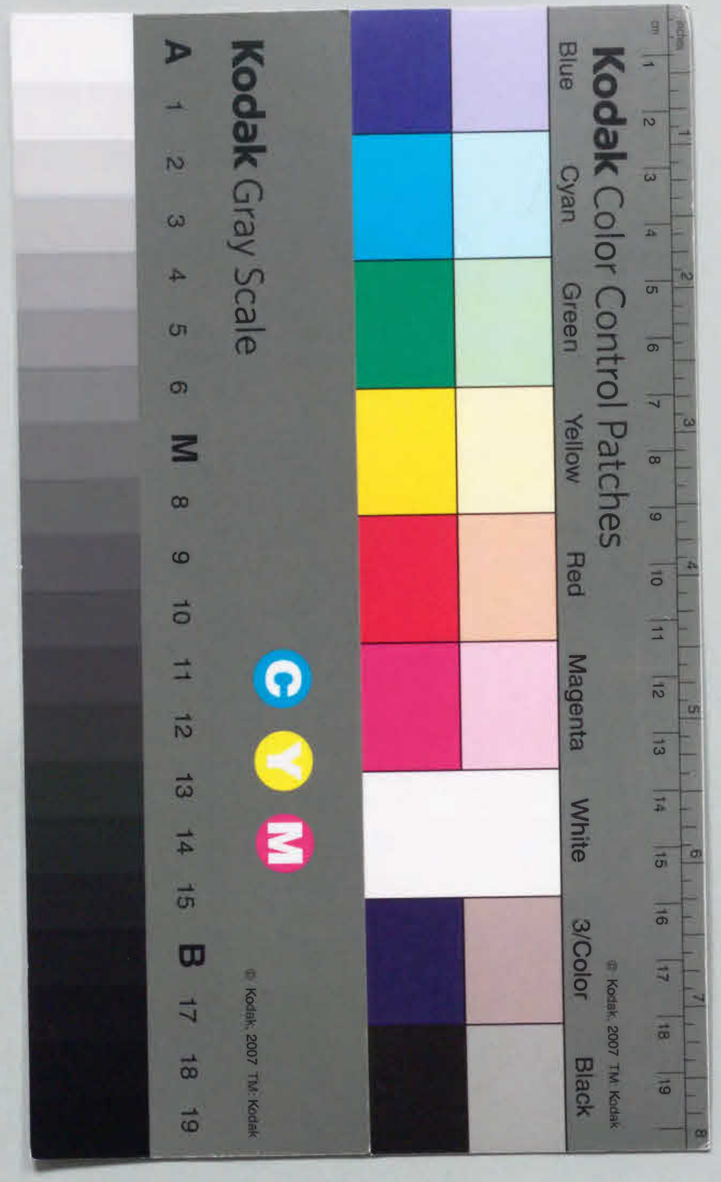

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論　文　目　録

報告番号	甲　工 乙　工 工　修　第　96　号	氏　名	赤　松　徹　也
学位論文題目	**Histochemical and Cytochemical Studies on Processing Protease PACE4 (SPC4)**		

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参考論文

主論文

① Akamatsu,T., Daikoku,S., Nagamune,H., Yoshida,S., Mori,K., Tsuji,A. and Matsuda,Y., Developmental expression of a novel Kexin family protease, PACE4E, in the rat olfactory system. Histochem. Cell Biol. (1997) in press

副論文

① Nagamune,H., Muramatsu,K., Akamatsu,T., Tamai,Y., Izumi,K., Tsuji,A. and Matsuda,Y., Distribution of he Kexin Family Proteases in Pancreatic Islets: PACE4C Is Specifically Expressed in B Cells of Pancreatic Islets. Endocrinology 136 (1995) 357-360

② Mori,K., Kii,S., Tsuji,A., Nagahama,M., Imamaki,A., Hayashi,K., Akamatsu,T., Nagamune,H. and Matsuda, Y., A novel human PACE4 isoform, PACE4E is an active processing protease containing a hydrophobic cluster at the carboxy terminus. J. Biochem. 121 (1997) 941-948

③ Nagamune,H., Muramatsu,K., Higashine,M., Akamatsu,T., Tamai,Y., Yonetomi,Y., Tsuji,A., Izumi,K., Sakaguchi,T., Yoshida,T. and Matsuda, Y., Cyclic AMP-inducible procalcitonin processing in thyroidal parafollicular cells is regulated by the Kexin family protease, PC1. In: Katunuma,N., Kido,H. and Travis,J.(eds) Medical Aspects of Proteases and Protease Inhibitors. IOS Press, Amsterdam. (1997) in press

④ Tsuji,A., Hine,C., Tamai,Y., Yonemoto,K., Mori,K., Yoshida,S., Bando,M., Sakai,E., Mori,K., Akamatsu,T. and Matsuda,Y., Genomic organization and alternative splicing of human PACE4 (SPC4), Kexin-like processing endoprotease. J. Biochem. (1997) in press

2

様式7

論 文 内 容 要 旨

報告番号	(甲 工) 乙 工 第 96 号 工 修	氏 名	赤 松 徹 也
学位論文題目	**Histochemical and Cytochemical Studies on Processing Protease PACE4 (SPC4)**		

インスリンに代表されるペプチドホルモン，神経ペプチドや増殖因子といった生理活性ペプチドや蛋白質の大部分はまず，不活性な前駆体蛋白質として合成され，特異的酵素 (プロセシング酵素) による前駆体蛋白質中の塩基性アミノ酸対部位 (LysArg, ArgArgのカルボキシ末端側) での限定切断を受けて生理活性を有する成熟体となる．従って，この限定切断は生理活性ペプチド・蛋白質生合成において必須の過程であり，そのようなプロセシングに関与する酵素の生理機能の解明は生物学的に極めて重要である．

1984年に酵母のプロセシング酵素であるKexin (EC3.4.21.61) が同定されて以後，哺乳類においてもKexinの相同体であるfurin，PC2，PC1/PC3，PACE4，PC4，PC5/PC6及びLPC/PC7/PC8からなる一群のKexinファミリープロテアーゼが同定されている．これらのKexinファミリープロテアーゼはCa^{2+}依存性のセリンプロテアーゼで，サチライシンに類似の触媒領域を持つことから順にサチライシン様プロ蛋白質変換酵素(Subtilisin- like Proprotein Convertase; SPC) SPC1-SPC7とも呼ばれる．

本論文はファミリーの1つPACE4 (SPC4) の生理機能の解明を目的として，その組織・細胞特異的発現を主として組織化学的手法により詳細に解析した研究成果である．PACE4は1991年にKieferらにより同定されたが，その後Tsujiら(1994) 及びMoriら (1997) により複数のアイソフォームの存在が明らかにされた．個々のPACE4アイソフォームの生理機能を解明するためにはまずアイソフォーム各々の組織・細胞特異的発現を詳細に解析することが必要不可欠であるにもかかわらず，これまで全く明らかにされていなかった．本研究では特に，プロセシング活性を有し，C末端構造の違いから生体内で異なる機能を果たすと考えられる2つのアイソフォーム，PACE4AとPACE4Eの中枢神経系における発現を，各々に特異的なジゴキシゲニン標識化アンチセンスcRNAプローブを用いた*in situ* ハイブリダイゼーション法により，成獣ラット脳及びその発生段階において詳細に解析し，アイソフォーム個々の発現部位をはじめて特異的に検出した．

成獣ラット脳では，2つのアイソフォームは広範囲に発現し，その発現は非常に類似していた．また，神経ペプチドの豊富な領域・細胞など特定の部位で特に強く発現しており，PACE4EとPACE4Aの一方または両方の神経ペプチド生合成への関与が示唆された．更に，種々の発生段階においてもPACE4EとPACE4Aはよく似た発現パターンを示し，発生過程が進み脳の層構造が形成されるにつれ明らかに特定の領域・細胞に局在化が認められた．このことはPACE4EとPACE4Aが増殖因子，神経栄養因子あるいは細胞接着分子などのプロセシングを介して中枢神経系の形成に深く関与していると考えられる．実際にPACE4アイソフォームがどの様な生理活性ペプチド・蛋白質と共存しているかも全く不明であったが，本研究において初めてニューロペプチドY，副腎皮質刺激ホルモンや接着分子L1などとの共存が明らかになった．以上より，PACE4アイソフォームが種々の生理活性ペプチド・蛋白質の活性化により中枢神経系の構造と機能の形成・維持に重要な役割を果たしていることが示唆され，PACE4の生理機能の解明において極めて重要な知見が得られた．

Histochemical and Cytochemical Studies on Processing Protease PACE4 (SPC4)

Tetsuya Akamatsu

Dissertation submitted to the Faculty of the Graduate School
of the University of Tokushima in partial fulfillment
of requirements for the degree of Doctor of Philosophy

Department of Biological Science and Technology /
Faculty of Engineering

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Color Figures

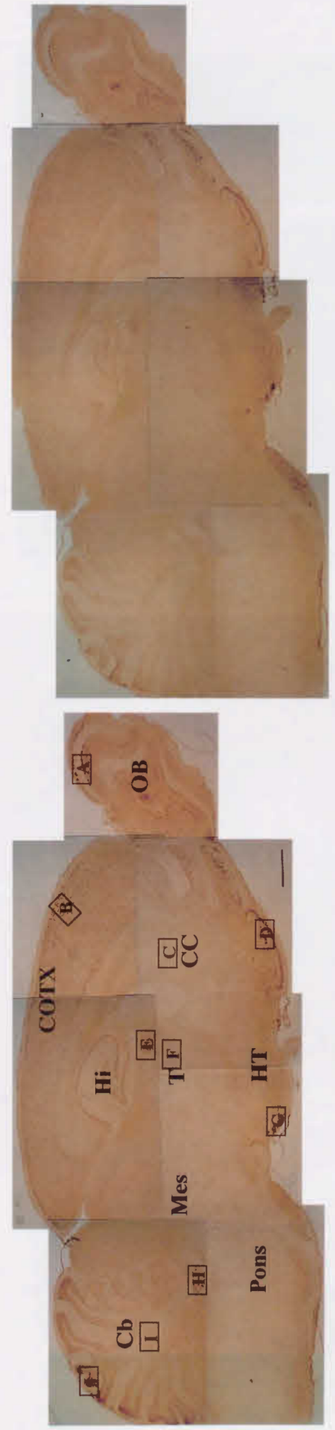

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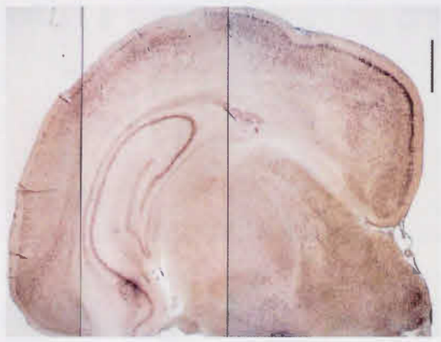
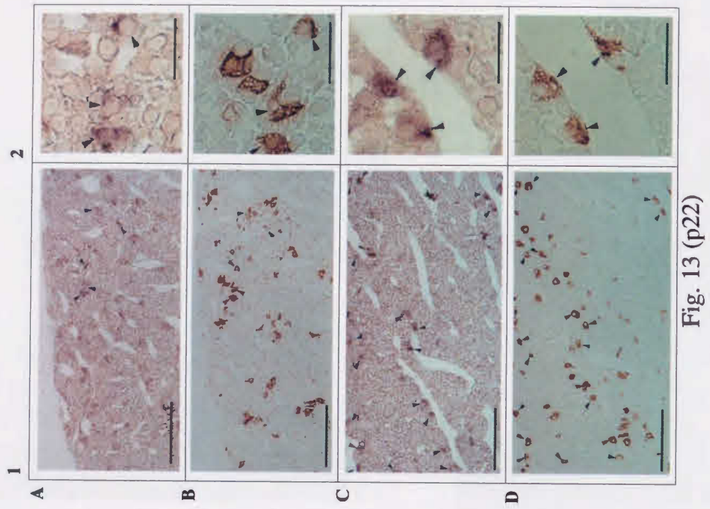
Fig. 9 (p13)

Fig. 7 (p11)





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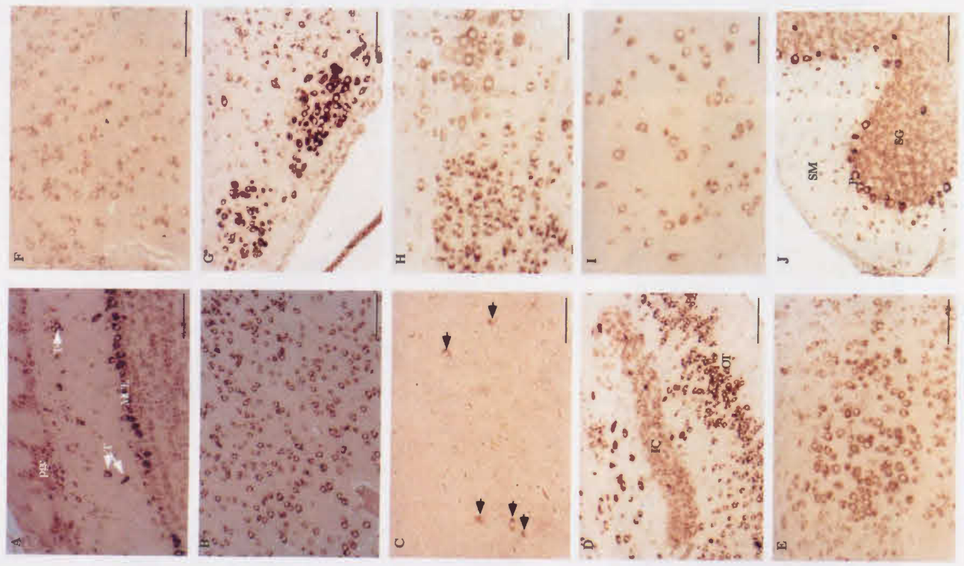

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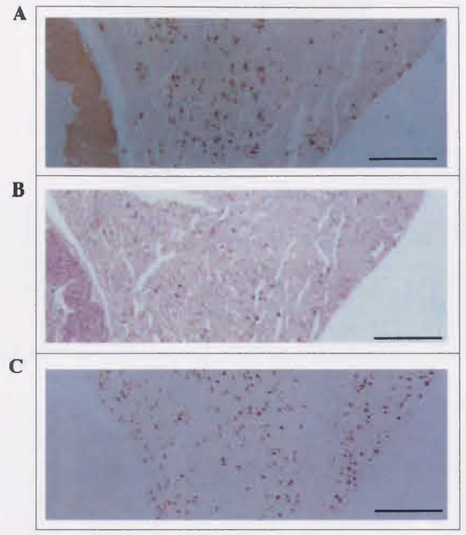

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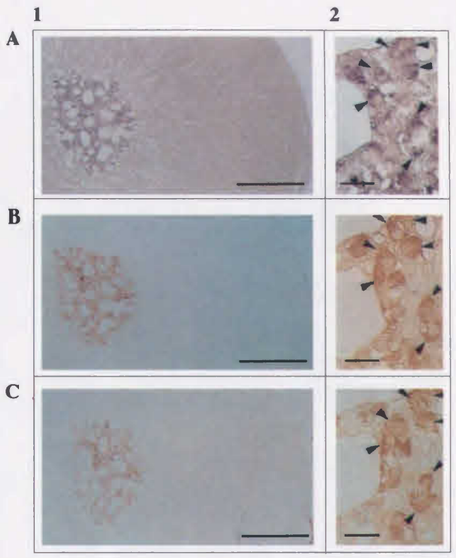
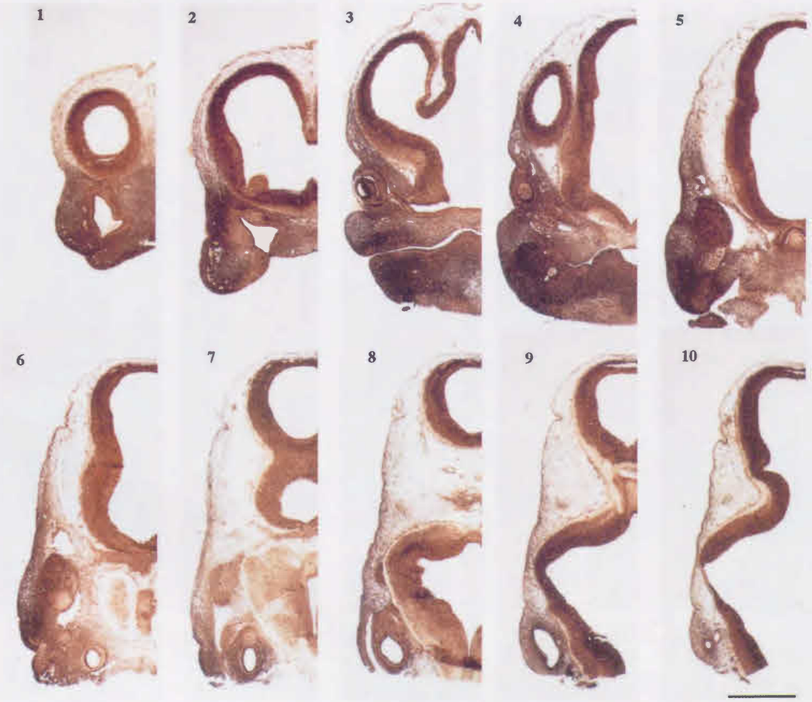


Fig. 14 (p23)

Fig. 15 (p25) [REFERENCE PAPER 2) Fig. 1]



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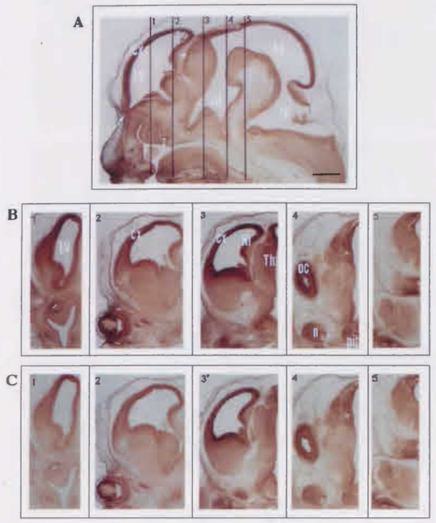

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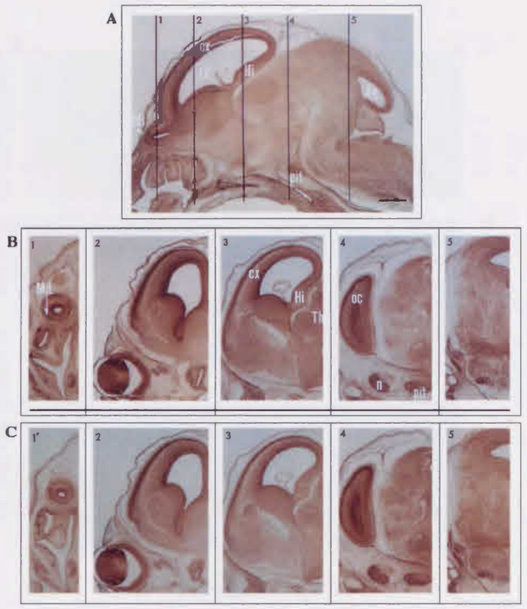

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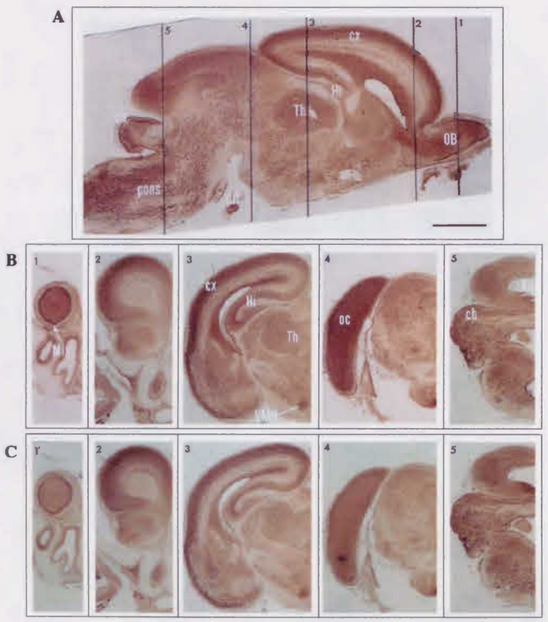

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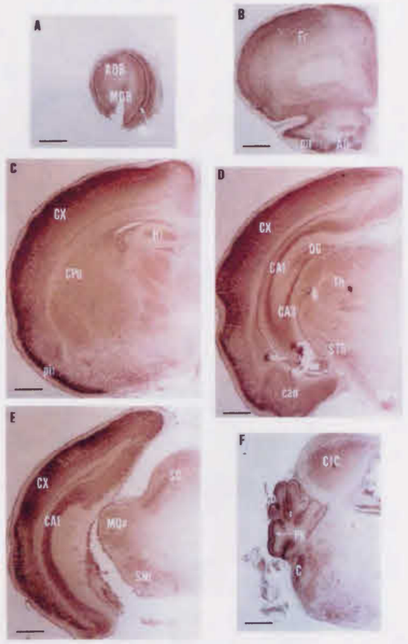

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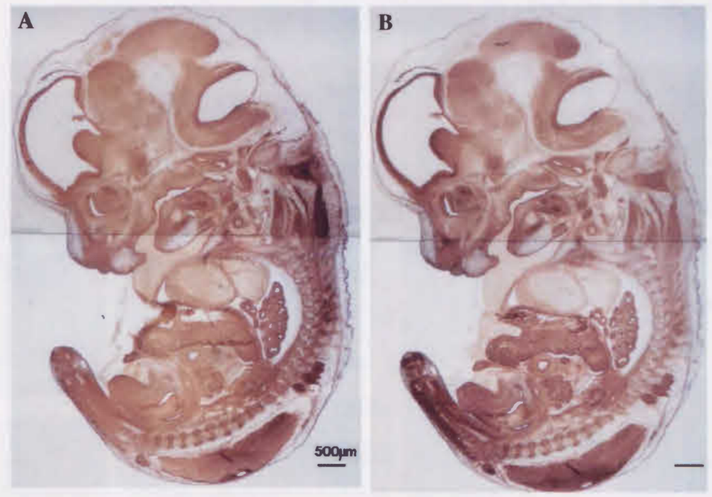

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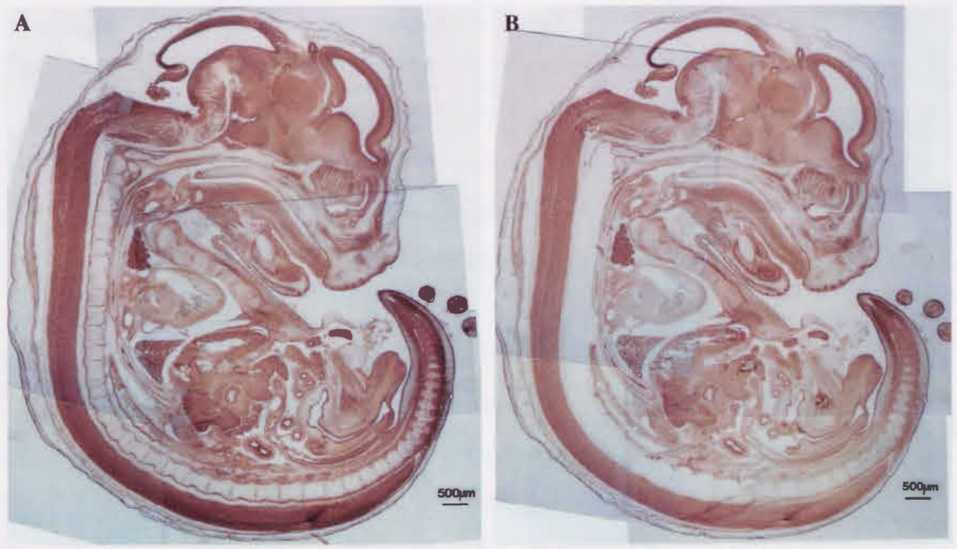

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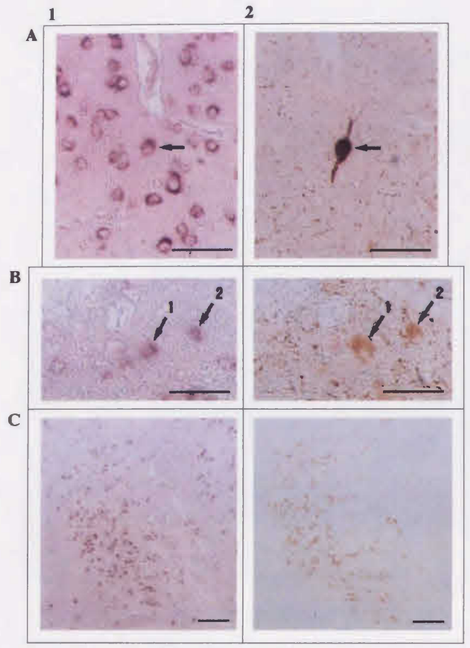

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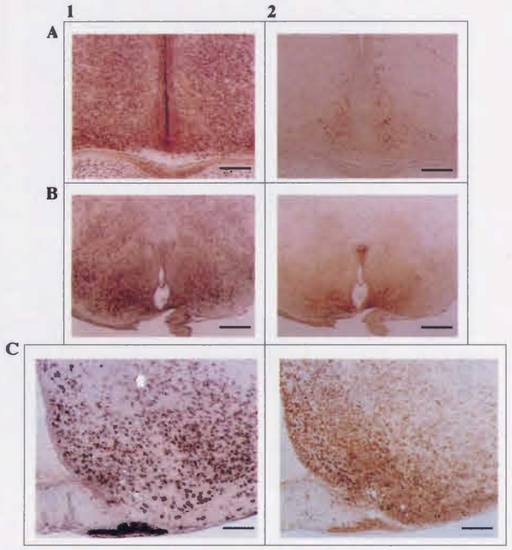

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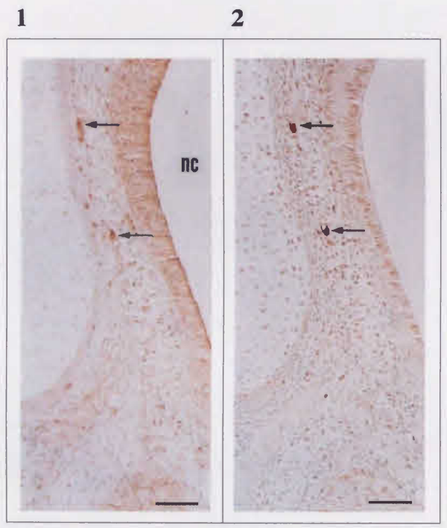

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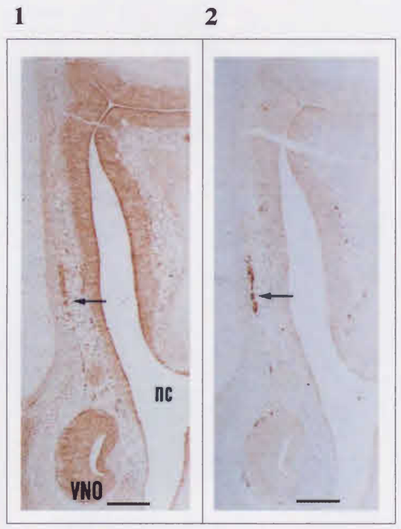

Fig. 27 (p43)

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CHAPTER I. Introduction

1. Biogenesis of biologically active peptides and proteins

In higher animals, it is thought that the sustaining homeostasis is regulated by the transfer of various informations at synapses in the central nervous system and the conversion of such signals into various biologically active peptides and proteins including peptide hormones, neuropeptides and growth factors. These bioactive peptides combine with their specific receptors on the cell surface to activate appropriate target cells that make specific physiological responses.

Most of these biologically active peptides and proteins are originally generated as the inactive form of large polypeptide precursors which contain more than one biologically active peptide. As shown in Fig. 1, the

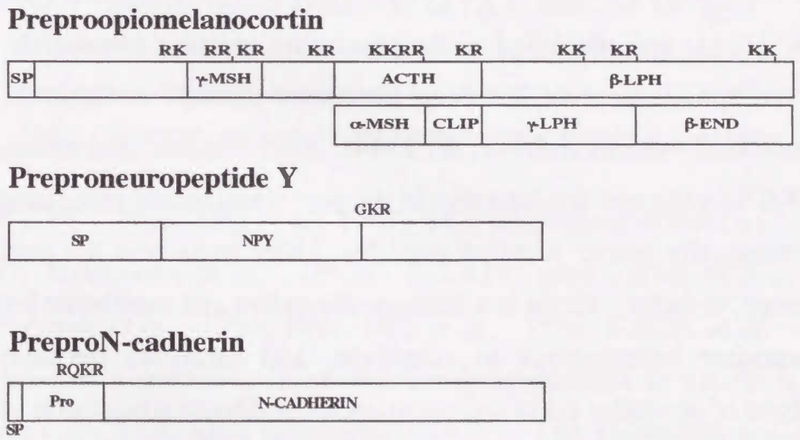

Fig. 1: Structure of prepropeptide hormones. Most bioactive peptides are flanked at the sites marked by paired basic amino acid residues. ACTH; adrenocorticotropic hormone, CLIP; corticotropin-like intermediate lobe peptide, END; endorphin, LPH; lipotropin, MSH; melanocyte stimulating hormone, NPY; neuropeptide Y.

biologically active peptides and proteins in the precursors are flanked at sites marked usually by paired basic amino acid residues (Douglass *et al.* 1984; Barr 1991). Therefore the proteolytic cleavage at paired basic amino acid sites is a key step in the production of biologically active peptides and proteins. Further, such proteolytic processing is extremely specific, and in some cases the same precursor is converted to different bioactive peptides by cell- and tissue-specific processing, or one biologically active peptide is processed by the interaction of several processing proteases (Douglass *et al.* 1984). Research into such processing proteases is very important for understanding the mechanisms of biogenesis of biologically active peptides and proteins.

2. Discovery of mammalian candidate processing proteases

In 1984, Kex2 protease of the yeast *Saccharomyces cerevisiae* (Kexin, EC3. 4. 21. 61) was identified as the processing protease responsible for the activation of peptide hormone precursor through analysis of *S. cerevisiae kex2* mutants (Julius *et al.* 1984). *Kex2* mutants have mutations in the KEX2 gene and are defective in the post-translational processing of two biologically active secreted peptides; killer toxin and the mating pheromone, α-factor. Kexin is a calcium-dependent and membrane bound endopeptidase homologous to subtilisin, and catalyzes the limited proteolysis of pro-killer toxin and pro-α-factor at dibasic sites (Julius *et al.* 1984; Fuller *et al.* 1988, 1989).

A homology search with Kexin revealed furin, a product of the human *fur* gene lying immediately upstream of the c-*fes/fps* protooncogene, as a candidate of mammalian processing protease (Schalken *et al.* 1987; Fuller

et al. 1989; Bresnahan *et al.* 1990). Further, additional Kexin/furin-related proteases (so called Kexin family proteases) in mammalian species have been identified by polymerase chain reaction and cDNA cloning (Fig. 2). These include PC2 (Smeekens and Steiner 1990), PC1 (also called PC3) (Seidah *et al.* 1991; Smeekens *et al.* 1991), PACE4 (Kiefer *et al.*

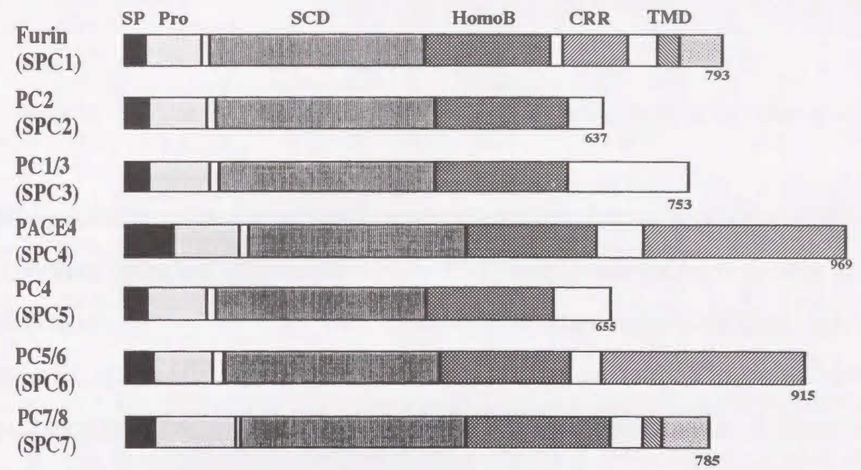

Fig. 2: Schematic representation of Kexin family proteases. SP; signal peptide, Pro; propeptide, SCD; subtilisin-like catalytic domain, Homo B; homologous domain B among Kexin family, CRR; cysteine-rich region, TMD; transmembrane domain, SPC; subtilisin-like proprotein convertase.

1991), PC4 (Nakayama *et al.* 1992), PC5 (also called PC6) (Lusson *et al.* 1993; Nakagawa *et al.* 1993a) and LPC (also called PC8 or PC7) (Meerabux *et al.* 1996; Bruzzaniti *et al.* 1996; Seidah *et al.* 1996). Because of a resemblance of their catalytic domains to subtilisin, these convertases have been designated subtilisin-like proprotein convertases (SPC); SPC1 to SPC7, respectively. Kexin family proteases have a common domain structure consisting of a signal peptide, a propeptide, a subtilisin-like catalytic domain and a homologous domain B (homo B). This is the minimum structure essential for the expression of protease

activity (Hatsuzawa *et al*. 1992). However, their carboxy-terminal structures are unique. Furin has a cisteine-rich region and a transmembrane domain, while, PACE4 and PC5/6 only have a long cisteine-rich region, and PC7/8 only has a transmembrane domain, in addition to the common domain. PC2, PC1/3 and PC4 have neither a cisteine-rich region nor a transmembrane domain.

3. General characterization of Kexin family proteases

Kexin family proteases are calcium-dependent serine proteases like yeast processing enzyme Kexin, and show structural similarity, especially in the subtilisin-like catalytic domain (SCD). Fig. 3 shows the conservation of sequences within the SCD. The active sites Asp, His, and Ser, as well as the catalytically important Asn residues are conserved and found at positions equivalent to those in subtilisins and Kexin. These convertases are known to be synthesized initially as zymogens, and the proteolytic removal of the N-terminal prosegment is necessary for expression of the processing activity. As shown in Fig. 3, the putative activation site, Arg-X-Lys-Arg, is completely conserved in all members. Among the Kexin family, furin had been well characterized in terms of its intramolecular autoproteolytic activation (Leduc *et al*. 1992; Rehemtulla *et al*. 1992). Autocatalytic maturation of pro-PC1/3 and pro-PC2 was also reported (Benjannet *et al*. 1993; Zhou and Lindberg 1993; Matthews *et al*. 1994).

Further, several isoforms of Kexin family protease have been identified. We identified two PACE4 isoforms, PACE4C and PACE4D, from a human placenta cDNA library (Tsuji *et al*. 1994). More recently,


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PACE4  120:LSSRGPHTFLRMDPQVKWLQQQEVKRRVKRQVR-SDPQALY-FNDPIWSNMWYLHC-GDK
Furin   78:SPHRPRHSRLQREPQVQWLEQQVAKRRTKR--DL--YQEP---TDPKFPQQWY------L
PC1/3   81:RSAFHITKRLSDDDRVIWAEQQYEKERSKRSALRD-SALNL-FNDPMWNQQWYLQDTRMT
PC2     80:RRSLHHKQQLERDPRVKMALQQEGFDRKKR-GYRDINEIDINMNDPLFTKQWYLINTGQA
PC4     81:TPHWGHRLRLKKDPKVRWFEQQTLRRRVKR--SL---VVP---TDPWFSKQWY------M
PC5/6   87:LSSRGTHSFISMEPKVEWIQQQVVKKRTKRDYDLSHAQSTY-FNDPKWPSMWYMHC-SQN
PC7/8  112:PIRQQVEAVLAGHEAVRWHSEQRLLRRAKRSVH---------FNDPKYPQQW---H-LNN

PACE4  177:NSRCRSEMNVQAAWKRGYTGKNVVVTILDDGIERNHPDLAPNYDSYASYDVNGNDYDPSP
Furin  125:SGVTQRDLNVKAAWAQGYTGHGIVVSILDDGIEKNHPDLAGNYDPGASFDVNDQDPDPQP
PC1/3  139:AALPKLDLHVIPVWQKGITGKGVVITVLDDGLEWNHTDIYANYDPEASYDFNDNDHDPFP
PC2    139:DGTPGLDLNVAEAWELGYTGKGVTIGIMDDGIDYLHPDLASNYNAEASYDFSSNDPYPYP
PC4    127:NKEIQQDLNILKAWNQGLTGRGVVISILDDGIEKDHPDLWANYDPLASYDFNDYDPDPQP
PC5/6  145:THPCQSDMNIEGAWKRGYTGKNIVVTILDDGIERTHPDLMQNYDALASCDVNGNDLDPMP
PC7/8  159:RRSPGRDINVTGVWERNVTGRGVTVVVVDDGVEHTIQDIAPNYSPEGSYDLNSNDPDPMP

PACE4  237:RYDASNENKHGTRCAGEVAASANNSYCIVGIAYNAKIGGIRMLD-GDVTDVVEAKSLGIR
Furin  185:RYTQMNDNRHGTRCAGEVAAVANNRVCGVGVAYNARIGGIRMLD-GEVTDAVEARSLGLN
PC1/3  199:RYDPTNENKHGTRCAGEIAMQANNHKCGVGVAYNSKVGGIRMLD-GIVTDAIEASSIGFN
PC2    199:RYTDDWFNSHGTRCAGEVSAAANNNICGVGVAYNSKVAGIRMLDQPFMTDIIEASSISHM
PC4    187:RYTPNDENRHGTRCAGEVSATANNGFCGAGVAFNARIGGIRMLD-GAITDIVEAQSLSLQ
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PC7/8  219:HPDVENGNHHGTRCAGEIAAVPNNGFCAVGVAYGSRIAGIRMLD-GPLTDSMEAVAFNKH

PACE4  296:PNYIDIYSASWGPDDDGKTVDGPGRLAKQAFEYGIKKGRQGLGSIFVWASGNGGREGDYC
Furin  244:PNHIHIYSASWGPEDDGKTVDGPARLAEEAFFRGVSQGRGGLGSIFVWASGNGGREHDSC
PC1/3  258:PGHVDIYSASWGPNDDGKTVDGPGRLAQKAFEYGVKQGRQGKGSIFVWASGNGGRQGDNC
PC2    259:PQLIDIYSASWGPTDNGRTVDGPRELTLQAMADGVNKGRGGKGSIYVWASGNGGSY-DDC
PC4    246:PQHIHIYSASWGPEDDGKTVDGPGLLTQEAFRRGVTKGRQGLGTLFIWASGNGGLHYDNC
PC5/6  264:PQHVHIYSASWGPDDDGKTVDGPAPLTRQAFENGVRMGRRGLGSVFVWASGNGGRSKDHC
PC7/8  278:YQINDIYSCSWGPDDDGKTVDGPHQLGKAALQHGVIAGRQGFGSIFVVASGNGGQHNDNC

PACE4  356:SCDGYTNSIYTISVSSATENGYKPWYLEECASTLATTYSSG--AFYERKIVTTDL--RQR
Furin  304:NCDGYTNSIYTLSISSATQFGNVPWYSEACSSTLATTYSSG--NQNEKQIVTTDL--RQK
PC1/3  318:DCDGYTDSIYTISISSASQQGLSPWYAEKCSSTLATSYSSG-D-YTDQRITSADL--HND
PC2    318:NCDGVASSMWTISINSAINDGRTALYDESCSSTLASTFSNGRKRNPEAGVATTDL--YGN
PC4    306:NCDGYTNSIHTLSVGSTTRQGRVPWYSEACASTFTTTFSSG--VVTDPQIVTTDL--HHQ
PC5/6  324:SCDGYTNSIYTISISSTAESGKKPWYLEECSSTLATTYSSG--ESYDKKIITTDL--RQR
PC7/8  338:NYDGYANSIYTVTIGAVDEEGRMPFYAEECASMLAVTFSGGDKMLRSIVTTDWDLQKGTG

PACE4  412:CTDGHTGTSVSAPMVAGIIALALEANSQLTWRDVQHL
Furin  360:STESHTGTSASAPLAAGIIALTLEANKNLTWRDMQHL
PC1/3  374:CTETHTGTSASAPLAAGIFALALEANPNLTWRDMQHL
PC2    376:CTLRHSGTSAAAPEAAGVFALALEANLGLTWRDMQHL
PC4    362:CTDKHTGTSASAPLAAGMIALALEANPLLTWRDLQHL
PC5/6  380:CTDNHTGTSASAPMAAGIIALALEANPFLTWRDVQHV
PC7/8  398:CTEGHTGTSAAAPLAAGMIALMLQVRPCLTWRDVQH-
```

Fig. 3: Conservation of active site residues of Kexin family proteases. The subtilisin-like catalytic domain of human PACE4 was compared with that of other human Kexin family members. Shaded letters indicate identical amino acid residues. Active site residues (D, H, N, S) are indicated by closed circles and the putative activation site (RXKR ↓) is underlined.

an additional PACE4 isoform, PACE4E, was identified from a human cerebellum cDNA library in our study (Mori *et al.* 1997). Nakagawa *et al.* (1993a, 1993b) reported two PC5/6 isoforms, PC5/6A and PC5/6B. PC5/6A and PC5/6B differ in the length of their C-terminal repeat structure of cysteine-rich motif and only PC5/6B has a transmembrane domain. In contrast, PACE4 isoforms have a unique C-terminus (Fig. 4). PACE4A, which was identified by Keifer *et al.* (1991) as PACE4, has the minimum structure essential for protease activity and the cysteine-rich region described above. PACE4B, which was identified as PACE4.1 (Keifer *et*

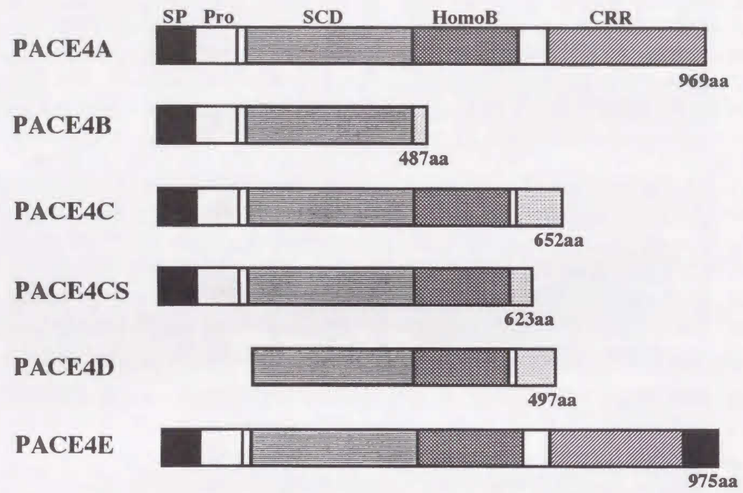

Fig. 4: Schematic representation of PACE4 isoforms. Abbreviations are same in Fig. 2 legend.

al. 1991) lacks a homo B domain, so this isoform is considered to be inactive. PACE4C protein has a short but certainly unique C-terminal structure in addition to the common domain structure. A carboxy terminally shortened version of PACE4C (PACE4CS) was recently reported by Zhong *et al.* (1996). PACE4D lacks a signal peptide and a propeptide, and may also be inactive. Interestingly, PACE4E contains a

hydrophobic cluster in its C-terminus, although its structure is most similar to that of PACE4A. No such hydrophobic cluster has been found in known other Kexin family proteases. Further, PACE4A and PACE4E are classified as type-I and type-II. Type-II isoforms lack a 39 bp nucleotide sequence just in front of the cysteine-rich region (Mori *et al.* 1997). The biogenesis and processing activities of several PACE4 isoforms were reported by Mori *et al.* (1997). PACE4A was first synthesized in a 110 kDa precursor form, and converted to a 103 kDa mature form as reported by Mains *et al.* (1997). While PACE4E was also synthesized as 112 kDa zymogen, and converted into a 105 kDa mature form. The maturation of these two PACE4 isoforms occurs with the same efficiency, however, the efficiency of their secretion differs (Mori *et al.* 1997). PACE4E seemed to be intracellularly retained probably because of its C-terminal hydrophobic cluster, although PACE4A was easily secreted. On the other hand, PACE4C synthesized as a 68 kDa species was not processed and secreted at all.

Further, both PACE4A and PACE4E exhibit processing activity at the dibasic amino acid residues of several precursor proteins, however, their cleavage efficiency somewhat differs. These observations suggest that PACE4 isoforms share physiological functions *in vivo*.

The tissue distribution of each Kexin family member was analyzed

Table 1. Tissue distribution of Kexin family proteases

1. Restricted Distribution
   A. Endocrine and nonendocrine cells
      (1) PACE4 : brain, **pituitary**, **heart**, duodenum, jujenum, colon, pancreatic islet B cells, liver, lung, kidney
      (2) PC5/6 : brain, pituitary, heart, **antrum**, **ileum**, liver, lung, spleen, kidney, ovary, adrenal gland
   B. Endocrine and neuroendocrine cells
      (1) PC1/3 : **brain**, **pituitary**, thyroid, **pancreatic islet B cells**, oviduct, adrenal gland
      (2) PC2 : **brain**, **pituitary**, thyroid, pancreatic islet A, B and D cells, adrenal gland, epididymis
      (3) PC4 : **testicular germ cells**, ovary
2. Ubiquitous Distribution
   (1) Furin
   (2) PC7/8 (LPC)

Tissues expressing a high level of mRNA are in bold.

by polymerase chain reaction and Northern blot analysis (Table 1. and Seidah *et al.* 1994), however, the physiological role of these convertases is largely unknown. A detailed understanding of the cell-localization of Kexin family proteases is necessary in order to clarify their physiological significance.

In this regard, there have been several studies on the cell-specific expression of Kexin family proteases by *in situ* hybridization histochemistry (Bloomquist *et al.* 1991; Day *et al.* 1992, 1993; Torii *et al.* 1993; Seidah *et al.* 1993; Beaubien *et al.* 1995). In the adult rat central nervous system (CNS), the distribution of PC2, PC1/3 and furin (Schafer *et al.* 1993) and more recently, PC5/6 and PACE4 mRNA (Dong *et al.* 1995) have been reported. However, neither study discriminated among isoforms in spite of the existence of plural isoform. Particularly, PACE4 has the most isoforms among Kexin family proteases. Recent studies of human PACE4 gene revealed that the isoforms are generated by alternative splicing from a single gene (Tsuji *et al.* 1997). Further, the extraordinarily large and complicated structure of this gene suggests that the expressions are highly regulated in a tissue- and cell-specific manner.

In the present study, we analyzed the tissue- and cell-specific expression of PACE4 isoforms in various rat organs. In particular, we focused on the expression of two active PACE4 isoforms, PACE4E and PACE4A, in not only adult but also embryonic rat CNS to gain insights into their physiological function.

CHAPTER II. Distribution of PACE4 isoforms in adult rat tissues

1. Central Nervous System (CNS)

Expression of PACE4E and PACE4A mRNAs in the adult rat CNS was examined by Northern blotting with specific probes whose sites are shown in Fig. 5. A PACE4E transcript of about 4.4kb was detected in the

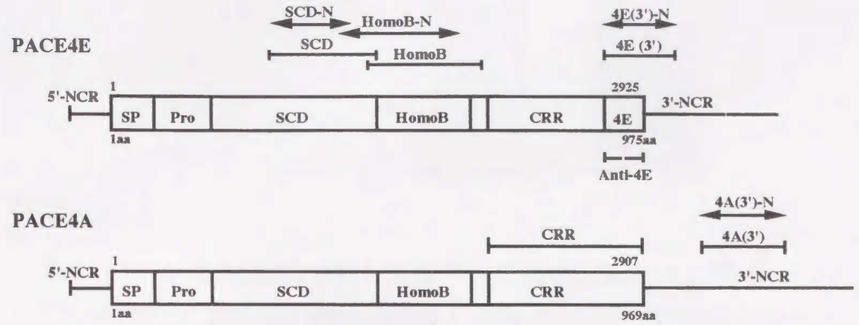

Fig. 5: Schematic representation of various probing sites. SCD-N, HomoB-N, 4E(3')-N, and 4A(3')-N are the sites of cDNA probe for Northern blotting. SCD, HomoB, CRR, 4E(3'), and 4A(3') are also the probing regions of cRNA probes for *in situ* hybridization. Anti-4E shows the site of specific antibody for PACE4E.

adult rat olfactory bulb, telencepharon, diencepharon-mesencepharon, cerebellum, pons and pituitary (data not shown and Akamatsu *et al.* 1997). On the other hand, the PACE4A transcript was also estimated to be 4.4kb in all CNS areas studied. Further, a PACE4 transcript of identical size was detected by two other probes specific for the subtilisin-like catalytic domain of PACE4 (SCD-N) and homologous domain B of PACE4 (Homo B-N) (data not shown). Thus, these PACE4 isoforms appear to be widely distributed in the adult rat CNS, and to have similar levels of expression.

The transcripts of PACE4E and PACE4A were detected in various regions of the adult rat brain by Northern blotting. To determine in detail the distribution of the cells expressing messages of these PACE4 isoforms in the adult rat brain, *in situ* hybridization was performed using a cRNA probe specific for PACE4E or PACE4A. These specific probes were

confirmed to hybridize only with the corresponding cDNA fragment(s) of PACE4E or PACE4A by Southern blot analysis (Fig. 6 A). Therefore these

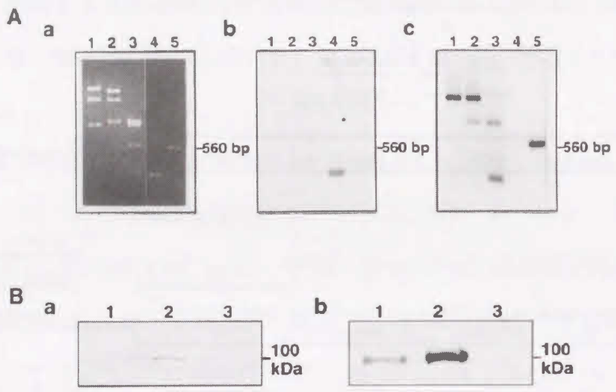

Fig. 6: Specificity of cRNA probes and antibodies. A: Southern blot analysis using specific cRNA probes. a: Ethidium bromide staining. b: Southern blot using PACE4E specific probe. c: Southern blot using PACE4A specific probe. These cRNA probes specifically recognized the correct cDNA fragment(s). Lane 1, HPL-11 digested by *Eco* RI; lane 2, HPL-11 digested by *Pst* I; lane 3, HPL-11 digested by *Eco* RI+*Pst* I; lane 4, the fragment of PACE4E specific region; lane 5, the probing region fragment of PACE4A. The size marker on the right is the 560 bp fragment from a λDNA-*Hind* III digest. B: Western blot analysis with total homogenate of COS-1 cells overexpressing PACE4 isoforms. a: Western blot using anti-PACE4E. b: Western blot using anti-SCD. Anti-PACE4E specifically detected PACE4E protein as a 100 kDa protein (a-2), but did not react with PACE4A (a-1). Lane 1, PACE4A-overexpressing cells; lane 2, PACE4E-overexpressing cells; lane 3, Mock-transfected cells.

specific cRNA probes can discriminate the cell-specific expression of two active PACE4 isoforms by *in situ* hybridization histochemistry. In *in situ* hybridization, the positive signals were detected by the antisense but not the sense probe. The results were shown in Fig. 7-10. The determination and nomenclature of the sites expressing PACE4E and PACE4A messages are based on Swanson (1992), Konig and Klippel (1963) and Paxinos (1995).

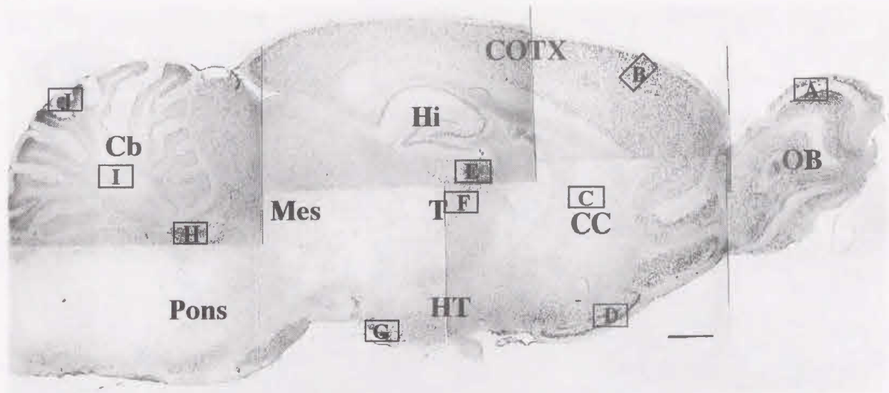

Fig. 7: *In situ* hybridization of PACE4E in adult rat brain cut sagittaly. Cb; cerebellum, CC; corpus callosum, COTX; cerebral cortex, Hi; hippocampus, HT; hypothalamus, Mes; mesencepharon, OB; olfactory bulb, T; thalamus. Boxed areas (A-J) are enlarged in Fig. 9 (A-J), respectively. Bar; 1mm.

Rhinencephalon

In the adult rat main olfactory bulb (MOB), PACE4E mRNA was most intensely expressed in the mitral cells which are large olfactory neurons arranged in almost a monolayer (Fig. 7 and Fig. 10 A). Additionally, the tufted cells and the periglomerular cells also expressed PACE4E mRNA intensely (Fig. 10 A), while the expression in the granule cells appeared weak. The expression pattern of PACE4E in the accessory olfactory bulb (AOB) was the same as in the MOB.

In the anterior olfactory nucleus, moderate to high levels of PACE4E mRNA expression were observed. Some other neurons and glial cells also expressed PACE4E mRNA, although the expression was weak.

Gene expression of PACE4A (Fig. 8) was analyzed and compared with that of PACE4E. The sites and intensity of signal were similar.

Among the cells expressing PACE4E and PACE4A, the mitral cells

Fig. 8: *In situ* hybridization of PACE4A in adult rat brain cut sagittaly.
Magnification is as for Fig. 7.

are the main output neurons in the OB, and have regenerating ability throughout the vertebrate lifetime. It is likely that the mitral cells continually generate the various kinds of biologically active peptides including neuropeptides, neurotropic factors and cell-adhesion molecules. The above two PACE4 isoforms may play a role in the activation of such bioactive peptides and proteins in these areas.

Telencephalon

Cortex

PACE4E and PACE4A mRNAs were widely, and similarly, distributed throughout the cerebral cortex (Fig. 7-9 and Fig. 10 B).

The pyramidal cells located in layer II-VI expressed low to high levels of PACE4E and PACE4A mRNAs. In layer VI, the expression of PACE4E and PACE4A seemed to be less intense. Further, in the temporal, the entorhinal and the piriform cortex, PACE4E and PACE4A was most strongly expressed in layer II.

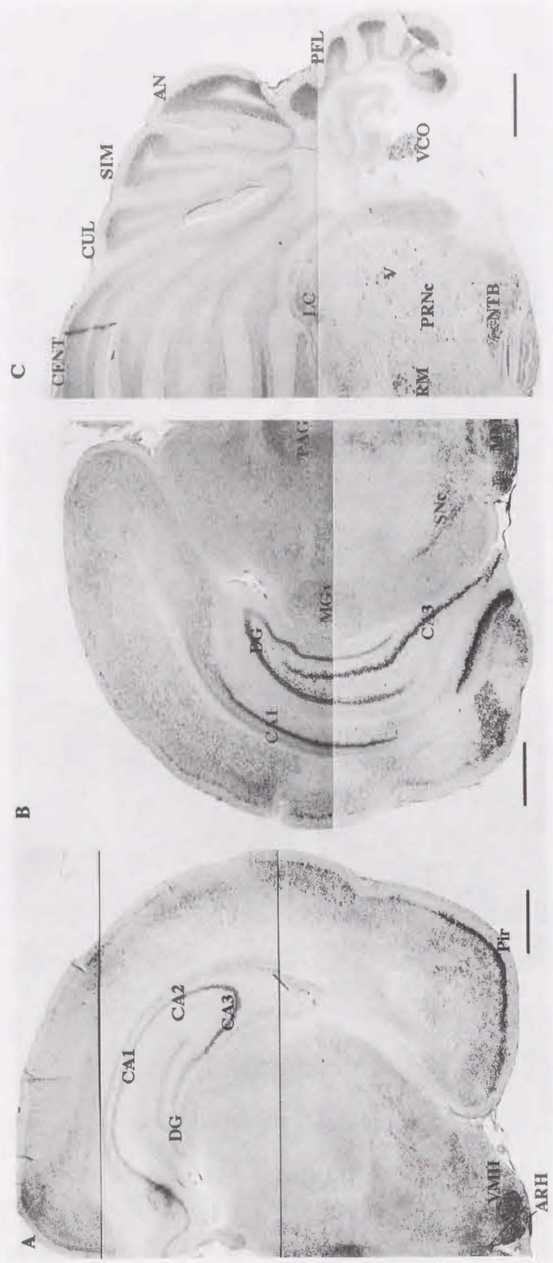

Fig. 9: *In situ* hybridization of PACE4E in the adult rat cerebrum (A), mesencepharon (B) and cerebellum (C). Bars; 1mm.

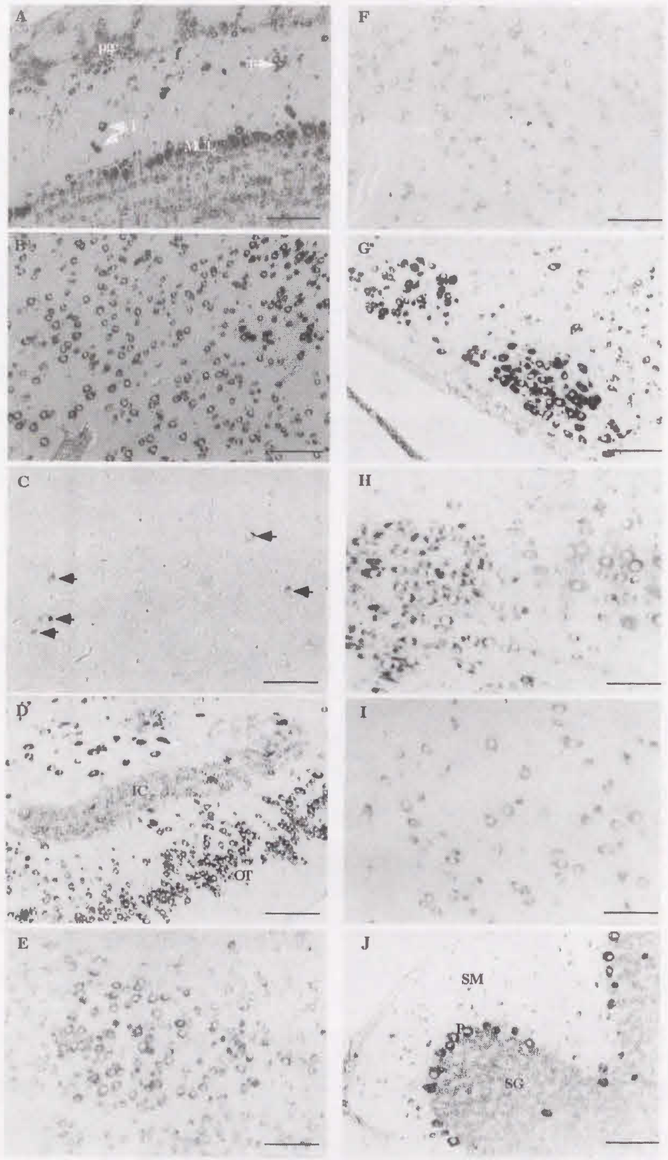

Fig. 10: High power views of panel A-J in Fig. 7. IC; islands of Calleja, MCL; mitral cell layer, OT; olfactory tubercle, P; Purkinje's cell, SG; stratum granulosum, SM; stratum moleculare, T; tufted cell, pgc; peri-glomerular cell. Bras; 100μm.

Hippocampal formation

Expression of PACE4E mRNA in the hippocampus was intense in the pyramidal cells of subfield CA1-CA3 of Ammon's horn, and most intense in subfield CA3 (Fig. 9 A). The granule cells of dentate gyrus (DG) expressed low levels of PACE4E mRNA. Additionally, some cells in the polymorph layer of DG, the stratum radiatum and the stratum oriens of the CA1 field expressed moderate levels of PACE4E message. In the subiculum, which expresses high levels of neurotensin/neuromedin N transcript (Alexander *et al.* 1989), PACE4E mRNA was also detected. All the above regions and cells expressed PACE4A mRNA.

Amygdala

Most of the amygdaloid body expressed low to high levels of PACE4E mRNA. The expression in the basolateral nucleus amygdala was particularly strong, whereas that in the lateral nucleus amygdala was very weak. These regions and cells also expressed PACE4A, though weakly so.

Other basal forebrain regions

In the nucleus caudatus putamen and the nucleus accumbens, a few neurons expressed PACE4E and PACE4A mRNAs at moderate to high levels, but in most cells the level was low or undetectable (Fig. 10 C). In contrast, intense expression of PACE4E and PACE4A mRNAs was observed in the olfactory tubercle. Islands of Calleja (Fig. 10 D) and the choroid plexus which express furin message but not PC1/3 and PC2 mRNAs (Schafer *et al.* 1993), expressed low levels of PACE4E and PACE4A mRNAs. Only furin and these two PACE4 isoforms were expressed in non-neuronal cells such as in the choroid plexus. Therefore,

these convertases were expected to play a critical role in glial and glial-like cells in the CNS.

Diencephalon

Epithalamus

Low to moderate levels of PACE4E mRNA expression were found in this region. In the medial habenula, the level of PACE4E mRNA expression was moderate, whereas in the lateral habenula it was very weak (Fig. 10 E). PACE4A showed a similar distribution. Amidated neuropeptides such as tachykinins are known to be expressed in the habenula (Burgunder and Young 1989).

Thalamus

In thalamus, the expression of PACE4E and PACE4A was weak to moderate (Fig. 10 F). The ventral posteromedial and posterolateral nucleus thalamus strongly expressed two active PACE4 isoforms, PACE4E and PACE4A. Further, the central medial nucleus thalamus, the paracentral nucleus thalamus and the rhomboid nucleus all expressed moderate levels of PACE4E and PACE4A mRNA.

Hypothalamus

In this region, both PACE4E and PACE4A mRNAs were strongly expressed (Fig. 10 G). The expression of both isoforms was intense in not only the arcuate nucleus hypo-thalamus (ARH) but also the ventromedial nucleus hypothalamus (VMH) (Fig. 9 A). In the lateral hypothalamic area and the dorsomedial nucleus hypothalamus, some cells strongly expressed PACE4E and PACE4A, while others showed weak expression. A high

level of PACE4E mRNA expression was observed in the supraoptic nucleus. Expression of PACE4A mRNA was also observed in this nucleus. The premammilary nucleus also expressed PACE4E and PACE4A mRNAs, at a moderate level.

The hypothalamic neurons generate various kinds of neuropeptides (Warden and Young 1988; Bondy *et al.* 1989; Lantos *et al.* 1995), including thyrotropin-releasing hormone, somatostatin, proopio-melanocortin, enkephalin and neuropeptide Y. Expression of PACE4E and PACE4A in this region suggests the participation of these convertases in the processing of the various neuropeptide precursors.

Metathalamus

In this region, a few cells expressed PACE4E and PACE4A messages, but the signal intensity was very weak.

Mesencepharon

In mesencepharon, the levels of expression of PACE4E and PACE4A mRNAs varied from low to high. The highest level of mRNA expression of both isoforms was found in the compact part of the substantia nigra (SNc; Fig. 9 B). In the reticular part of the substantia nigra, few neurons expressed both PACE4 isoforms intensely. Moderate to high levels of PACE4E and PACE4A mRNA expression were observed in the medial geniculate complex (MGv), periaqueductal gray (PAG), interstitial nucleus of Cajal and red nucleus (Fig. 9 B).

Medial mammilary nucleus (MM) was one of the sites where the messages of PACE4E and PACE4A were abundantly expressed (Fig. 9 B and Fig. 10 G).

Although the expression of PACE4E and PACE4A mRNA was very weak in the superior colliculus, both mRNAs were abundantly expressed in the inferior colliculus.

Pons

PACE4E and PACE4A were strongly expressed in the brain stem, especially in the pons. The sites expressing the highest levels of PACE4E and PACE4A mRNA were the locus coeruleus (LC), the mesencephalic nucleus of the trigeminal, the motor nucleus of the trigeminal nerve (V), the nucleus of the trapezoid body (NTB) and the nucleus raphe magnus (RM) (Fig. 9 C and Fig. 10 H). The messages of both isoforms were also detected in the medial to large size neurons in the pontine reticular nucleus (caudal part) (PRNc). Additionally, the ventral cochlear nucleus (VCO) expressed both convertases at high levels (Fig. 9 C).

Cerebellum

The cerebellum has a well laminated structure. The expression of PACE4E and PACE4A mRNA was observed in all areas, i.e. the culmen lobule (CUL), central lobule (CENT), simple lobule (SIM), ansiform lobule (AN) and paraflocculus (PFL), as shown in Fig. 9 C.

In the cortex cerebelli (stratum moleculare), only the small neurons expressed PACE4E (Fig. 10 J) and PACE4A. These are the outer stellate cells. The highest level of mRNA expression for both isoforms was observed in the stratum gangliosum which consists of Purkinje's cells (Fig. 10 J). In the stratum granulosum, the small granular cells expressed PACE4E and PACE4A mRNAs at low to moderate levels.

Messages for both PACE4 isoforms were detected in the large

neurons of the corpus medullare (Fig. 10 I).

Dong *et al.* (1995) examined the distribution of PACE4 mRNAs in the adult rat brain by *in situ* hybridization histochemistry, and found wide-spread low-level, and restricted, to some areas of the CNS, high-level expression. However, discrepancies exist in their findings. For example, they deteced PACE4 mRNA in the hippocampus by Northern blotting but not by *in situ* hybridization. Additionally, the mitral cells of the olfactory bulb where we detected PACE4E and PACE4A mRNAs at high levels expressed no PACE4 messages in their experiment. Therefore, we examined the expression of PACE4 mRNAs in the mitral cells of the olfactory bulb and hippocampus by *in situ* hybridization using universal probes for PACE4 isoforms and found the messages primarily in the mitral cells and some other cells of the olfactory bulb and in the pyramidal cell layer of the hippocampus. We further confirmed that the above cells expressing PACE4E mRNA were immunoreactive for PACE4E-specific antibody which was completely absorbed with antigen (data not shown). Dong *et al.* (1995) prepared frozen sections without any fixation and stored them at -80 °C until *in situ* hybridization histochemistry, whereas we immediately fixed the tissues with Bouin's solution. This may be the fundamental difference between the two studies. In the present study, we found that Bouin's fixation increases the sensitivity of our *in situ* hybridization histochemistry, relative to fixation with formaldehyde or paraformaldehyde, on both paraffin and frozen sections.

The distribution of both PACE4E and PACE4A mRNA was widespread in the CNS, however, the expression levels varied considerably among the various brain regions. In well-laminated structures of brain

such as the olfactory bulb, hippocampus and cerebellum, the highest level of mRNA expression or a restricted mRNA expression of these PACE4 isoforms was observed in the particular cell layer. Further, the isoforms tend to be expressed in large neurons in the adult rat CNS. In the hypothalamus, studies have shown the distribution of a good many neuropeptides (Warden and Young 1988; Lantos *et al.* 1995). PACE4E and PACE4A mRNAs were expressed in neuropeptide(s)-rich hypothalamic neurons including in the supraoptic nucleus (Bondy *et al.* 1989) and arcuate nucleus. Other neuropeptide(s)-rich regions such as the subiculum (Alexander *et al.* 1989), habenula (Burgunder and Young 1989), substantia nigra and cerebellar Purkinje's cells (Osborne *et al.* 1992) also expressed PACE4E mRNA intensely. Thus, PACE4(s) with processing activity probably catalyze the maturation of propeptide hormone(s). Additionally, mRNA expression of PACE4E was observed not only in neuronal cells but also in non-neuronal cells. Only furin had previously been shown to be expressed in both neuronal and non-neuronal cells (Shafer *et al.* 1993; Day *et al.* 1993). PACE4s and furin are thought to be involved in processing events in non-neuronal cells in the CNS.

2. Pituitary and adrenal gland

In situ hybridization histochemistry using the cRNA probe specific for the cysteine rich region of PACE4 (CRR, shown in Fig. 5), which recognizes PACE4A and PACE4E, suggested the distribution of one or both PACE4 isoforms in the adult rat pituitary and adrenal gland.

The sequence of this probing region is highly conserved among rat and human (80.4% identity; Johnson *et al.* 1994). This probe, based on the rat PACE4A cDNA, was found to cross-hybridize with human PACE4

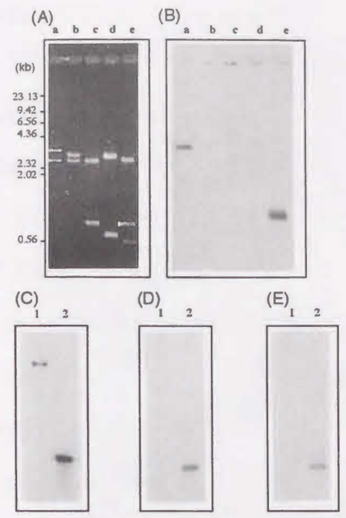

Fig. 11: Specificty of PACE4-CRR probe. A; Ethidium bromide staining. Lane a; human (h) PACE4A cDNA, lane b; h-PACE4C cDNA, lane c; rat (r) PACE4A-5' region, lane d; r-PACE4A-Homo B, lane e; rPACE4A-CRR. B; The membrane corresponding to A was hybridized with r-PACE4A-CRR probe at 42C. This probe cross-hybridized with hPACE4A. C-E; Same hybridization was performed under high stringency conditions (C; hybri 42C-wash 42C, D; hybri 60C-wash 60C, D; hybri 65C-wash 65C).
Lane 1; hPACE4A, lane 2; rPACE4A-CRR

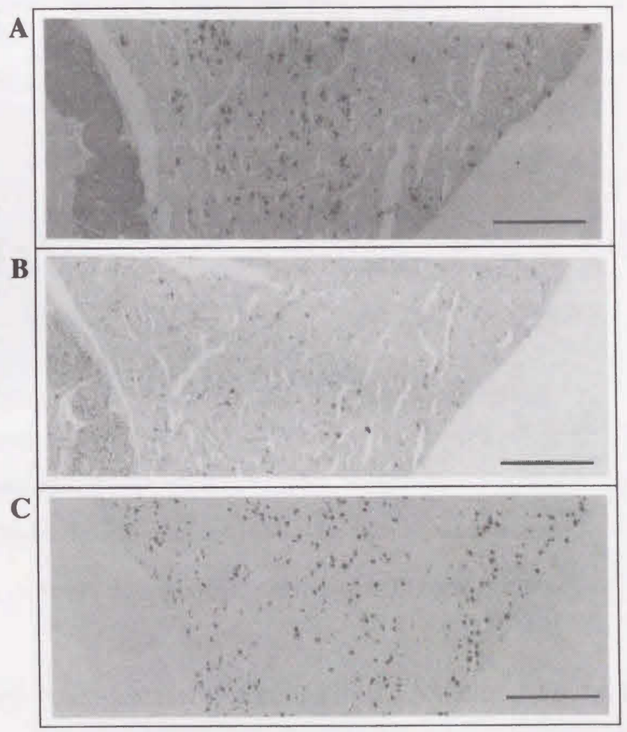

Fig. 12: Localization of PACE4, ACTH and LHβ in the adult rat pituitary.
Serial sections were stained by *in situ* hybridization for PACE4 (B) and by immunohistochemistry for ACTH (A) or LHβ (C). Bars: 500μm.

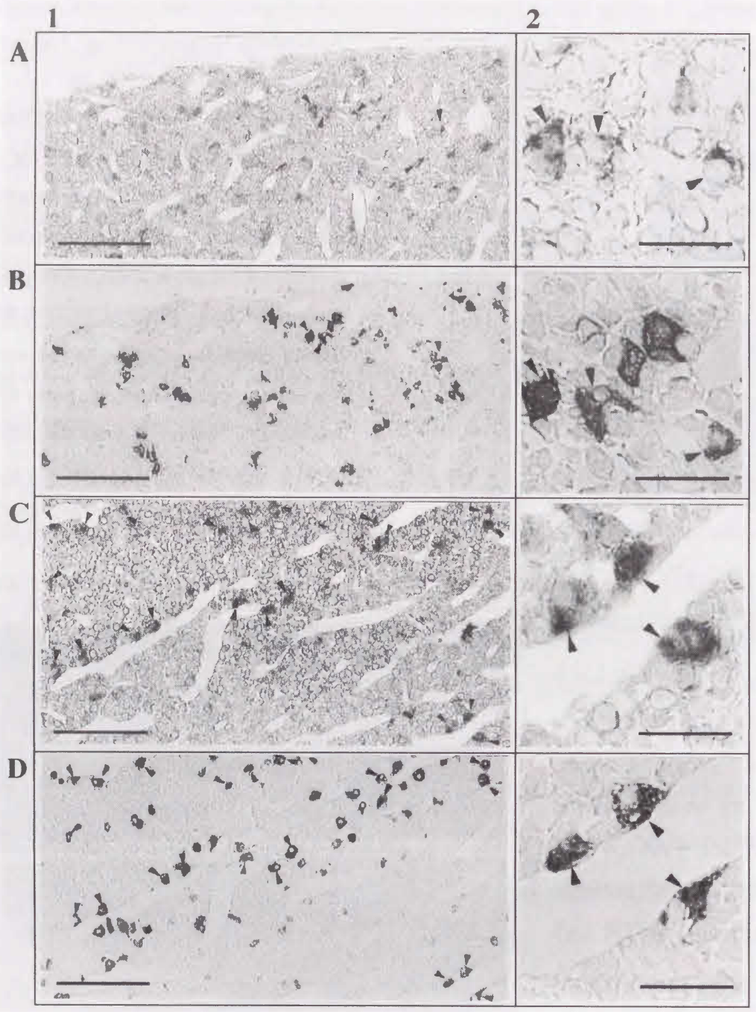

Fig. 13: Expression of PACE4 mRNAs in the adult rat anterior pituitary lobe. Distribution of PACE4 messages (A and C) was compared with that of ACTH -immunoreactive cells (B) or LHβ-immunoreactive cells (D). Arrowheads in panel A/B and C/D showed the identical cells. Bars: 1; 100μm, 2; 25μm.

(PACE4A and PACE4E) under low stringency hybridization conditions (Fig. 11 B, C), but to specifically hybridize only with rat PACE4 under high stringency conditions (Fig. 11 D, E) by means of Southern blot

analysis.

Therefore, this probe was expected to detect both PACE4A and PACE4E mRNA by *in situ* hybridization under high stringency condition even if unidentified homologue(s) exist.

As shown in Fig. 12 B, moderate to high level expressions of PACE4 mRNA(s) were observed in the anterior lobe and the intermediate lobe of pituitary. In the anterior lobe, the cells expressing PACE4 mRNA(s) were scattered. Comparative distribution with PACE4 transcript(s) and several bioactive peptides by *in situ* hybridization and immunohistochemistry revealed that PACE4 isoform(s) was expressed in most of gonadotroph and in part of the corticotroph in the anterior pituitary lobe (Fig. 12, 13). Previously, Johnson *et al.* (1994) reported that the expression of PACE4

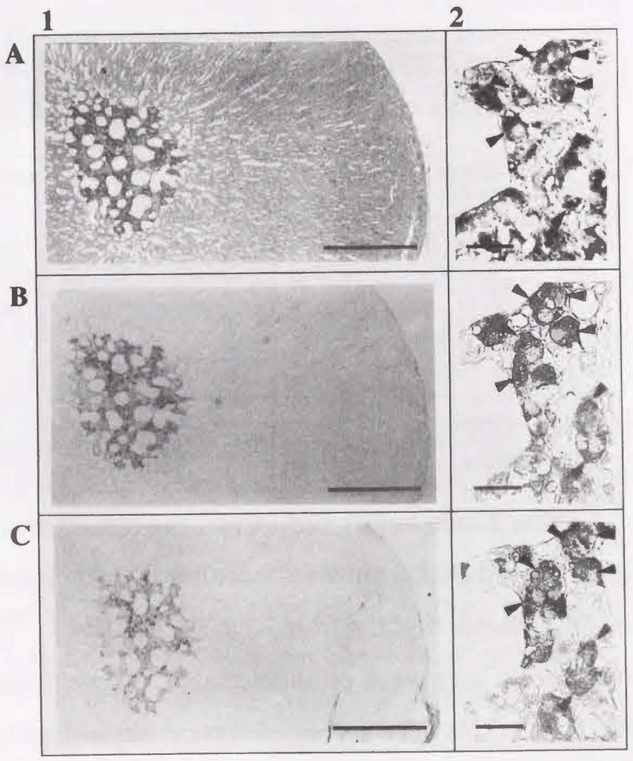

Fig. 14: Localization of PACE4, NPY and pro-NPY in the adult rat adrenal gland. Serial sections were stained by *in situ* hybridization for PACE4 (A) and by immunohistochemistry for NPY (B) or pro-NPY (C). Bars: 1; 500 μm, 2; 50μm.

mRNAs in the rat anterior pituitary lobe was regulated by thyroid status. However, it is still unknown whether the PACE4 isoforms are expressed under similar regulation by thyroid hormones.

Additionally, PACE4A and/or PACE4E mRNAs were expressed only in some cells in the adrenal medulla, and not at all in the adrenal cortex (Fig. 14 A).

3. Ovary

In the ovary, the messages of PACE4 were detected in the theca folliculi interna, follicular epithelial cells, stratum granulosum, oocyte and corpus luteum. However, these expression were only detectable in secondary follicles or the small antral follicles, and not in large antral follicles such as the Graafian follicle.

Table 2. Summary of mRNA distribution of each Kexin family in the adult rat ovary analyzed by *in situ* hybridization

	EOF			GF			CL	SO
	TFI	SG	O	TFI	SG	O		
PC2	++	++	+	++	-	+	+++	+
PC1/3	+	+	+	+	-	+	+	±
furin	±	+	±	-	-	±	+	±
PACE4	+++	++	+	+	±	+	++	+
PC6	±	±	++	-	-	+	+	±

CL; Corpus luteum, EOF; Early ovallian follicle, GF; Graafian follicle, O; Oocyte, SG; Stratum granulosum, SO; Stroma ovarii, TFI; Theca folliculi interna
Positivity; + < ++ < +++ (-; negative, ±; weakly positive)

Other Kexin family proteases were also expressed in the ovary (Table 2). PC2 and PC1/3 showed a similar distribution to, but their expression levels differed slightly from, that of PACE4. Expression of furin and PC5/6 were very weak or undetectable. These results suggest that PACE4, PC2 and PC1/3 play a very important physiological role in ontogeny and differentiation including oogenesis, oocyte maturation and fertilization,

activating bioactive peptide(s) such as activin and inhibin.

4. Pancreas

Further we analyzed the distribution of not only PACE4 isoforms but also other Kexin family members in the adult rat pancreatic islets by immunohistochemistry using antibodies specific for each convertase (Nagamune *et al.* 1995). Interestingly, PACE4C was specifically expressed

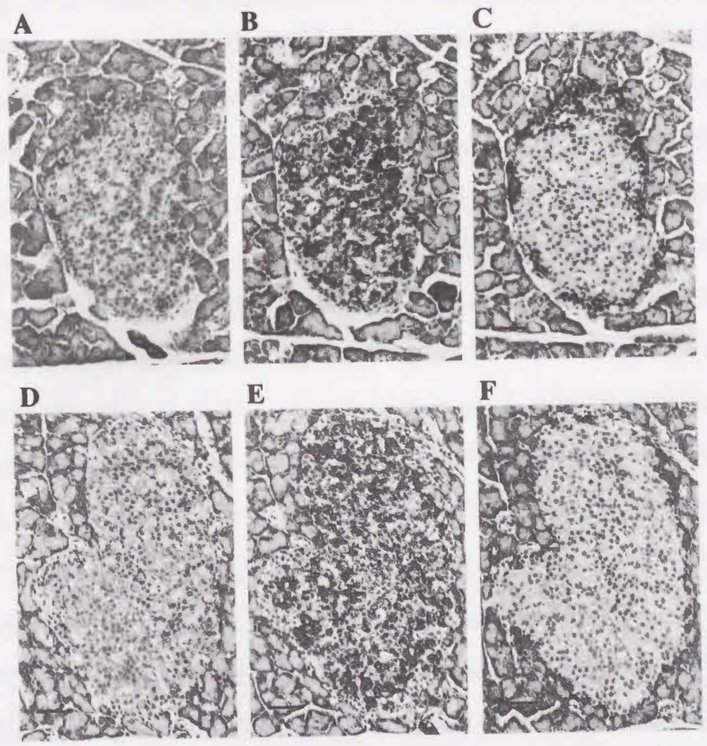

Fig. 15: Immunohistochemical detection of PACE4C in pancreatic islets. Serial sections (A-C and D-F) were immunostained, then counter stained with Mayer's hemalum solution. A; anti-somatostatin, B; anti-PACE4C, C; anti-glucagon, D; anti-insulin, E; anti-PACE4C, F; anti-pancreatic polypeptide. Bars: 50μm.

in B cells of the islet (Fig. 15 B, E), whereas PACE4A was hardly found in any cells of the islet. PC1/3 was localized only in B cells like PACE4C. On the other hand, PC2 was more abundantly distributed in A and in some D cells than in B cells. PC5/6 and furin were weakly and evenly expressed in the islets.

Among Kexin family proteases, only PC2 and PC1/3 have been focused on the processing of pancreatic peptide hormones such as insulin without their precise localization in the islet cells (Bailyes *et al.* 1992; Bennett *et al.* 1992; Smeekens *et al.* 1992; Galanopoulou *et al.* 1993; Rouille *et al.* 1994). Our findings suggest that these convertases share roles in islet cells. PC2 is likely involved in the prohormone processing in A and D cells, while B cell-specific processing event(s) are speculated to be catalyzed by PC1/3, PACE4C and PC2.

Although the ultrastructural localization of PC1/3 and PC2 in rat islet cells was recently demonstrated by Tanaka *et al.* (1996), little is known on the intracellular localization of PACE4C. Clarifying the physiological function of PACE4C in pancreatic B cells is important to understanding its ultrastructural localization in the cells.

5. Thyroid

By immunohistochemical analysis, PACE4 isoforms were undetectable in the adult rat thyroid. Among the Kexin family, PC1/3 and PC2 were distributed only in the parafollicular cells which secrete the peptide hormone, calcitonin, and not in the follicular cells (Nagamune *et al.* 1997). Furin was mainly distributed in the follicular cells, and expression of PC5/6 was weakly detectable in both follicular and parafollicular cells.

CHAPTER III. Developmental expression of PACE4 isoforms in rat tissues

We were further interested in the expressions of PACE4E and PACE4A in rat development. During embryogenesis, cell-growth, migration, differentiation and patterning largely depends on cell-cell interactions, especially in the CNS. Many biologically active peptides including growth factors, cell-adhesion molecules and receptor proteins are likely involved in such biological phenomena. Zheng *et al.* (1994) reported the expression of PC1 and PC2 was initiated at E13.5 in the rat CNS, while furin mRNA in the CNS was undetectable at this stage. Expression of PACE4 mRNA in the developing nervous system was first shown by Constam *et al.* (1996). They reported high levels of PACE4 mRNA expression in the floor plate of the neural tube at E11.5 of mouse embryo, although they did not analyze the mRNA distribution of PACE4 in the further developing CNS. These findings suggest that PACE4 plays the most important role in the development of CNS among the Kexin family proteases. Therefore, we analyzed the cell-specific expression of two active PACE4 isoforms, PACE4E and PACE4A, in prenatal and postnatal rat CNS by *in situ* hybridization histochemistory, and investigated their mRNA distribution in other organ primordiums to gain insights into their physiological significances not only in CNS development but also in the ontogenesis.

The determination and nomenclature of the sites expressing PACE4 isoforms in the developmental rat brain are based on Paxinos *et al.* (1991), Schambra *et al.* (1992) and Paxinos (1995).

1. CNS

1) Expression of PACE4E and PACE4A mRNA in the developing rat CNS

Northern blot analysis using total RNA isolated from rat embryonic brains at various stages of development revealed messages for both PACE4 isoforms in all the brains (Fig. 16). And their sizes were identical with that

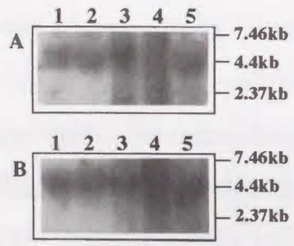

Fig. 16: Northern blot analysis using cRNA specific probe for PACE4E (A) or PACE4A (B). Total RNA (10μg/lane) isolated from embryo heads (lane 1; E13.5) or brains (lane 2; E15.5, lane 3; E17.5, lane4; E18.5, lane 5; E20.5) was analyzed.

detected in various adult rat brain regions (Akamatsu *et al.* 1997). In the course of development, the expression level of PACE4E and PACE4A was slightly increased.

2) Mapping of the expression sites of the novel active PACE4 isoform, PACE4E, in the course of development of rat CNS

Day 13.5 of gestation (E13.5)

At this stage, high levels of PACE4E mRNA expression were observed throughout the CNS (Fig. 17). PACE4E mRNA was detected in the cortical neuroepithelium (cx), basal telencephalic plate (bt), hippocampal formation neuroepithelium (hi), thalamus (Th), posterior hypothalamic neuroepithelium (ph), pons (PN), cerebellar neuroepithelium (cb) and medulla (ME). The Rathke's pouch also expressed PACE4E. Further,

Fig. 17: Distribution of PACE4E mRNA in the rat brain on day 13.5 of gestation. Various cross sections (1-10) were hybridized with antisense cRNA probe specific for PACE4E. Bar: 50μm.

PACE4E message was intensely expressed in the visual system such as the optic vesicle, neural retina and lens.

Expression of PACE4A mRNA was also found in the above regions (data not shown).

E15.5

Moderate to high level expression of PACE4E mRNA was observed in the cerebra l cortex on E15.5 (Fig. 18 A, B) . Especially, the cortical

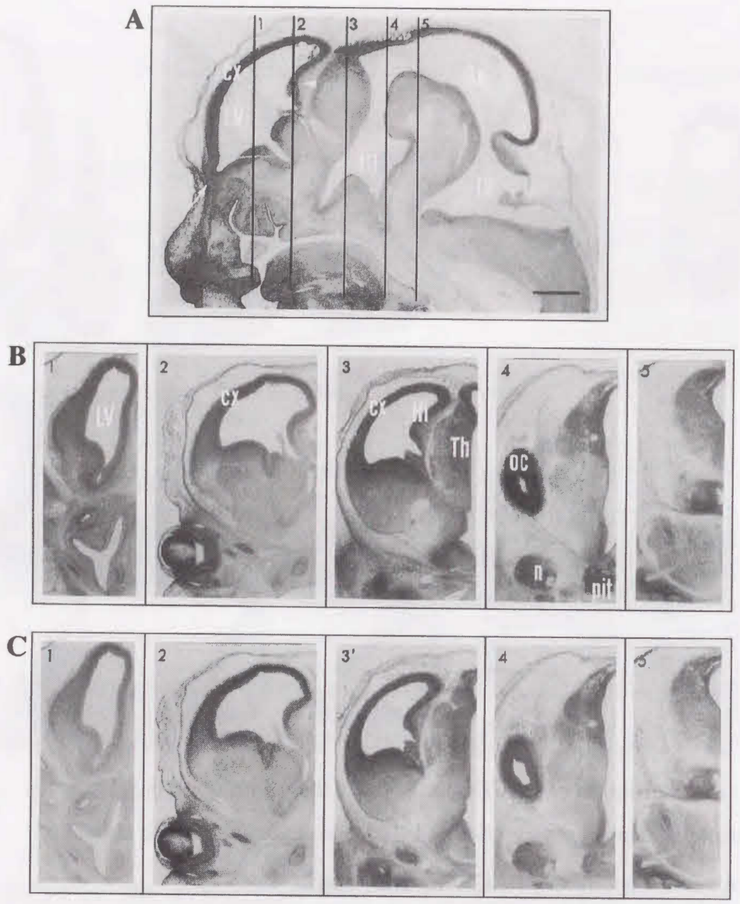

Fig. 18: Comparison of mRNA distribution of PACE4E (A and B) and PACE4A (C) in the rat brain on embryonic day 15.5. A; sagittal section, B and C; frontal sections at the sites numbered from 1 to 5 in A. 3' means the section adjacent to 3. Bar in A: 500μm, Magnification of B and C is the same as for A.

PACE4E message (Fig. 18 A, B-2, 3). PACE4E mRNA was also detected in the hippocampal primordium with the expression level highest in the hippocampal formation neuroepithelium (hi) (Fig. 18 B-3).

Additionally, the lateral habenular nucleus (LHb), the mediodorsal thalamic nucleus and the ventral posterolateral/posteromedial thalamic nucleus expressed PACE4E mRNA intensely.

In the hypothalamic region, the ventromedial hypothalamic nucleus (VMH) intensely expressed message for PACE4E. In contrast, the expression of PACE4E mRNA in the amygdala was very weak. Rathke's pouch (pit) and the trigeminal ganglion (n) showed moderate to high level expression of PACE4E mRNA (Fig. 18 B-4).

The sites expressing another PACE4 isoform, PACE4A, coincided remarkably with those of PACE4E (Fig. 18 C).

Interestingly, both isoforms were strongly expressed in the visual system (Fig. 18 A-2, B-2).

E17.5

On E17.5, the greatest change in PACE4E mRNA expression was in the olfactory bulb. Mitral cells had appeared and were intensely expressing PACE4E message (Fig. 19 A, B-1). The cortical wall of the forebrain vesicle indicated regional differences in laminar structures, and the highest level of PACE4E mRNA expression was observed in the cortical plate (CxP) and cortical neuroepithelium (cx) (Fig. 19 A, B-2, 3). But the subventricular (SubV) and the intermediate (ICx) cortical layer expressed low to moderate levels of PACE4E message.

In the hippocampal primordium, the formation of the pyramidal cell layer was found at this stage. PACE4E mRNA was most intensely expressed in the hippocampal formation neuroepithelium (hi) and the pyramidal cells (Fig. 19 B-3). In addition to the thalamus and hypothalamus, the amygdala where PACE4E was expressed very weakly on E-

15.5 expressed PACE4E mRNA.

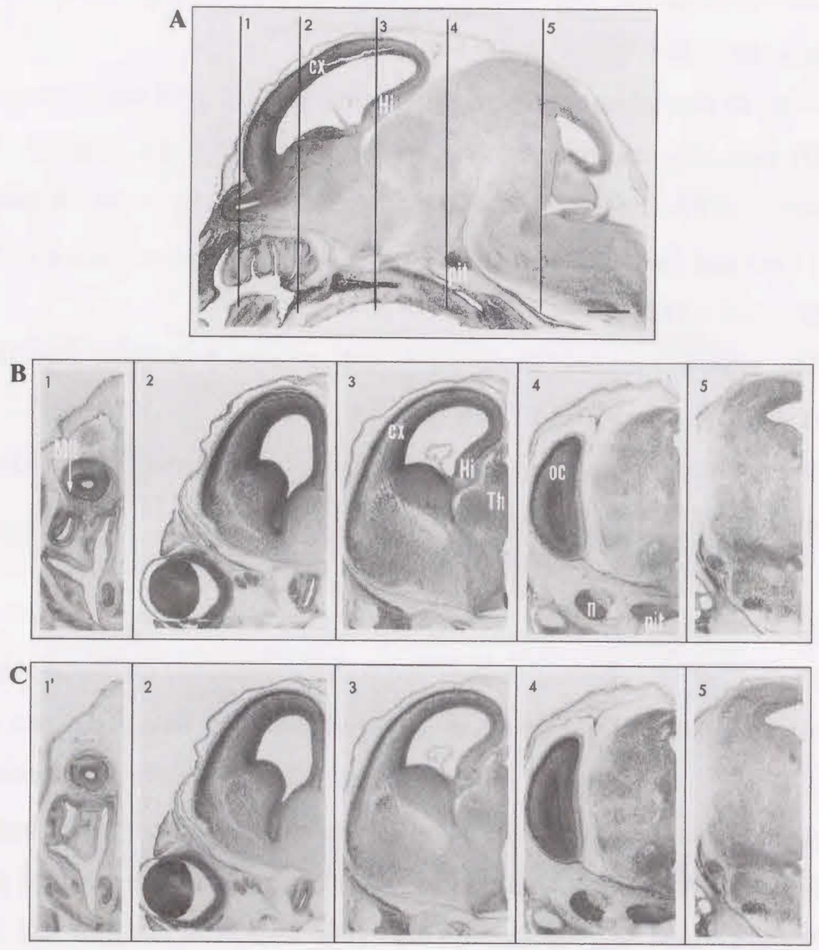

Fig. 19: Comparison of mRNA distribution of PACE4E (A and B) and PACE4A (C) in the rat brain on embryonic day 17.5. A; sagittal section, B and C; frontal sections at the sites numbered from 1 to 5 in A. 1' means the section adjacent to 1. Bar in A: 500μm, Magnification of B and C is as for A.

In Rathke's pouch (pit), PACE4E mRNA expression was intense in the anterior lobe, but undetectable or low level in the intermediate and

posterior lobes (Fig. 19 B-4).

The expression pattern for PACE4A mRNA was the same as that for PACE4E (Fig. 19 C).

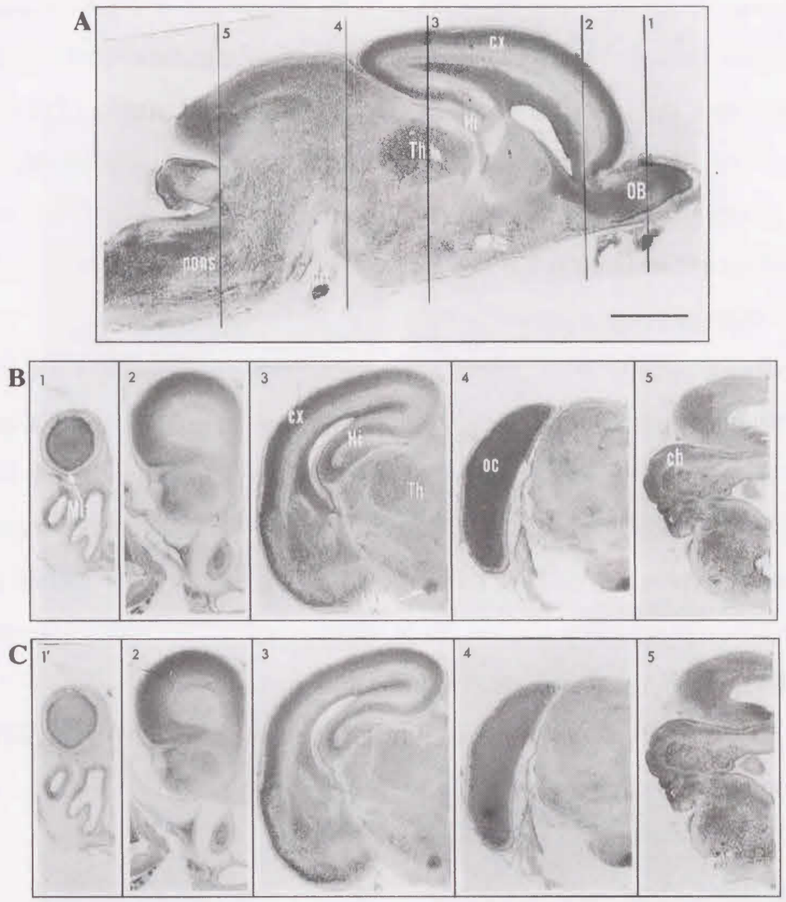

Fig. 20: Comparison of mRNA distribution of PACE4E (A and B) and PACE4A (C) in the rat brain on embryonic day 20.5. A; sagittal section, B and C; frontal sections at the sites numbered from 1 to 5 in A. 3' means the section adjacent to 3. Bar in A: 500μm, Magnification of B and C is the same as that of A.

E20.5

In the olfactory bulb, PACE4E mRNA was most abundantly expressed in the mitral cells, though it was also detected in the olfactory bulb neuroepithelium (obn) at moderate levels (Fig. 20 A, B-1). The message of PACE4E was expressed throughout the cerebral cortex, including the frontal cortex (Fr), cortical plate (CxP), parietal cortex (Par), piriform cortex (pir), occipital cortex (Oc) and temporal cortex (Te) (Fig. 20 A, B-2, 3). The cortical neuroepithelium (cx) also expressed PACE4E mRNA intensely. In the hippocampal formation, PACE4E mRNA was expressed in the pyramidal layer (CA1-CA3), but was undetectable in the granular layer of dentate gyrus (GrDG) (Fig. 20 B-3).

Moderate to high level expression of PACE4E message was found in various thalamic and hypothalamic regions; the ventromedial hypothalamic nucleus showing the highest level of expression. In the amygdala, the level of PACE4E mRNA expression varied among areas. Additionally, conspicuous expression of PACE4E mRNA was found in the Prukinje cell layer of the cerebellum (Fig. 20 A, B-5). Further, a high level of expression of PACE4E mRNA was observed in pons and medulla (Fig. 20 A).

PACE4A mRNA was expressed in all the above regions (Fig. 20 C), but at somewhat different levels.

Postnatal day 2 (2-day-old newborn)

The distribution of PACE4E and PACE4A mRNAs in the rat brain of newborns differed little to that of adults. Expression of PACE4E mRNA was most marked in the cerebellar Prukinje's cells (Fig. 21 F).

In our experiment, we found the spacio-temporal expression of PACE4E and PACE4A mRNA in the course of development of the rat

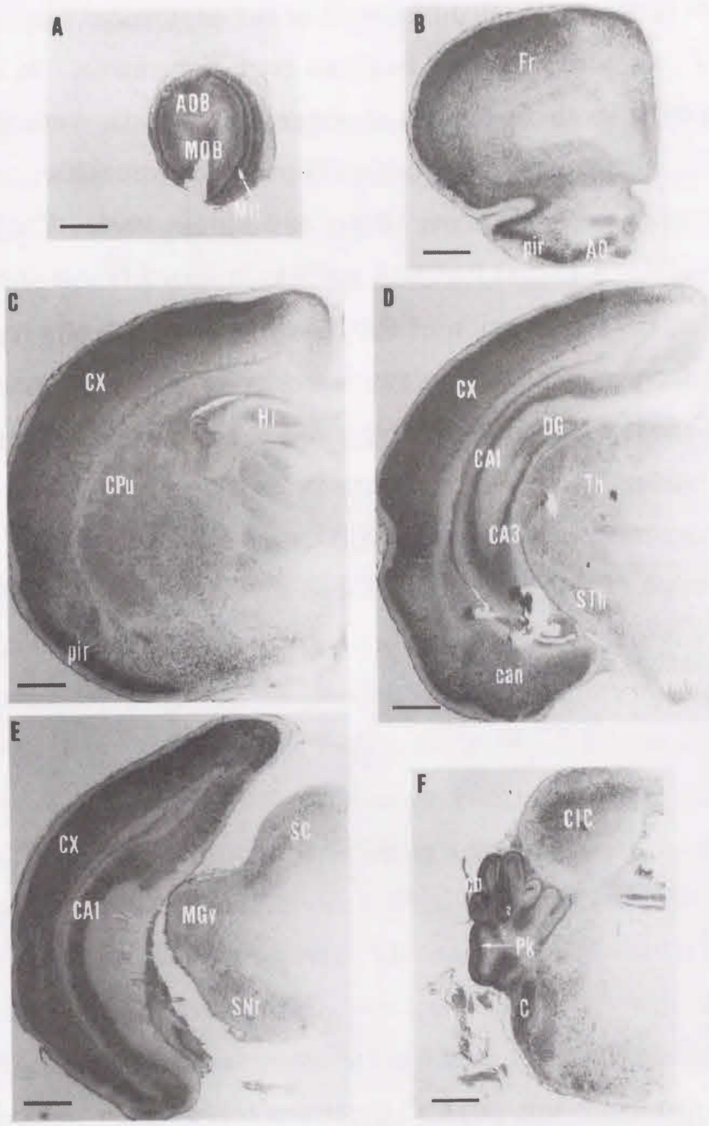

Fig. 21: Distribution of PACE4E mRNA in the rat brain on postnatal day 2. Various frontal sections (7μm-thick) were hybridized with PACE4E-specific antisense cRNA probe. Bars: 555μm.

CNS described above. On E13.5, the highest levels of PACE4E and PACE4A messages occurred in the CNS. As development proceeded

(E15.5-E20.5), the regional differences in the expression levels of the mRNAs of two active PACE4 isoforms were remarkable. In regions expressing high levels of PACE4 messages such as the cortical plate, cortical neuroepithelium and hippocampal formation neuroepithelium, cell-growth and migration were active (Bayer and Altman 1995). The intense expression of PACE4E and PACE4A mRNAs in such differentiating cells suggests that the transcription of PACE4 gene is specifically regulated during development. This was further supported by the finding that the 5'-promoter region of human PACE4 gene has E box elements (Tsuji *et al.* 1997), the binding sites of neural determination factors such as basic helix-loop-helix factor (Akazawa *et al.* 1995; Shimizu *et al.* 1995).

Expression of PACE4E and PACE4A in the developing CNS keeps pace with the establishment of the brain structure. The maturation of the dendro-dendritic contacts between the neurons and the target neurons are necessary for normal development of the CNS. These events are probably regulated by many regulatory molecules such as growth factors, cell-adhesion molecules and receptor proteins. Previously, Redies and Takeichi (1993) showed the expression of N-cadherin mRNA during development of the mouse brain. Interestingly, the expression pattern was very similar to that of both active PACE4 isoforms. N-cadherin is a Ca^{2+}-dependent cell-cell-adhesion molecule, and has the consensus sequence -Arg-X-Lys-Arg- at the maturation site. All Ca^{2+}-dependent cell-adhesion molecules are synthesized as precursor polypeptides, and converted to mature form by limited proteolysis at the dibasic site described above (Ozawa and Kemler 1990). These findings strongly suggest that Ca^{2+}-dependent cell-adhesion molecules, especially N-cadherin, are most possible physiological substrate(s) for PACE4 isoforms.

2. Other embryonic tissues

We were also interested in the mRNA distribution of PACE4E and PACE4A in other developing rat tissues, because PACE4(s) was shown to be widely distributed in adult tissues by Northern blot analysis. Adjacent sagittal sections of whole embryos were hybridized with cRNA probes specific for PACE4E or PACE4A.

On day 15.5 of gestation, PACE4E and PACE4A mRNAs were detected in several organ primordia (Fig. 22), including the heart, lung,

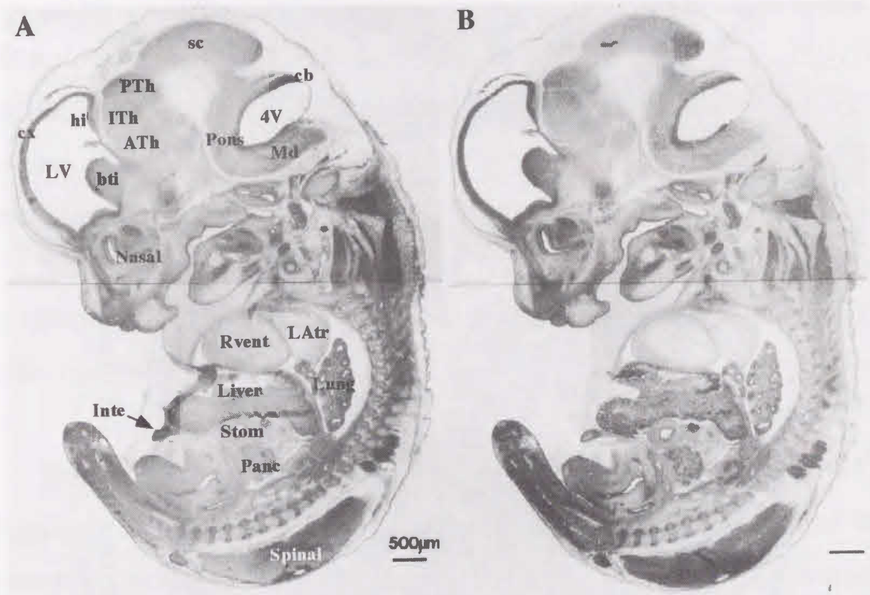

Fig. 22: Distribution of PACE4E (A) and PACE4A (B) mRNAs in the rat embryo on E15.5. Ath: anterior thalamus; Inte: intestine; ITh: intermediate thalamus; LAtr: left atrium; LV: lateral ventricle; Md: medulla; Nasal: nasal cavity; Panc: pancreas; PTh: posterior thalamus; Rvent: right ventricle; Spinal: spinal cord; Stom: stomach; 4V: 4th ventricle; bti: basal telencephalic plate, interm; cb: cerebellar neuroepithelium; cx: cortical neuroepithelium; hi: hippo - campal formation neuroepithelium; sc: superior colliculus neuroepithelium.

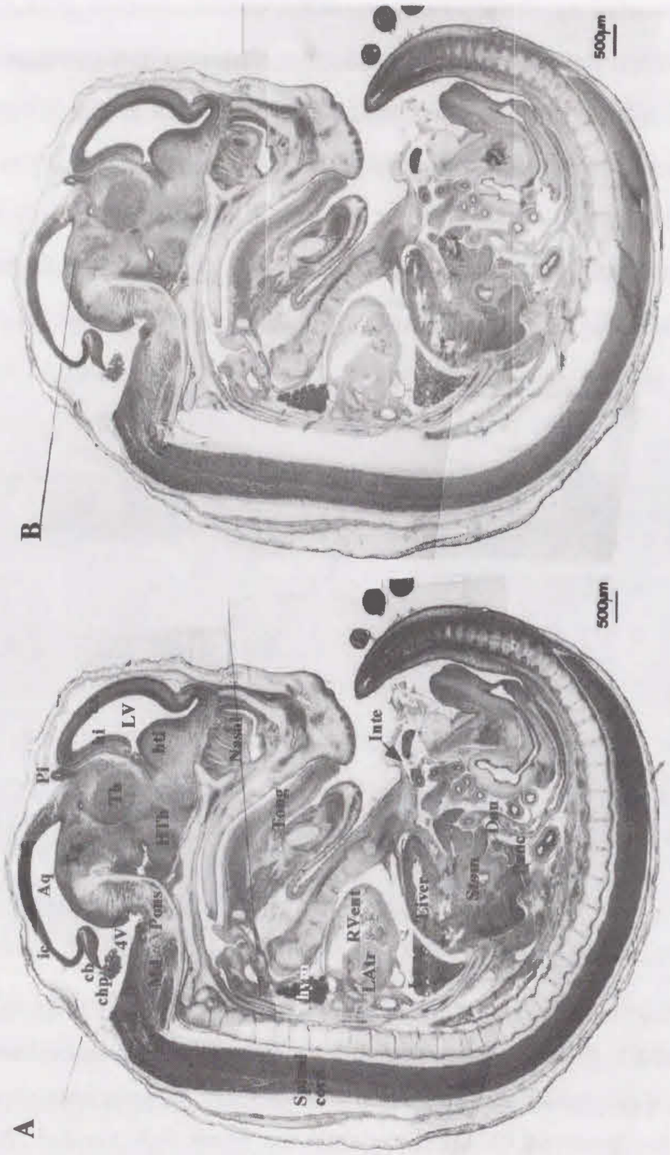

Fig. 23: Distribution of PACE4E (A) and PACE4A (B) mRNAs in the rat embryo on E17.5. Bars; 500μm

liver, pancreas, duodenum and intestine.

In the lung, both PACE4 isoforms were intensely expressed in the bronchial epithelium and the splanchnic mesenchyme of lung bud, but weakly expressed in particular cells in the heart. In the mucosa of the stomach, duodenum and intestine, moderate level expression of PACE4E and PACE4A messages were observed. In the liver, low to high level mRNA expression of PACE4E and PACE4A was observed.

Further, the mRNA of these PACE4 isoforms distributed in the adrenal primordium and metanephros.

At E17.5, the expressions of PACE4E and PACE4A strengthened in the various organ primordia described above (Fig. 23). Interestingly, the highest level mRNA expression of both PACE4E and PACE4A was in the thymus gland. The spinal cord also expressed PACE4E and PACE4A messages intensely.

These observations suggest that PACE4E and PACE4A play a very important role not only in the development of the CNS but also in embryogenesis.

Recently, Constam *et al.* (1996) showed the expression of PACE4 transcripts during mouse limb development and their co-localization with several bone morphogenetic proteins (BMPs) suggesting their participation in the activation of BMPs. In our experiment, we also observed mRNA expression of PACE4E and PACE4A in developing rat limb buds. Therefore, BMPs are thought to be one of possible substrates for PACE4 isoforms *in vivo*.

CHAPTER IV. Co-localization of PACE4 isoform(s) and Bioactive

peptides

To clarify the physiological functions of PACE4 isoforms *in vivo*, it is necessary to identify their physiological substrate(s). Therefore, we analyzed the comparative distribution of PACE4 isoform(s) and several bioactive peptides by *in situ* hybridization and immunohistochemistry.

1. Neuropeptide Y (NPY)

NPY-immunoreactive cells were widely distributed throughout the adult rat CNS (Lantos *et al.* 1995). In the cerebral cortex, several pyramidal neurons expressed NPY, as well as PACE4E mRNA (Fig. 24 A). Co-expression of processing protease PACE4E and NPY was also observed in regions such as the ventral posterolateral nucleus thalamus (VPL), arcuate nucleus hypothalamus (ARH) and locus coeruleus (LC) (Fig. 24 B, C). And, NPY in ARH co-localized with PACE4E in the course of development (Fig. 25).

Interestingly, NPY was expressed in the cells migrating from the vomeronasal organ (VNO) to the forebrain, and co-localized with PACE4E in these cells (Fig. 26).

Additionally, co-localization of PACE4 isoform(s) and NPY was observed in the adrenal medulla (Fig. 14).

These observations strongly suggest that NPY is a candidate substrate for PACE4E and PACE4A. As the pro-NPY converting enzyme in the rat superior cervical ganglion neurons, PC2 was reported by Paquet *et al.* (1996). However, NPY is widely distributed in the rat CNS and also localized in the adrenal medulla. Therefore, it is possible that PACE4E

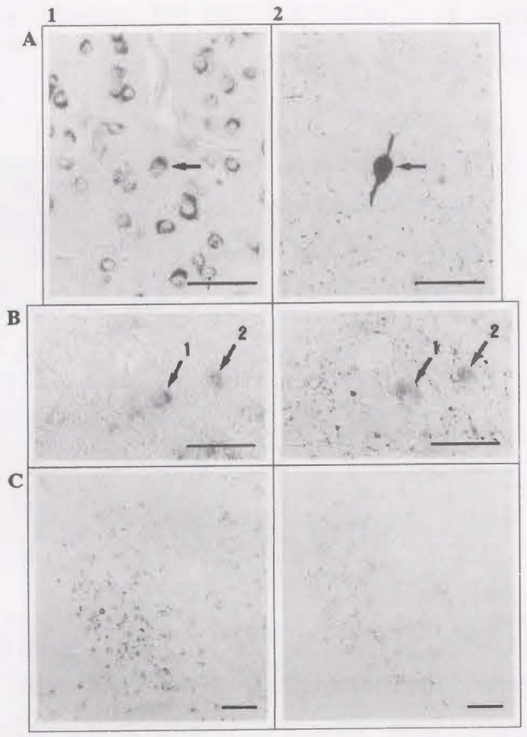

Fig.24: Co-localization of PACE4E (1) and NPY (2) in various regions of the adult rat brain. Arrows in A and B indicate identical cells. A; Pyramidal cell in cerebral cortex, B; Locus coeruleus, C; Thalamus (VPL). Bars: A and B; 50μm, C; 100μm.

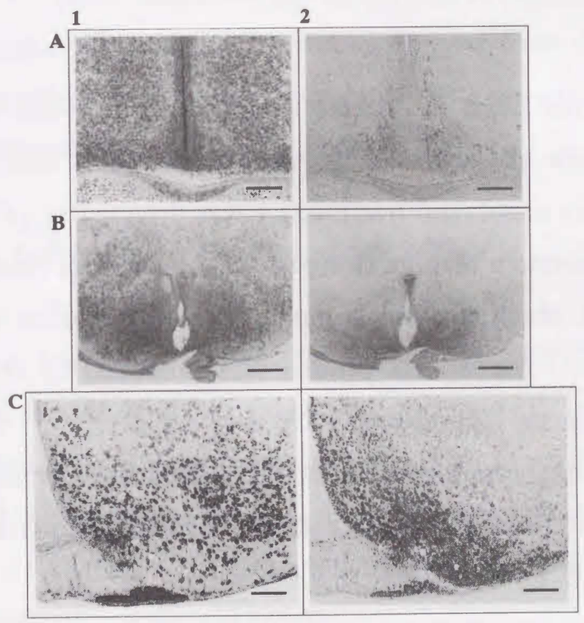

Fig. 25: Developmental co-localization of PACE4E (1) and NPY (2) in the rat arcuate nucleus hypo-thalamus (ARH). A; E17.5, B; NB2, C; adult. Bars: 100μm.

and/or PACE4A catalyzes the activation of pro-NPY in some cells expressing mature NPY.

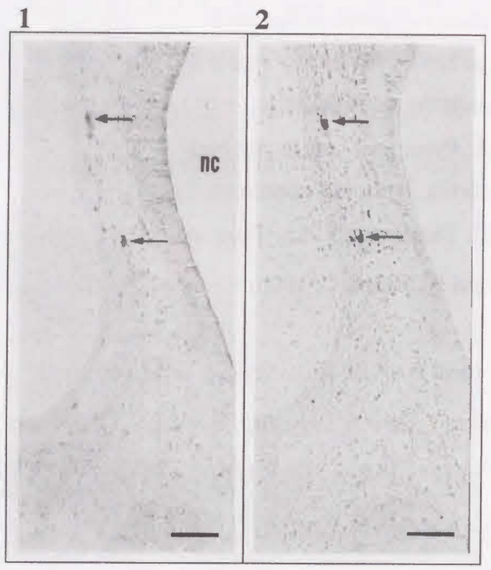

Fig. 26: Co-localization of PACE4E (1) and NPY (2). The cells migrating from the vomeronasal organ to the forebrain expressed both PACE4E and NPY (arrows). nc; nasal cavity. Bars: 50μm.

2. Adhesion molecule L1

Cell-adhesion molecule L1 is a 200kDa glycoprotein involved in neuronal migration, adhesion and neurite outgrowth (Miura *et al.* 1991). In the embryonic rat brain membrane fraction, L1 was detected as two major bands by means of western blotting (Miura *et al.* 1992). One band is about a 200kDa protein which is an intact molecule, while the other is about an 80kDa protein. The 80kDa protein was produced by the proteolytic cleavage of L1 at the dibasic amino acid site.

L1-immunoreactive cells were distributed in the fiber bundles derived from the VNO at the E17.5 stage like NPY (Fig. 27-2) and they also expressed PACE4E (Fig. 27-1).

The mechanism of the migration of gonadotropic hormone-releasing

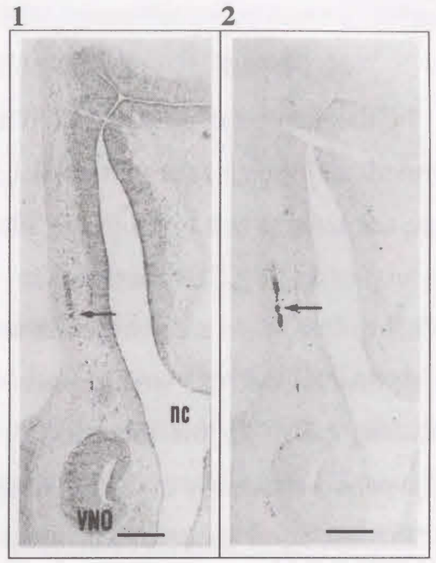

Fig. 27: Co-localization of PACE4E (1) and adhesion molecule L1 (2). The cells migrating from the vomeronasal organ (VNO) to the anterior pole of forebrain vesicle expressed PACE4E and L1 (arrow). nc; nasal cavity. Bars: 100μm.

hormone (GnRH)-producing cells from the olfactory epithelium and the epithelium of VNO to the forebrain has been well characterized. Neural cell adhesion molecule plays a key role in such mechanisms. Our findings suggest that the NPY neurons derived from the VNO migrate to the anterior pole of the forebrain vesicle with the guidance of L1, and PACE4E regulates the biological activity of L1.

3. Calcitonin gene-related peptide (CGRP)

CGRP-immunoreactive neurons were distributed in the restricted CNS areas. In the peripeduncular nucleus (PP), subparafascicular nucleus thalamus (SPF) and external medial part of parabrachial nucleus, (PBme), CGRP seemed to be co-localized with PACE4E (data not shown).

At E17.5, CGRP-immunoreactive facial nucleus (7) also expressed PACE4E mRNA and protein.

4. Adrenocorticotropic hormone (ACTH)

In the adult rat pituitary, PACE4 mRNAs were expressed in the intermediate lobe and the particular cells in the anterior lobe (Fig. 12 B). There are several cell types in the anterior pituitary lobe. To identify the cells expressing PACE4 messages in the anterior lobe, the distribution of PACE4 mRNAs was compared with that of ACTH. As shown in Fig. 13 A and B, PACE4 mRNAs were expressed in part of the corticotroph (ACTH producing cell). In the intermediate lobe, ACTH-immunoreactive cells completely coincided with the cells expressing PACE4.

Processing of proopiomelanocortin (POMC), a precursor polypeptide of ACTH, is thought to be catalyzed by PC1/3 and PC2 (Benjannet *et al.* 1991). However, POMC contains various bioactive peptides such as lipotropin (β-LPH and γ-LPH), melanocyte stimulating hormone (α-MSH and γ-MSH) and β-endorphin, all of which are generated by the limited proteolysis at the dibasic site in a cell-specific manner (Fig. 1 and Douglass *et al.* 1984). Therefore, PACE4 may be involved in the production of ACTH or other peptide hormones derived from POMC in the pituitary gland.

5. Luteinizing hormone (LH)

We also compared the localization of PACE4 mRNAs and LH in the anterior pituitary lobe. LH-immunoreactive gonadotrophs were scattered all over the anterior lobe, and most gonadotrophs expressed PACE4 messages (Fig. 13 C, D).

Because LH is not processed by limited proteolysis, expression of

PACE4 mRNAs in the gonadotrophs may suggest the existence of unidentified biologically active peptide(s) which requires proteolytic processing at the paired basic amino acid site.

6. Insulin

One PACE4 isoform, PACE4C, was specifically expressed in B cells in the pancreatic islets (Fig. 15 B and E, Nagamune *et al.* 1995, and also see CHAPTER II. 2-3)). In B cells of the islets, it is well known that insulin is expressed. In terms of processing of insulin, PC1/3 and PC2 have been studied without knowledge of their precise distribution in the islets (Bailyes *et al.* 1992; Bennett *et al.* 1992; Smeekens *et al.* 1992). Immunohistochemical analysis revealed that PC1/3, PC2 and PACE4C were expressed in B cells. These findings suggest that PACE4C may also be involved in the maturation of B cell specific hormones such as insulin and amylin.

CHAPTER V. Summary and conclusion

We have described the histochemical and cytochemical findings on prohormone/proprotein convertase PACE4.

In the rat CNS, two active PACE4 isoforms, PACE4E and PACE4A were expressed throughout the lifetime. However, spatial and temporal differences in their expression patterns were observed in the course of the development of CNS. Early in the development, PACE4E and PACE4A mRNAs were expressed throughout the CNS at high levels. As development proceeded, the expressions became particularly intense in the

cells whose growth and migration were active, and were obviously localized in particular areas and cells such as the mitral cells in the olfactory bulb, the pyramidal cells of subfield CA1-CA3 of Ammon's horn in the hippocampus and the Purkinje's cell layer in the cerebellum.

The spacio-temporal expression of these active PACE4 isoforms suggests that PACE4E and/or PACE4A plays an important physiological role in the differentiation and establishment of the CNS.

Further, both isoforms were expressed in other organ primordia suggesting their participation in embryogenesis.

In conclusion, the proteolytic activity of PACE4E and/or PACE4A is probably involved in not only morphogenesis but also ontogeny. An understanding of the physiological substrate(s) for these PACE4 isoforms is of great interest to the aspects of ontogenesis. Further, the ultrastructual localization of PACE4E and PACE4A must be determined to clarify their own physiological functions *in vivo*. They are the future problems.

ACKNOWLEDGMENTS

I would like to thank Prof. Yoshiko Matsuda for her interest in and support of this research project and my graduate education.

I am also grateful to Dr. Shigeo Daikoku for the gift of anti-LHb antibody, his help and valuable discussions concerning developmental studies.

I wish to express my gratitude to the members of my committee, Prof. Sumihare Noji and Prof. Shoji Kaneshina, for comments on this work.

I wish to thank Dr. Eric Grouzmann (Ctr Hospitalier Universitaire Vaudois Div of Hypertension, Switzerland) for the gift of anti-NPY and anti-pro-NPY (C-Franking Peptide of NPY) antibodies, and Dr. Keiichi Uyemura (Keio University School of Medicine, Tokyo) for the gift of anti-L1 antibody.

Thanks are also due to Dr. Akihiko Tsuji, Dr. Hideaki Nagamune, and Dr. Sumihare Noji for help and advice in many aspects of this work.

I am grateful to Mrs. Yuka Sasaki and Mrs. Tomomi Segawa for excellent secretarial assistance and Mr. Shigeru Yoshida, Miss Emi Imamura, Mr. Kazuya Murasato and Mr. Katsuhiro Mizuno for excellent technical assistance.

Finally, I wish to extend my sincere thanks to all my collaborators, especially a postgraduate student, Kenji Mori, in Prof. Yoshiko Matsuda's laboratory (B-1 group, Department of Biological Science and Technology) who helped to make this work a reality.

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Dissertation submitted to the Faculty of the Graduate School
of the University of Tokushima in partial fulfillment
of requirements for the degree of Doctor of Philosophy

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Akamatsu, T., Daikoku, S., Nagamune, H., Yoshida, S., Mori, K., Tsuji, A., and Matsuda, Y.

1997

Histochemistry and Cell Biology
vol. 108 (No.2) : 95-103

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様式 9

論文審査の結果の要旨

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学位論文題目

Histochemical and Cytochemical Studies
on Processing Protease PACE4(SPC4)

審査結果の要旨

生理活性ペプチドやタンパク質の活性化に関わるプロセシングプロテアーゼであるPACE4（SPC4）の組織，細胞における分布状態を本酵素を認識する特異的プローブを用いて in situ hybridization 及び特異抗体を用いての免疫組織化学的方法ではじめて明らかにした．さらに脳神経系の構築に本酵素が主要な役割を果たしていることを発生過程での分布の解析から明らかにした．つづいて，生理活性ペプチドであるニューロペプチドYとの共存関係を明らかにしたことにより中枢神経系の組織構築のメカニズムを解明した．

以上本研究は，生理活性ペプチド生成の主要ステップであるプロセシングプロテアーゼの生物学的役割を明らかにしたもので本論文は博士（工学）の学位授与に値するものと判定する．

なお，本論文の審査には，辻明彦助教授の協力を得た．