proprotein convertases, PC2 and furin, in order to gain information about the enzymatic synthesis of proSAAS-related peptides. Lastly, in order to assess the potential contribution of PC2 to physiological processing of proSAAS, we studied *in vivo* processing in the brains of wild-type and PC2 null mice.

Materials and methods

Radioimmunoassay

LS40 antiserum was raised in rabbits against the sequence Tyr-LENPSPQAPA (Fig. 1) coupled to keyhole limpet hemocyanin using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, and the radioimmunoassay (RIA) used [I]^{125}YLENPSPQAPA as a tracer. For the QERARAEAQEAED RIA, he LS38 antiserum was raised in rabbits against the sequence QERARAEAQEAED coupled to keyhole limpet hemocyanin, and the RIA used [I]^{125}QERARAEAQEAED as a tracer. The IC_{50} of the LENPSPQAPA RIA was 0.15 ± 0.02 pmol and that of the QERARAEAQEAED assay was 52 ± 6.7 fmol. The range of the standard curves for both the RIAs was 0.01–10 pmol, and were, respectively, generated with the peptides YLENPSPQAPA and QERARAEAQEAED.

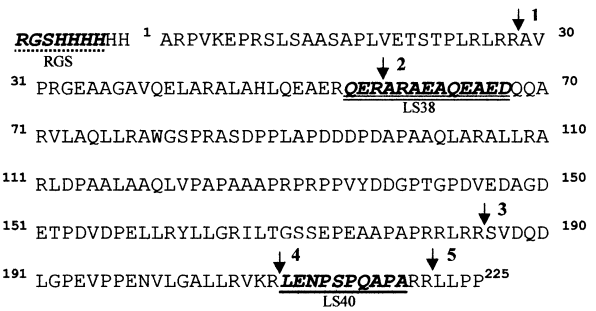

Fig. 1 Sequence of the recombinant proSAAS. Arrows represent the different cleavage sites, numbered 1 to 5. The dotted underlined sequence corresponds to the RGS epitope, the double underlined sequence to the QERARAEAQEAED epitope, and the single underlined sequence to the LENPSPQAPA epitope.

One hundred microliters of antiserum (of a working dilution 1 : 750 for both LS40 and LS38 antisera) and 100 μL of [I^{125}] peptide diluted in RIA buffer [0.1 M Na phosphate, 50 mM NaCl, 0.1% NaN_3, 0.1% heat-treated bovine serum albumin (BSA), pH 7.4] were added to 100 μL of the samples [either in RIA buffer; or in Tris–BSA if to be digested with trypsin/carboxypeptidase B (CPB)]. RIAs were incubated overnight at 4°C. The antibody-bound radiolabel was then separated from the free labeled peptide by addition of 100 μL of 7.5% carrier γ-globulin (in 0.1 M phosphate buffer, pH 7.5) and 1 mL of 25% polyethylene glycol, centrifugation for 20 min at 950 ***g*** at 4°C and aspiration of the supernatant, as described previously (Fortenberry *et al*. 1999). Radioactivity in pellets was estimated by gamma spectroscopy. All samples were assayed in duplicate.

Tissue extraction

We first tested various solutions for efficiency in extracting SAAS-immunoreactivity (ir) peptides: brains were extracted in acid mix (1 M acetic acid, 20 mM HCl, 0.1% β-mercaptoethanol), 1 M acetic acid, 0.1 M HCl, or boiling water. The highest yield of LENPSPQAPA-ir, as assessed using either western blotting or RIA, was achieved using 1 N acetic acid. Mouse tissues and brain areas were homogenized immediately following dissection in 10 vol. ice-cold 1 M acetic acid (100 vol. for the pituitary). The homogenization of entire organs was performed with a polytron, whereas brain areas were homogenized with a sonic dismembrator (Fisher Scientific), for 5 s on ice. The homogenates were frozen on dry ice and then thawed. Aliquots of one-tenth of the volume of each sample were taken for protein determination. The remaining 90% of the samples was centrifuged for 25 min at 3800 *g* at 4°C. Supernatants were frozen on dry ice and then lyophilized overnight. For the RIA, the tissue extracts were resuspended in 10 vol. (or 100 vol. for the pituitary) of Tris–BSA (100 mM Tris–HCl, 0.01% BSA, 0.1% NaN_3, pH 8.0), and centrifuged 20 min at 3800 *g* at 4°C. Supernatants were harvested for assay by RIA.

Trypsin/CPB digestion

In order to reveal cryptic LENPSPQAPA-immunoreactive peptides, duplicate samples (80 μL for the liver, brain, spleen, kidney and duodenum extracts, 50 μL for the pancreas, 25 μL for the adrenals and 30 μL duplicates for the brain areas) were digested using trypsin and CPB prior to RIA for LENPSPQAPA. Samples were incubated with 0.02 mg/mL of trypsin (Sigma, St Louis, MO, USA) for 2 h at 37°C in a final volume of 100 μL. Twenty microliters of CPB (Worthington Biochemical Corporation) (18 μg/mL) were then added to each sample. After 25 min of incubation at 37°C, the reaction was stopped by boiling. Digestion was carried out in the same tubes used for RIA.

Protein assay

Acid homogenates were solubilized by the addition of Triton X-100 to a final concentration of 1%, and 45 μL of 1 M NaOH was added

to 15 μL of solubilized samples. Five microliters of this solution were then assayed in 800 μL of H_2O and 200 μL of Bradford reagent (Bio-Rad). The standard curve was generated with BSA, and the optical density was measured at 595 nm. Each sample was assayed in duplicate.

Production of recombinant enzymes

Purified recombinant mouse PC2 was obtained from Chinese hamster ovary (CHO) cells as described previously (Lamango *et al*. 1996). Recombinant soluble mouse furin was purified from the conditioned medium of CHO cells (Cameron *et al*. 2000a).

Expression and purification of full-length proSAAS [1–225]

Full-length proSAAS was amplified via polymerase chain reaction (PCR) from the plasmid pBluescript using the following primers: 5′−CGGGGTACCGCGAGGCCCGTGAAGGAGCCC-3′ directed toward the 5′-end and 5′-GCGAAGCTTATTATTGAGGGCT-CAGG-3′ directed toward the 3′-end. The PCR-amplified product was subcloned into the pQE30 vector (Qiagen), which contains an N-terminal His tag, between the *Kpn*I and *Hin*dIII sites. Recombinant proteins were purified as described previously (Martens *et al*. 1994) with slight modifications. Briefly, bacteria were grown at 37°C until A_{600} reached 0.8–0.9. Protein expression was induced by adding isopropyl β-D-thiogalactopyranoside (Sigma) to a final concentration of 1 mM, and bacteria were then incubated at 30°C with shaking for 16 h. The bacterial pellet was resuspended in buffer A (6 M guanidinium chloride, 10 mM HEPES, pH 7.5) and incubated for 2 h at 10°C, to allow solubilization of the protein. The suspension was then centrifuged at 2700 *g* and the supernatant removed and incubated with Ni-NTA (Qiagen) beads for 2 h at 10°C. The protein was gradually refolded along a gradient of 100% A to 100% B (10 mM HEPES, 0.1 M NaCl) for 2 h. The proteins were eluted in 250 mM imidazole, 7-min fractions were collected (at a flow rate of 0.4 mL/min) and the purity assessed on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Fractions containing the recombinant protein (fractions 7–10) were pooled. To remove the imidazole, and to further purify the protein, the Ni-NTA eluate was subjected to reverse-phase chromatography on a Vydac C_4 column. Each sample was loaded onto the column at a flow rate of 1 mL/min and the column was washed in buffer A [0.1% trifluoroacetic acid (TFA)] until a stable UV_{280} baseline was achieved. The proteins were eluted from the column with 80% acetonitrile (ACN) in 0.1% TFA using the gradient described previously for the purification of proenkephalin (Lindberg and Zhou 1995). Fractions were dried and observed by Coomassie Brilliant Blue staining on 15% polyacrylamide gels; aliquots were also tested for PC1 inhibition. Fractions containing full length SAAS (1–225) were pooled (fractions 41–43), lyophilized and resuspended in water for subsequent experiments.

Enzyme digestion

In order to analyze proSAAS cleavage products by Western blotting, 32 μg (1280 pmol) of recombinant proSAAS [1–225] were diluted in 300 μL of 2× furin buffer (HEPES 200 mM, 10 mM $CaCl_2$, 0.2% Brij, pH 7), or 2× PC2 buffer (200 mM Na acetate, 10 mM $CaCl_2$, 0.2% octyl glucoside, pH 5), and the volume was brought to 600 μL with H_2O. A 100-μL aliquot was removed to analyze the starting material. Ten microliters of purified furin (0.024 mg/mL) or 10 μL of proPC2 (0.2 mg/mL) were added. One-hundred-microliter aliquots were incubated for either 5, 15, 30, 120 and 240 min for the furin digestion, or for 30, 60, 120, 180 and 360 min for the PC2 digestion. The reactions were stopped by the addition of 25 μL of 5× Laemmli loading buffer (0.75% Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.006% bromophenol blue), following by boiling.

Western blotting

Thirty microliters of sample were loaded per lane, and each sample was loaded onto four independent 15% polyacrylamide SDS gels. The standard used was prestained low molecular mass (Amersham). One of the gels was Coomassie Brilliant Blue-stained, and the other three were transferred to nitrocellulose membranes (0.2 μm, Bio-Rad). Two of the membranes were incubated for 30 min in milk [5% non-fat dry milk and 0.02% NaN_3 in Tris-buffered saline (TBS)], and then incubated overnight at 4°C with LS40 or LS38 antisera diluted 1 : 1000 in milk. Three washes in TBS containing 0.05% Tween were then performed prior to 1 h incubation with the secondary antibody, goat anti-rabbit IgG coupled to alkaline phosphatase (Sigma), at a dilution of 1 : 10 000. Two washes in TBS–Tween 0.05% and one wash in TBS were then performed before developing the blots with 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt/*p*-nitroblue tetrazolium chloride (BCIP/NBT) color development solution (Bio-Rad).

The same procedures were followed with the third membrane, but the incubations were performed in blocking solution for monoclonal antibody (3% BSA, 0.3% Triton X-100, 0.02% Na azide, 1% goat serum in TBS) and the washes were performed in 0.2% BSA, 0.3% Triton X-100, 0.02% NaN_3, 0.5% goat serum in TBS. The primary antibody used was the RGS monoclonal mouse antibody (QIAGEN Inc., Chatsworth, CA, USA), raised against the sequence RGSHHHH. The RGS antibody was used as a 1 : 1000 dilution in blocking solution. Goat antimouse IgG (H + L)-alkaline phosphatase conjugate (Bio-Rad) was used as a secondary antibody; blots were developed with the BCIP/NBT color developing solution from Bio-Rad.

Size separation of proSAAS processing products

In order to analyze the *in vitro* proSAAS cleavage products by high-pressure gel permeation chromatography (HPGPC), 16 μg (640 pmols) of recombinant proSAAS [1–225] were diluted in 150 μL of furin buffer or PC2 buffer, and the volume was brought to 300 μL with H_2O. A first 50-μL aliquot was taken to analyze the starting material. Ten microliters of furin (0.012 mg/mL) or of proPC2 (0.1 mg/mL) were then added, and the 50 μL aliquots were incubated at 37°C for 15 min and 6 h for the furin digestions, or for 1 and 6 h for the PC2 digestions. The reactions were stopped by immediately adding 200 μL of HPGPC buffer (32% ACN, 0.1% TFA) and freezing. Each sample was then thawed and centrifuged for 10 min at 3800 *g*; no pellet was observed. One-hundred microliters of supernatant were separated by HPGPC as described previously (Fortenberry *et al*. 1999).

In order to analyze the proSAAS cleavage products present in the brains of wild-type or PC2 null mice, lyophilized brain extracts were resuspended in 300 μL of HPGPC buffer and centrifuged for 10 min at 3800 *g*. One-hundred microliters were then separated by HPGPC, with a flow rate of 0.5 mL/min; 1-min fractions were collected. Fifty microliters of 0.5% BSA, 0.1% NaN_3 were added to each fraction. Fifty microliters of fractions 20–50 were then

removed in duplicate, lyophilized overnight, digested with trypsin/CPB in Tris–BSA, and subjected to the LENPSPQAPA RIA. For the QERARAEAQEAED RIA, the samples were lyophilized and directly resuspended in RIA buffer.

Results

Distribution of proSAAS derived peptides

In order to investigate the tissue distribution of proSAAS-derived peptides various tissues were analyzed using a new RIA directed against the LENPSPQAPA epitope, which is located in the C-terminal portion of proSAAS (residues 210–219) (Fig. 1). The gut (duodenum) and brain contain the highest levels of LENPSPQAPA-ir peptides (Fig. 2a). The adrenals exhibit a significant level of immunoreactivity for LENPSPQAPA when digested by trypsin/CPB; in the pancreas and the spleen, only low amounts of proSAAS-derived peptides are detected. The other tissues studied contain little or no LENPSPQAPA-ir peptides.

The trypsin/CPB digestion removes the C-terminal residues 221–225, which exposes the LENPSPQAPA epitope and reveals cryptic immunoreactive peptides. In the gut (duodenum), cleavage of the C-terminal peptide at residue 221 (RR cleavage, see Fig. 1) seems to occur naturally, because the trypsin/CPB digestion did not reveal significant additional immunoreactive peptide. In the brain, the C-terminal RR cleavage is apparently not as complete as in the gut, because the LENPSPQAPA-ir detected in the brain is ≈ threefold lower if the extracts are not treated with trypsin/CPB (Fig. 2a). Differences between gut and brain processing have been noted for other neuropeptides, such as cholecystokinin (Cain *et al*. 1997).

In order to investigate the brain distribution of the proSAAS-derived peptides, brain areas were analyzed using the LENPSPQAPA RIA following treatment with trypsin/CPB (Fig. 2b). ProSAAS-derived peptides with LENPSPQAPA immunoreactivity were detected mostly in the hypothalamus and pituitary. The medulla oblongata and thalamus contain lower amounts of the peptides, whereas only background levels were detected in the other brain areas studied.

In vitro processing of proSAAS by PC2

Recombinant His-tagged proSAAS was generated in

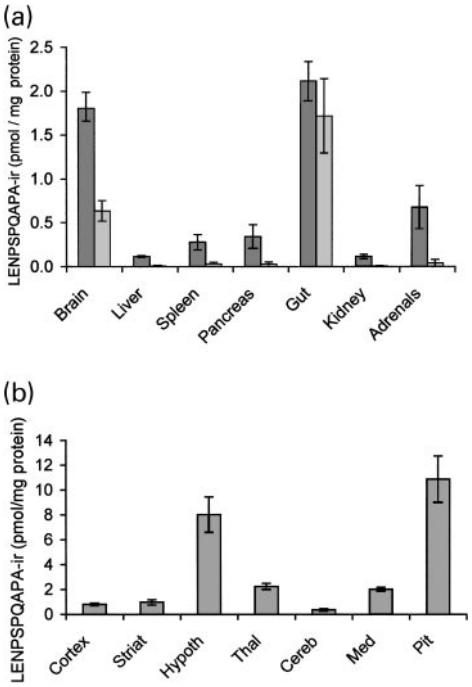

Fig. 2 Distribution of LENP-ir proSAAS-derived peptides. Mouse tissues ($n = 3$ for each tissue) and brain areas ($n = 3$ for each brain area) were homogenized in 1 N acetic acid, frozen and thawed, centrifuged and the clear supernatant lyophilized. The extracts were resuspended in Tris–BSA, and replicate aliquots were digested with trypsin and CPB (black) prior to assay of LENPSPQAPA-ir by RIA, or left undigested (gray). (a) Tissue distribution of LENPSPQAPA-ir peptides (with or without trypsin/CPB digestion). (b) Brain distribution or LENPSPQAPA-ir peptides (with trypsin/CPB digestion). Striat, striatum; Hypoth, hypothalamus; Thal, thalamus; Cereb, cerebellum; Med, medulla oblongata; Pit, pituitary.

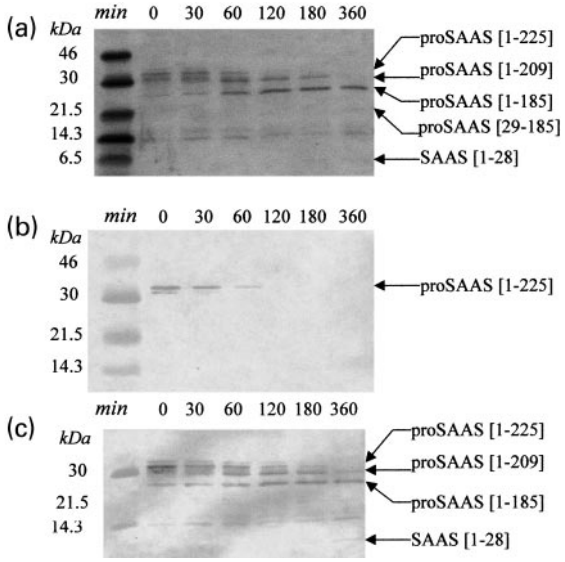

Fig. 3 *In vitro* processing of proSAAS by PC2: western blotting and Coomassie Brilliant Blue gel. Recombinant His-tagged proSAAS [1–225] was incubated with recombinant mouse PC2 for 30, 60, 120, 180 or 360 min. The reaction was stopped by adding 5× Laemmli sample buffer concentrate and boiling the samples for 3 min. Samples were then loaded on a 15% gel, and blotted with two different antisera, directed against a C-terminal epitope (LENPSPQAPA) or against the extreme N-terminus (RGS); or stained with Coomassie Brilliant Blue. (a) Coomassie Brilliant Blue-stained gel. (b) Western blotting using LS40 (LENPSPQAPA) antiserum. (c) Western blotting using RGS antiserum.

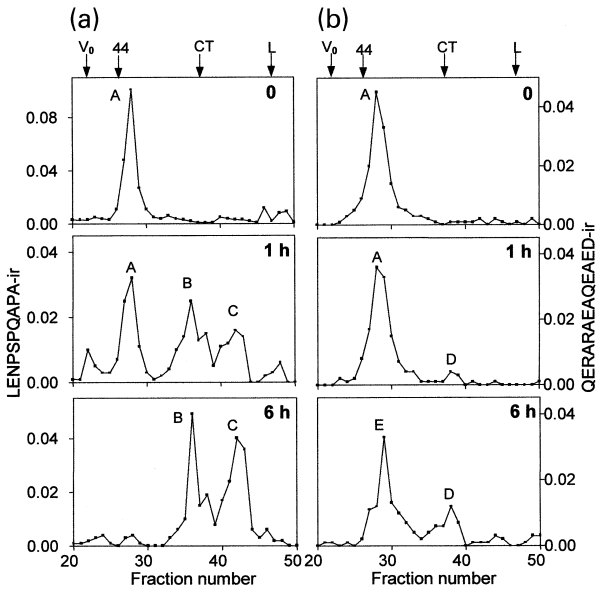

Fig. 4 *In vitro* processing of proSAAS by PC2: RIA of size-fractionated reaction mixtures. Recombinant His-tagged proSAAS [1–225] was incubated with purified mouse recombinant PC2 for 1 or 6 h. The reaction was stopped by adding 32% ACN and 0.1% TFA buffer, and freezing. Samples were then thawed and centrifuged. The cleavage products were separated by HPGPC and analyzed by RIA to an internal portion of proSAAS (QERARAEAQEAED), or submitted to trypsin and CPB digestion prior to assay by RIA for the LENPSPQAPA epitope. Arrows indicate the position of the standards used: Chicken ovalbumin 44 kDa (Bio-Rad) (44), SAAS C-terminal peptide [186–225] (C-terminal), LENPSPQAPA (L), and the cleavage products: recombinant proSAAS [1–225] (A); C-terminal peptide [186–225] (B); C-terminal peptide [209–225] (C); proSAAS fragment [29–57] (D); and proSAAS fragment [1–185] (E). (a) LENPSPQAPA RIA; (b) QERARAEAQEAED RIA.

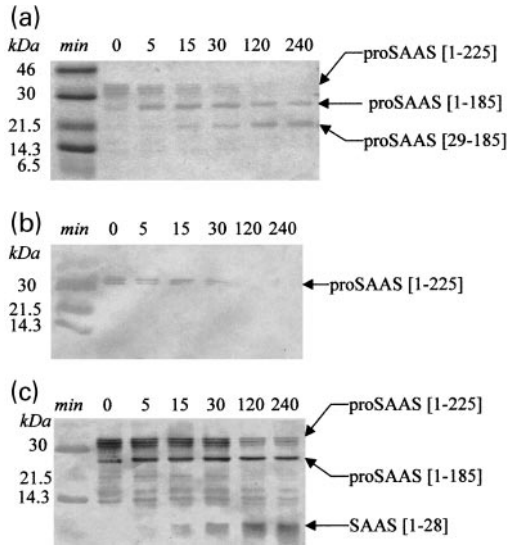

Fig. 5 *In vitro* processing of proSAAS by furin: western blotting and Coomassie Brilliant Blue gel. Recombinant His-tagged proSAAS [1–225] was incubated with recombinant mouse furin for 5, 15, 30, 120 or 240 min, and analyzed by western blotting or Coomassie Brilliant Blue staining as in Fig. 2. (a) Coomassie Brilliant Blue gel; (b) western blotting using LS40 (LENPSPQAPA) antiserum; (c) western blotting using RGS antibody.

bacteria and purified using a metal ion affinity column and reverse-phase high-performance liquid chromatography. This material was incubated with recombinant mouse proPC2 (Lamango *et al*. 1996) which activates immediately in the low pH of the reaction buffer to form the catalytically active species PC2 (Lamango *et al*. 1999). Cleavage products were either analyzed by Western blotting using various antisera, or subjected to RIAs against the LENPSPQAPA or QERARAEAQEAED epitopes after separation by HPGPC. Western blotting of the recombinant proSAAS [1–185] was also performed using the various antisera in order to determine its migration position (not shown). Figure 3 shows the Coomassie Brilliant Blue gel (Fig. 3a) and the Western blots of the reaction mixtures with the LS40 (LENPSPQAPA) and RGS antisera (Figs 3b and c). Our results, using antiserum directed against the LENPSPQAPA epitope (Fig. 3b), reveal that PC2 accomplished rapid removal of this epitope, as demonstrated by its disappearance, by cleavage either within the C-terminal peptide (site #4, see Fig. 1) or at the furin site (site #3, see Fig. 1). The use of a commercially available antibody directed against the extreme N-terminus of His-tagged proSAAS (RGS) (Fig. 3c) revealed two bands. The first corresponds to a proSAAS-derived peptide that does not contain the LENPSPQAPA epitope (because this band does not appear on the LS40 blot), and which is bigger than proSAAS [1–185]: we conclude that this represents the fragment proSAAS [1–209]. The other band was identified as proSAAS [1–185] by comigration with the recombinant His-tagged proSAAS [1–185]. These data demonstrate that the C-terminal peptide is cleaved very rapidly by PC2, both internally and at the furin site. Much slower cleavage occurs at the N-terminal portion of the protein, as indicated by the late appearance of a small RGS-ir band. The Coomassie Brilliant Blue gel (Fig. 3a) yielded the same results as the RGS blot. No additional information was obtained using the LS38 QERARAEAQEAED antiserum, because it appeared to be very poor for Western blotting.

In order to identify the PC2-mediated cleavages that occur within the C-terminal peptide and in the internal portion of the proSAAS, RIAs of size-fractionated reaction mixtures were performed (Fig. 4). The peak obtained at time 0 with both RIAs (peak A) corresponds to full-length His-tagged proSAAS [1–225], which is the starting material. After 6 h, two LENPSPQAPA-ir peaks were identified: the first corresponding to intact C-terminal peptide (residues 186–225; peak B), as shown by the co-elution with the standard; and the second to the

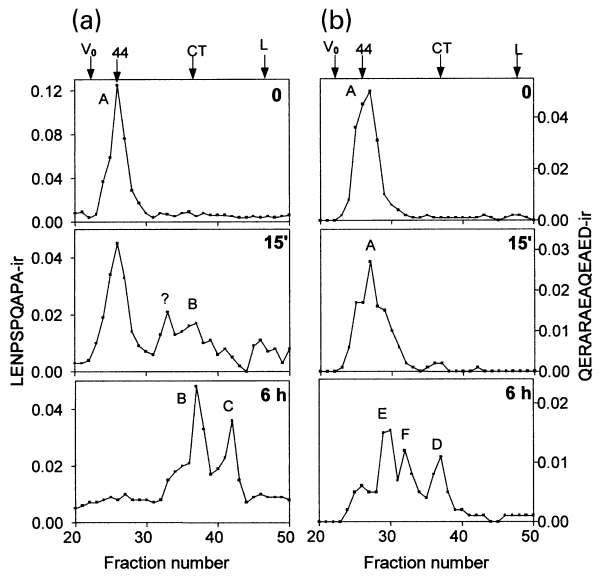

Fig. 6 *In vitro* processing of proSAAS by furin: RIA of size-fractionated reaction mixtures. Recombinant His-tagged proSAAS [1–225] was incubated with recombinant mouse furin for 15 min or 6 h, separated by HPGPC, and analyzed by RIA as in Fig. 3. Arrows indicate the position of standards and of cleavage products, as in Fig. 3. (F) represents the proSAAS-derived fragment [29–185]. (a) LENPSPQAPA RIA; (b) QERARAEAQEAED RIA.

C-terminal peptide fragment [209–225] (peak C; Fig. 4a). In agreement with the western blotting results, the RIA to the LENPSPQAPA epitope demonstrates that PC2 can both remove and internally cleave the SAAS C-terminal peptide. Our results using the RIA to the QERARAEAQEAED epitope (Fig. 4b), which spans a potential furin cleavage site (site #2, see Fig. 1), revealed the appearance of a small peak after 1 or 6 h (peak D) that was tentatively identified as the proSAAS-derived peptide [29–57] based on its size and immunoreactive profile. After 6 h of digestion, conversion of intact proSAAS to a slightly smaller protein is evident (peak E); given the Western blotting results, this is likely to reflect removal of the C-terminal portion of proSAAS, residues 186–225, from intact proSAAS.

To summarize, PC2 can process the C-terminal peptide very efficiently *in vitro* by rapid removal from the precursor, as well as by internal cleavage. PC2 can also remove the N-terminal portion of proSAAS by cleaving between residues 28 and 29. However, this cleavage is not complete after 6 h, and is much slower than removal of the C-terminal peptide.

In vitro processing of proSAAS by furin

Recombinant His-tagged proSAAS was also incubated with recombinant soluble mouse furin (Cameron *et al*. 2001), and the cleavage products analyzed by western blotting using various antisera (Fig. 5). Figure 5(a) depicts the Coomassie Brilliant Blue-stained gel. The LS40 antiserum revealed very rapid loss of this epitope by furin, because the LENPSPQAPA-ir band corresponding to intact proSAAS disappeared after 30 min; no other band was observed on the LS40 blot (Fig. 5b). Our results using antiserum directed against the extreme N-terminus of recombinant proSAAS (RGS) (Fig. 5c) showed that the intact inhibitory C-terminal peptide is initially removed (as demonstrated by the appearance of a band corresponding to proSAAS [1–185]). A second furin-mediated cleavage occurred between residues 28 and 29, as indicated by the emergence of a small RGS-ir band after 30 min of incubation; this corresponds to the N-terminal fragment [1–28]. In addition, the Coomassie Brilliant Blue gel (Fig. 5a) revealed another band that does not appear on any of the blots, which from its size is likely to correspond to the proSAAS-derived fragment [29–185].

Furin-mediated cleavages of proSAAS were then analyzed by RIAs directed against the LENPSPQAPA or the QERARAEAQEAED epitopes, following separation of the cleavage products by HPGPC (Fig. 6). The starting material appeared as a peak in fraction 25 with both RIAs (peak A). After 15 min of digestion, the RIA to the LENPSPQAPA epitope revealed one peak (peak B) which corresponds to the position of intact C-terminal peptide (Fig. 6a). Another peak appears after a longer incubation (peak C). This peak was identified as the C-terminal peptide fragment [209–225] by co-elution with a C-terminal peptide/furin digestion cleavage product (not shown). These data indicate that furin can also internally cleave the C-terminal peptide. A third small peak of immunoreactivity against the LENPSPQAPA epitope remains unidentified. The RIA to the internal portion of proSAAS (Fig. 6b), revealed the appearance of two QERARAEAQEAED-ir peaks which could correspond to the proSAAS-derived peptides [1–185] (peak E) and [29–185] (peak F), according to the western blotting results. A small peak tentatively identified as the proSAAS-derived peptide [29–57] (peak D) was generated more slowly.

Like PC2, furin performs very efficient processing of the C-terminal portion of proSAAS, first by rapid removal of the C-terminal peptide, and then by internal cleavage at the consensus furin site. A slower N-terminal cleavage is also performed by furin, probably at the furin site following the SAAS sequence, between residues 28 and 29. Another furin-mediated cleavage generates an LENPSPQAPA-ir peptide larger than the C-terminal peptide, which remains unidentified. Neither PC2 nor furin cleaved the RR site at the C-terminus.

Processing of proSAAS in mouse brain

PC2 appears to cleave proSAAS efficiently *in vitro*; in order to assess the potential contribution of PC2 to physiological processing of proSAAS, we investigated processing in the brains of wild-type and PC2 null mice. Peptides in brain

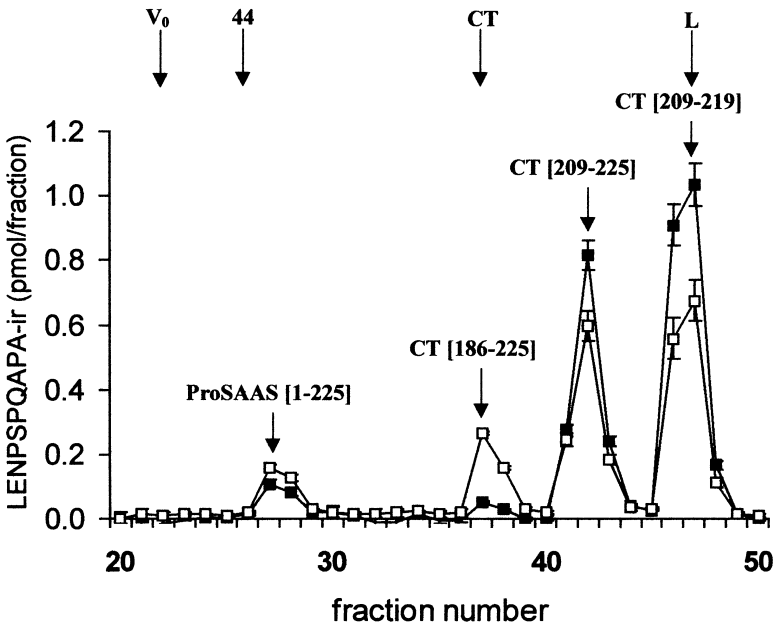

Fig. 7 Processing of proSAAS in mouse brain. Brains of wild-type mice ($n = 3$) (■) or PC2 KO mice ($n = 3$) (□) were extracted in 1 N acetic acid, and the extracts resuspended in 32% ACN and 0.1% TFA buffer. Cleavage products were then separated by HPGPC and digested with trypsin and CPB to expose epitopes prior to assay of LENPSPQAPA-ir by RIA; digestion was required for the detection of all peaks except the last. The standards are the same as in Fig. 3.

extracts were separated by HPGPC, digested by trypsin/CPB and analyzed by RIA against the LENPSPQAPA epitope (Fig. 7). The data obtained with the wild-type animals show the presence of four peaks: the first, a very small peak, eluted in the position of the entire proSAAS molecule, and the second, also a relatively small peak, was identified as the C-terminal peptide [186–225] by co-elution with synthetic standard. The two other peaks, which contain much more LENPSPQAPA-immunoreactivity than the first two peaks, most likely correspond to cleavage products of the C-terminal peptide: fragments [209–225] and [209–219] (which co-elutes with the standard), and indicate extensive processing of the C-terminal peptide in wild-type mice. In the brains of PC2 null mice, the same peaks were obtained, but the profile was different. The peak identified as the entire proSAAS molecule remained very small, whereas the peak corresponding to the intact C-terminal peptide [186–225] was larger than in the wild-type mouse. The two peaks

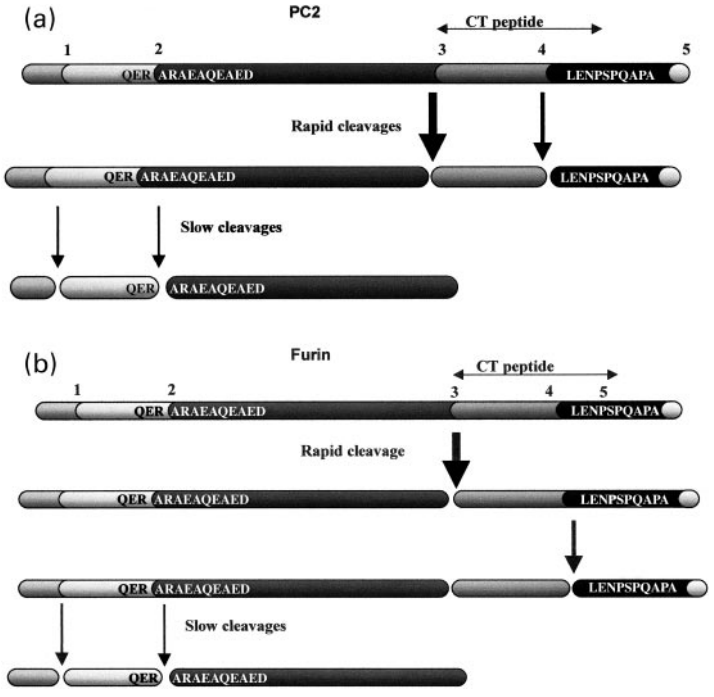

Fig. 8 Proposed model of proSAAS processing by PC2 and furin. The numbers correspond to the cleavage sites shown in Fig. 1. The size of the arrows corresponds to the extent of the cleavage: large arrow, > 75%; intermediate arrow, 50–75%; small arrow, < 50%. (a) proSAAS processing by PC2; (b) proSAAS processing by furin.

corresponding to the cleavage products of the C-terminal peptide contained less immunoreactivity than in the wild-type brain. The different profile of proSAAS processing demonstrates that the C-terminal peptide can still be removed, but is less efficiently internally cleaved, in the brains of PC2 null mice. Therefore, PC2 may be partially responsible for this internal cleavage *in vivo*.

Discussion

ProSAAS, a novel protein discovered using a mass spectrometry-based screen for peptides enriched in Cpe^{fat}/Cpe^{fat} mouse brain (Fricker *et al*. 2000), is a granin-like protein which exhibits structural and functional homology to the 7B2 protein (Cameron *et al*. 2000b), and appears to be a potent inhibitor of PC1 (Cameron *et al*. 2000b; Qian *et al*. 2000).

The tissue distribution of proSAAS-derived peptides with immunoreactivity towards the C-terminus of this precursor (LENPSPQAPA-ir peptides) is consistent with the neuroendocrine expression of its mRNA described previously (Fricker *et al*. 2000). The highest-expressing tissues are the brain, pituitary and gut, which exhibit a high concentration of LENPSPQAPA-ir on a per mg protein basis. These data imply that proSAAS may play an important role in peptide processing in these tissues. The high amounts of LENPSPQAPA-ir peptides in the gut are in agreement with the known abundance of PC1 in the gastrointestinal tract (Scopsi *et al*. 1995). The distribution of proSAAS between the brain, pituitary and gut is also consistent with that of chromogranins A and B (Fischer-Colbrie *et al*. 1985; Curry *et al*. 1991) and secretogranin III (Hutton *et al*. 1988).

Within the brain, the distribution of LENPSPQAPA-ir peptides differs from the expression of proSAAS mRNA described previously; whereas proSAAS mRNA shows a very widespread pattern of expression in the brain (Fricker *et al*. 2000), we found that the hypothalamus was clearly the highest-expressing region. The richness of the hypothalamus and pituitary are not surprising given the abundant expression of PC1 in these tissues (Day *et al*. 1992; Zheng *et al*. 1994). The high level of expression in the hypothalamus is also consistent with its known role as a repository for a great number of neuropeptides. Lower amounts of immunoreactivity against proSAAS-derived peptides are detected in the cortex, striatum and thalamus, whereas the cerebellum does not contain significant levels LENPSPQAPA-ir; this profile is in complete agreement with the expression pattern of PC1 described previously (Winsky-Sommerer *et al*. 2000). We conclude that the distribution of proSAAS-derived peptides is consistent with the distribution of PC1. Fine mapping of various brain areas by immunocytochemistry is required to establish whether areas exist which express one protein in the absence of the other.

Our study of *in vitro* processing of recombinant proSAAS by recombinant purified proprotein convertases indicated that furin is able to efficiently process proSAAS at the furin consensus cleavage site, and to additionally cleave the SAAS C-terminal peptide internally to a fragment which most likely corresponds to cleavage following the sequence LLRVKR, the inhibitory hexapeptide (Cameron *et al*. 2000b). This cleavage would expose the inhibitory peptide to further digestion by carboxypeptidases. While this furin-mediated cleavage is unlikely to occur while the peptide is bound to PC1, for which it has a higher affinity (Cameron *et al*. 2000b), the ability of furin to mediate this internal cleavage event may have physiological implications for the removal of surplus inhibitory C-terminal peptide within the secretory pathway. Furin is also able to remove the RGS epitope by performing an N-terminal cleavage event; we predict that this event most likely occurs at the furin consensus site following the SAAS sequence. Like furin, PC2 is also able to efficiently process proSAAS at its C-terminus, both by removing the C-terminal peptide as well as by cleaving the C-terminal peptide internally. Under these *in vitro* conditions, we did not observe PC2-mediated production of a C-terminal peptide fragment cleaved at the RR site (residues 220 and 221). Similarly to furin, PC2 is also able to remove an N-terminal peptide, but this cleavage is much slower than removal of the C-terminal peptide. PC1 processing was not tested, because PC1 is potently inhibited under these conditions. Regarding processing by PC1, we reported previously that the iodinated SAAS C-terminal peptide can be cleaved by PC1 *in vitro* to generate a product that would correspond to C-terminal peptide [186–208]; however, this requires a large excess of enzyme (Cameron *et al*. 2000). Taken together, our data indicate that the proSAAS precursor can be efficiently processed *in vitro* by both furin and PC2. Processing patterns are summarized in Fig. 8.

Studies performed using brain extracts prepared from wild-type and PC2 null mice are generally consistent with the results obtained *in vitro.* Analysis of size-separated proSAAS-derived peptides from wild-type mouse brain indicates that the SAAS C-terminal peptide is also efficiently internally cleaved *in vivo*. However, the major proSAAS cleavage product with LENPSPQAPA-ir detected in the brain is the peptide [209–220], which is not generated by PC2 or furin under *in vitro* conditions. Thus, more complete digestion can be obtained *in vivo* than under our *in vitro* conditions, most likely due to the more favorable secretory granule milieu. In the brains of PC2 null mice, the amount of processed C-terminal peptide is reduced, which is consistent with the results obtained *in vitro*. Because PC2 is able to efficiently internally cleave the C-terminal peptide, the lack of this enzyme results in diminution of processing of this peptide. However, we do not observe a complete abolition of this internal cleavage event in the PC2 null mouse; possibly other enzymes (such as furin and PC1

itself) are still able to process the C-terminal peptide internally.

In summary, we have shown that the proSAAS precursor can be efficiently processed by furin and by PC2 at multiple cleavage sites, generating a greater number of potentially important peptide fragments than its functional relative, pro7B2. Further experiments are required to provide a definitive identification of all the products in these reaction mixtures, and to identify other enzymes responsible for proSAAS processing *in vivo*.

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References

Cain B. M., Wang W. and Beinfeld M. C. (1997) Cholecystokinin (CCK) levels are greatly reduced in the brains but not the duodenums of Cpe (fat)/Cpe (fat) mice: a regional difference in the involvement of carboxypeptidase E (Cpe) in pro-CCK processing. *Endocrinology* **138**, 4034–4037.

Cameron A., Appel J., Houghten R. A. and Lindberg I. (2000a) Polyarginines are potent furin inhibitors. *J. Biol. Chem.* **275**, 36741–36749.

Cameron A., Fortenberry Y. and Lindberg I. (2000b) The SAAS granin exhibits structural and functional homology to 7B2 and contains a highly potent hexapeptide inhibitor of PC1. *FEBS Letts.* **473**, 135–138.

Curry W. J., Johnston C. F., Hutton J. C., Arden S. D., Rutherford N. G., Shaw C. and Buchanan K. D. (1991) The tissue distribution of rat chromogranin A-derived peptides: evidence for differential tissue processing from sequence specific antisera. *Histochemistry* **96**, 531–538.

Day R., Schafer M. K.-H., Watson S. J., Chrétien M. and Seidah N. G. (1992) Distribution and regulation of the prohormone convertases PC1 and PC2 in the rat pituitary. *Mol. Endocrinol.* **6**, 485–497.

Fischer-Colbrie R., Lassmann H., Hagn C. and Winkler H. (1985) Immunological studies on the distribution of chromogranin A and B in endocrine and nervous tissues. *Neuroscience* **16**, 547–555.

Fortenberry Y., Liu J. and Lindberg I. (1999) The role of the 7B2 CT peptide in the inhibition of prohormone convertase 2 in endocrine cell lines. *J. Neurochem.* **73**, 994–1003.

Fricker L. D., McKinzie A. A., Sun J., Curran E., Qian Y., Yan L., Patterson S. D., Courchesne P. L., Richards B., Levin L., Mzhavia N., Devi L. A. and Douglass J. (2000) Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. *J. Neurosci.* **20**, 639–648.

Hutton J. C., Peshavaria M., Johnston C. F., Ravazzola M. and Orci L. (1988) Immunolocalization of betagranin: a chromogranin A-related protein of the pancreatic B-cell. *Endocrinology* **122**, 1014–1020.

Lamango N. S., Apletalina E., Liu J. and Lindberg I. (1999) The proteolytic maturation of prohormone convertase 2 (PC2) is a pH-driven process. *Arch. Biochem. Biophys.* **362**, 275–282.

Lamango N. S., Zhu X. and Lindberg I. (1996) Purification and enzymatic characterization of recombinant prohormone convertase 2: stabilization of activity by 21 kDa 7B2. *Arch. Biochem. Biophys.* **330**, 238–250.

Lindberg I. and Zhou Y. (1995) Overexpression of neuropeptide precursors and processing enzymes. *Methods Neurosci.* **23**, 94–108.

Martens G. J. M., Braks J. A. M., Eib D. W., Zhou Y. and Lindberg I. (1994) The neuroendocrine polypeptide 7B2 is an endogenous inhibitor of prohormone convertase PC2. *Proc. Natl Acad. Sci. USA* **91**, 5784–5785.

Muller L. and Lindberg I. (1999) The cell biology of the prohormone convertases PC1 and PC2. *Prog. Nucleic Acid Res.*

Qian Y., Devi L. A., Mzhavia N., Munzer S., Seidah N. G. and Fricker L. D. (2000) The C-terminal region of proSAAS is a potent inhibitor of prohormone convertase 1. *J. Biol. Chem* **275**, 23596–23601.

Rouillé Y., Duguay S. J., Lund K., Furuta M., Gong Q., Lipkind G., Oliva A. A. J., Chan S. J. and Steiner D. F. (1995) Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convertases. *Front. Neuroendocrinol.* **16**, 322–361.

Scopsi L., Gullo M., Rilke F., Martin S. and Steiner D. F. (1995) Proprotein convertases (PC1/PC3 and PC2) in normal and neoplastic human tissues: their use as markers of neuroendocrine differentiation. *J. Clin. Endocrinol. Metab.* **80**, 294–301.

Seidah N. G. and Chrétien M. (1999) Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Res.* **848**, 45–62.

Westphal C. H., Muller L., Zhou A., Bonner-Weir S., Schambelan M., Steiner D. F., Lindberg I. and Leder P. (1999) The neuroendocrine protein 7B2 is required for peptide hormone processing *in vivo* and provides a novel mechanism for pituitary Cushing's disease. *Cell* **96**, 689–7000.

Winsky-Sommerer R., Benjannet S., Rovère C., Barbero P., Seidah N. G., Epelbaum J. and Dournaud P. (2000) Regional and cellular localization of the neuroendocrine prohormone convertases PC1 and PC2 in the rat central nervous system. *J. Comp. Neurol.* **424**, 439–460.

Zheng M., Streck R. D., Scott R. E., Seidah N. G. and Pintar J. E. (1994) The developmental expression in rat of proteases furin, PC1, PC2, and carboxypeptidase E: implications for early maturation of proteolytic processing capacity. *J. Neurosci.* **14**, 4656–4673.