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Research report

Differentially expressed olfactomedin-related glycoproteins (Pancortins) in the brain

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Abstract

Messenger RNA differential display is conducted to search for genes that are expressed in a region-specific pattern in the rodent brain. Eleven novel gene fragments are isolated. One of these genes which we call *pancortin*, based on its predominant mRNA expression in the cerebral cortex of the adult, is studied. These pancortin cDNA clones are grouped into four different types of cDNA, designated as pancortin-1 to -4. All pancortin cDNAs share a common sequence in the middle of their structure, having two alternative sequences at both 5′- and 3′-ends, respectively. Deduced amino acid sequence shows that all pancortins have sequences of hydrophobic amino acids at N-terminus and no obvious membrane spanning regions. In situ hybridization histochemistry using oligonucleotide probes specific for 5′- and 3′-end variable parts has revealed that these four pancortin mRNAs are expressed differentially in the adult rodent brain. Robust expression of pancortin-1 and -2 mRNA is observed in the cerebral cortex (including the hippocampus and the olfactory bulb). However, little of pancortin-3 and -4 mRNA is observed there. In the cortex, some neurons are stained by an antibody raised against Pancortin.

Abbreviations: 3V, third ventricle; AA, anterior amygdaloid area; aca, anterior commissure, anterior part; Acb, accumbens nucleus; ACo, anterior cortical amygdaloid nucleus; AD, anterodorsal thalamic nucleus; AHiAL, amygdalohippocampal area, anterolateralpart; AHiPM, amygdalohippocampal area, posteromedial part; AHy, anterior hypothalamic area; AM, anteromedial thalamic nucleus; AOB, accessory olfactory bulb; AOE, anterior olfactory nucleus, external part; AOL, anterior olfactory nucleus, lateral part; AOP, anterior olfactory nucleus, posterior part; APir, amygdalopiriform transition area; APT, anterior pretectal area; AV, anteroventral thalamic nucleus; BL, basolateral amygdaloid nucleus; BM, basomedial amygdaloid nucleus; CA1, field CA1 of Ammon's horn; CA2, field CA2 of Ammon's horn; CA3, field CA3 of Ammon's horn; cc, corpus callosum; Cg, cingulate cortex (anterior cingulate cortex); CG, central (periaqueductal) grey; Cl, claustrum; CL, centrolateral thalamic nucleus; CM, central medial thalamic nucleus; CPu, caudate putamen (striatum); DG, dentate gyrus; DLG, dorsal lateral geniculate nucleus; DMD, dorsomedial hypothalamic nucleus, diffuse part; En, endopiriform nucleus; Ent, entorhinal cortex; Epl, external plexiform layer of the olfactory bulb; f, fornix; Fr, frontal cortex; FStr, fundus striata; gcc, genu of the corpus callosum; Gl, glomerular layer of the olfactory bulb; GP, globus pallidus; GrA, granular cell layer of the accessory olfactory bulb; Hb, habenular nucleus (lateral and medial); IAM, interanteromedial thalamic nucleus; ic, internal capsule; IGr, internal granular layer of the olfactory bulb; IPl, internal plexiform layer of the olfactory bulb; La, lateral amygdaloid nucleus; LH, lateral hypothalamic area; LOT, nucleus of the lateral olfactory; LP, lateral posterior thalamic nucleus (pulvinar); LSD, lateral septal nucleus, dorsal part; LV, lateral ventricle; MDC, mediodorsal thalamic nucleus, central part; Me, medial amygdaloid nucleus; MGV, medial geniculate nucleus, ventral part; Mi, mitral cell layer of the olfactory bulb; ml, medial lemniscus; MT, medial terminal nucleus of the accessory optic tract; MP, medial mammillary nucleus, posterior part; MPT, medial pretectal nucleus; opt, optic tract; OT, nucleus of the optic tract; ox, optic chiasm; Pa, paraventricular hypothalamic nucleus; Pir, piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus; PMCo, posteromedial cortical amygdaloid nucleus; Po, posterior thalamic nucleargroup; PoDG, polymorph layer of the dentate gyrus; PT, paratenial thalamic nucleus; PVA, paraventricular thalamic nucleus, anterior part; Re, reuniens thalamic nucleus; Rh, rhomboid thalamic nucleus; RS, retrosplenial cortex (posterior cingulate cortex); Rt, reticular thalamic nucleus; S, subiculum; scc, spelnium of the corpus callosum; SI, substantia innominata; sm, stria medullaris of the thalamus; SNC, substantia nigra, compact part; SNR, substantia nigra, reticular part; SO, supraoptic hypothalamic nucleus; st, stria terminalis; TT, tenia tecta (anterior hippocampal rudiment); Tu, olfactory tubercle; VDB, nucleus of the ventricle limb of the diagonal band (Broca); vhc, ventral hippocampal commissure; VL, ventrolateral thalamic nucleus; VMH, ventromedial hypothalamic nucleus; VPL, ventroposterior thalamic nucleus, lateral part; VPM, ventroposterior thalamic nucleus, medial part

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Immuno-electron microscopic study has revealed that Pancortin-like immunoreactive products are localized mainly in the endoplasmic reticulum and not in the Golgi apparatus indicating that Pancortins are the endoplasmic reticulum-anchored proteins. Our results suggest that each Pancortin is differentially regulated and may perform different functions in the brain.

Keywords: Cortex; Differential display; Glycoprotein; Olfactomedin; Rough endoplasmic reticulum (rER)

1. Introduction

The central nervous system (CNS) in higher animals is composed of many regions which are both functionally and histologically distinct. What makes one region functionally distinct from others? How different is one region from another? A factor expressed exclusively or predominantly in a specific region appears to be associated with such specificity [3]. A well-known example is the G-protein-coupled receptors, one of which is expressed exclusively in the neurons of the olfactory epithelium and related to olfactory functions [5]. The search for and analyses of genes expressed in a region-specific manner in the CNS are one of the applicable strategies in identifying specific factors or in studying region-specific neuronal functions at the molecular level.

Much effort has been made to search for genes which are expressed in a region-specific manner in the brain. The subtraction method is one strategy used for this purpose [1,3,21,22,24]. Recently, an innovative technique, the messenger RNA (mRNA) differential display method [9,10], has been developed. This method enables us to compare, identify and acquire genes expressed only under some specific conditions and/or regions [4,6,11]. With this method, we have been searching for a gene expressed in the pons during the neuronal circuit formation period. One such gene, which we call *pancortin*, based on its predominant mRNA expression in the cerebral cortex of the adult, is studied in our research.

2. Materials and methods

2.1. mRNA differential display

2.1.1. Animals

Adult female Wistar rats, weighing approximately 300 g, and newborn pups were used. Rats were purchased from a local vendor (Nippon Clea, Japan). They were housed in a room kept at a constant temperature and humidity and were provided with food and water ad libitum. Diurnal lighting conditions were maintained with lights on from 08:00 to 20:00 h. Pups were delivered from housed Wistar rats which were obtained 2 days prior to full-term pregnancy. Pups were used for experiments within 24 h of their delivery (P0).

2.1.2. Isolation of a complementary DNA fragments

We employed the mRNA differential display method to search for genes expressed locally in the rat basilar pons at P0. First, polyadenylated (poly A(+)) RNA was extracted using a micro Fast Track kit (Invitrogen, USA) from the basilar pons and cerebral cortex of 3 P0 littermates. We used sets of two primers to display mRNAs: one being anchored to polyadenylate tail of a subset of mRNAs, and the other being short and arbitrary, as described. Briefly, complementary DNAs (cDNAs), synthesized with the former primer from poly A(+) RNAs extracted from the basilar pons and cerebral cortices, were subjected to polymerase chain reaction (PCR) using both primers in the presence of [α-^{35}S]dATP; then the amplified DNAs were separated by electrophoresis on a polyacrylamide sequencing gel. Amplified cDNA fragments were visualized by autoradiography as bands in order of their molecular size. Some of these bands appeared only in the basilar pons-derived lane and not in the cerebral cortices-derived one. These bands correspond to cDNA fragments of genes which are expressed in the P0 basilar pons but not in the P0 cerebral cortices. We extracted these DNAs from the gel, subcloned them into the pBluescript plasmid (Stratagene, USA) and determined their nucleotide sequences using a DNA sequencer (370A, Applied Biosystems, USA) with Taq DyeDeoxy™ Terminator Cycle Sequencing kit (Applied Biosystems, USA).

2.1.3. Tissue preparation (for in situ hybridization histochemistry with cRNA probe)

Adult rats were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal, 50 mg/kg b.wt.), and perfused transcardially with saline followed by ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) (3 ml/g b.wt.). After perfusion, brains were removed, postfixed with the same fixative at 4°C for 1–3 days and immersed in 30% sucrose in 0.1 M PB at 4°C for 2–3 days. They were then frozen with powdered dry ice and 16-μm-thick sections were made on a cryostat. Sections were thaw-mounted onto glass-slides coated with TESTA (3-amino propyltriethoxysilane, Sigma, USA) and stored at −80°C until use.

2.1.4. DNA databank computer searches

The nucleic acid databases (GenBank, October, 1993) were searched by DNASIS program (Hitachi, Japan). Among the obtained 17 DNA fragments, 11 DNA fragments turned out to be novel; they were processed for in situ hybridization histochemistry. After in situ hybridization histochemistry, one of these fragments (#103) was selected based on its expression pattern and processed by a cDNA library screening. We checked the DNA database before submission, then found that the sequences of appar-

ent pancortin homologue in rat have been reported (U03414–U03417), although no other information on its subcellular localization and distribution of individual pancortin is available at present [2]. The cloning of pancortins has been reported in the meeting (Fourth IBRO World Congress of Neuroscience, A1.24, 1995).

2.1.5. In situ hybridization histochemistry (ISHH) with cRNA probe

Tissue sections were fixed with formaldehyde, digested with proteinase K, acetylated with acetic acid anhydride, then dehydrated. [α-^{35}S]UTP-labeled single-stranded RNA synthesized with the *Eco*RI-digested template plasmid containing #103 DNA fragment and T3 RNA polymerase, and that synthesized with the *Xho*I-digested template and T7 RNA polymerase were used as antisense and sense probes, respectively. Details of hybridization and washing procedures are described elsewhere [23]. Hybridization signals were visualized by macro-autoradiogram (film autoradiogram) and micro-autoradiogram (emulsion autoradiogram).

2.1.6. Identification of positive hybridization signals

Positive regions and cells were identified based on the accumulation of silver grains. In the case of micro-autoradiography, only cells with a grain density of at least 3 times higher than the background density were considered to be positive throughout this study [13]. Based on this criteria, however, we found a limited number of cells and/or regions difficult to identify as either positive or negative. Such cells and/or regions were regarded as negative in this study.

2.1.7. Northern blot analysis

Poly A(+) RNA was extracted from P0 rat brain using a micro Fast Track kit (Invitrogen, USA), then denatured and separated by electrophoresis on agarose–formaldehyde gels. RNA was transferred onto a nylon membrane (Immobilon, Dupont, USA) and hybridized with a ^{32}P-labeled DNA probe. Filters were washed in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) and 1% SDS (sodium dodecyl sulfate) at 60°C for 1 h before being processed for autoradiography. Film autoradiogram was carried out following the conventional procedure.

2.2. Isolation and analysis of pancortin cDNAs

2.2.1. Animals (for oligonucleotide ISHH)

Eight to 10-week-old male ICR mice were purchased from a local vender (Keari, Japan). They were treated in the same manner as animals for mRNA differential display (see Animals in mRNA differential display section).

2.2.2. Tissue preparation (for oligonucleotide ISHH and immunohistochemistry)

Adult mice were deeply anesthetized by sodium pentobarbital (Nembutal, 50 mg/kg b.wt.). Brains were removed without perfusion, frozen with powdered dry ice, then 16-μm-thick sections were made on a cryostat. Sections were thaw-mounted onto glass-slides coated with TESTA (3-amino propyltriethoxysilane, Sigma, USA) and stored at −80°C until use.

2.2.3. Screening of mouse cDNA library

A P0 mouse whole-brain cDNA library constructed in lambda ZAP II (purchased from Stratagene, USA) was screened using #103 DNA fragment labeled with ^{32}P-labelled dCTP by the BcaBest labeling kit (Takara, Japan).

2.2.4. ISHH with oligonucleotide probes

To examine fragment-specific distribution of each fragment such as A1, A2, C1, and C2 (see Section 3.3; Fig. 2), ISHH with synthetic oligonucleotides was carried out. The following synthetic oligonucleotide probes were labeled with [α-^{35}S]dATP by a 3′-end-labeling method.

A1: TGAAGATCGGGGTCGTGCTAAGCACCATGG-CCATGATCACC AACTGGATGT
A2: TGTCCGAGAGGCAGTCGCGCCGGAACCGGG-ACCATAAATA TGCAGA
C1: TTGAACTTGCGTACCCTGTAGCTAGATCGTG-AGGTGTCCGT CTGTTATCC
C2: GCATGCGACTCTAGATGCATCCCATGAATA-GTTTGAACCCTTGTCAATGC

These probes were complementary to the mRNA sequence coding for A1, A2, C1 and C2 parts, respectively. Tissue sections were treated in basically the same way as with the cRNA probes, excluding the proteinase K treatment. Details of the procedures were described previously except for the employed washing temperature (65°C) [18].

2.3. Analysis of Pancortin protein

2.3.1. Preparation and characterization of the monoclonal antibody

Fourteen-mer peptide (KFKQVEESHKQHLA of part B) was synthesized, then conjugated with KLH (keyhole limpet hemocyanin; Peptide Laboratory, Osaka, Japan). Female Balb/c mice were maintained and immunized with the peptide conjugates. Primary immunization with the synthetic peptide–KLH conjugate (0.1 mg peptide) emulsified in complete Freund's adjuvant (Gibco) was followed by 22 and 34 day booster injections (0.1 mg peptide) in incomplete Freund's adjuvant (Gibco). Sixty-four days after the first injection, blood was collected and resulting antiserum was checked by dot blot. Monoclonal antibody was raised based on the published method [20]. Specificity of the antibody was checked by Western blot analysis.

2.3.2. Western blot analysis

Homogenized protein extracts were prepared from various ICR mouse brain tissues (the olfactory bulb, neocor-

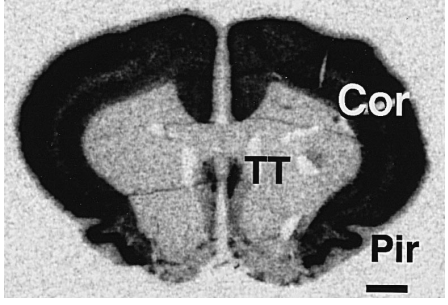

Fig. 1. Hybridization signals of #103 (pancortin fragment) mRNA in the rat brain. Macro-autoradiogram: frontal sections. Positive signals are observed in the cortex (Cor), including the piriform cortex (Pir) and the tenia tecta (TT). Scale bar: 1 mm.

tex, hippocampus and thalamus from the adult and the neocortex and thalamus from P0 pups) with sample buffer (125 mM Tris-HCl, pH 6.8, 5% mercaptoethanol, 2% SDS, 0.1% glycerol). Aliquotes (100 μg) were resolved by electrophoresis on 12.5% SDS–polyacrylamide gels and transferred onto nitrocellulose filters by electroblotting. Culture medium of the hybridomas-producing anti-Pancortin antibody was diluted 1:1 with 0.2 M PB, then used as incubation solution containing a primary antibody. The nitrocellulose filter was incubated with this solution after pre-incubation with 3% bovine serum albumin. Then the filter was incubated with the secondary antibody conjugated with alkaline phosphatase (Bio-Rad, diluted 1:2000) after washing (BioRad, Immunoblot kit). Immunoreactivity was visualized with the alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) with NBT (4-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate).

2.3.3. Immunohistochemistry

Eight-week-old adult ICR mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, 50 mg/kg b.wt.), then perfused transcardially with saline followed by ice-cold 4% Zamboni's fixative (0.21% picric acid and 4% paraformaldehyde in 0.1 M sodium phosphate buffer). After perfusion, brains were removed, postfixed with the same fixative at 4°C for 1 day and embedded in paraffine. Five-μm-thick frontal sections were made using a microtome. After paraffine was removed from tissues by immersion in xylene and ethanol, a conventional immunohistochemical procedure was performed. The culture medium of the hybridomas-producing anti-Pancortin antibody was diluted 1:1 with 0.2 M PB, then used as incubation solution containing a primary antibody. ABC kit (Vectastain) was used for further procedures and immunoreactivity was visualized with DAB (3,3′-diaminobenzidine).

2.3.4. Immuno-electron microscopic studies

Eight-week-old adult ICR mice were anesthetized by sodium pentobarbital, then perfused with Zamboni's solution containing 0.05% glutaraldehyde. Brains were post-

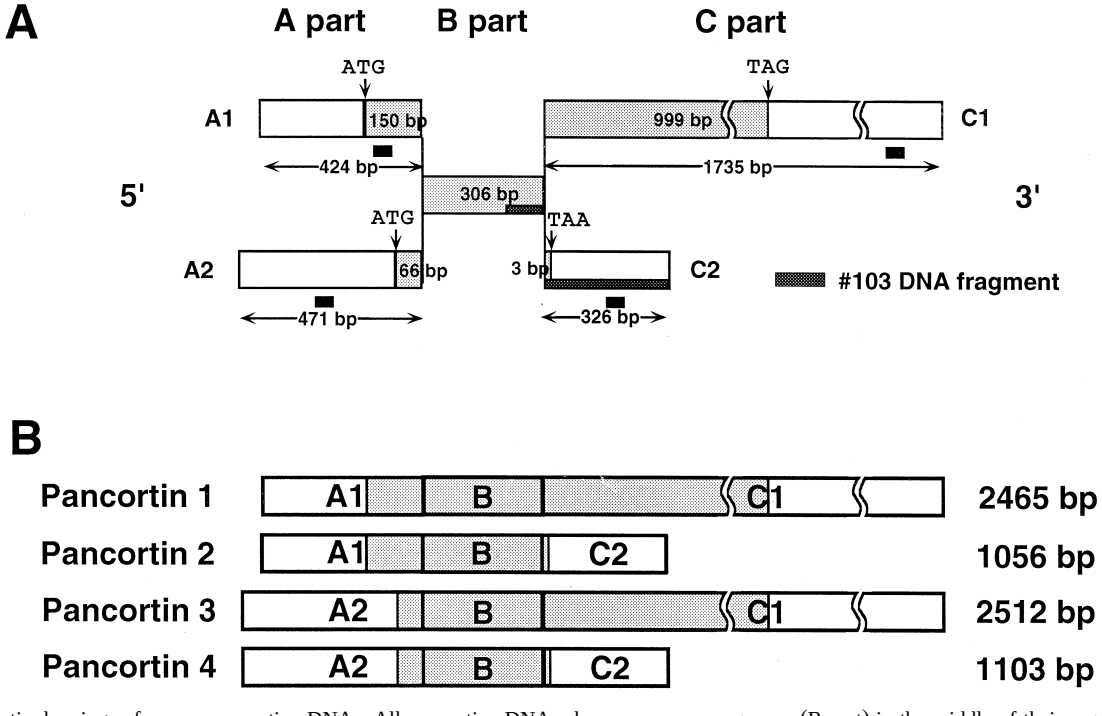

Fig. 2. Schematic drawings of mouse pancortin cDNAs. All pancortin cDNAs share a common sequence (B part) in the middle of their sequence, having two alternative sequences at both 5′- (A1 and A2) and 3′-ends (C1 and C2), respectively. Possible open reading frame is shown in gray squares. A: black squares are showing the positions where synthetic oligonucleotide probes hybridize, corresponding regions of #103 DNA fragment is also indicated. B: schematic representation of each pancortin cDNAs (pancortin-1 to -4).

fixed overnight in the same fixative, 50-μm-thick frontal sections were cut on a microslicer (Dosaka EM) and processed for further immunohistochemical procedures. After the reaction with DAB, sections were postfixed with 1% osmium tetroxide, dehydrated with alcohol, treated with propylene oxide, then embedded in Durcupan (Fluka). Ultra-thin sections were cut on an ultramicrotome (Reichert–Jung), and stained with uranyl acetate. Following lead staining, sections were observed under an electron microscope.

2.3.5. Terminology

Terminology was based on the atlas of Paxinos and Watson [15].

3. Results

3.1. Isolation of a gene which is expressed predominantly in the cortex

We isolated 17 gene fragments through several screenings using the mRNA differential display method (see Section 2). Among them, 11 fragments turned out to be novel after sequencing and DNA Data Base inquiry (GenBank, October, 1993) by DNASIS program (Hitachi, Japan). ISHH with macro-autoradiogram was carried out to examine their regional distribution in the brain. Among 11 novel candidates, one DNA fragment designated as #103 showed strong hybridization signals in the cortex of the adult rat (Fig. 1) as well as in the pons of the newborn rat. Since the strong hybridization signals are observed in all over the cortex ('pan' cortex) and appear to be confined in the cortex, we name the gene that is recognized by #103 fragment *pancortin*. To characterize this *pancortin* gene, Northern blot analysis and cDNA cloning were performed.

3.2. Analysis of #103 fragment

In order to determine the size of pancortin mRNA, Northern blot analysis was carried out using #103 fragment as a probe. The #103 fragment produced two obvious bands, approximately 1 and 2.5 kb in size, respectively (data not shown). This suggests that the *pancortin* gene has a sibling.

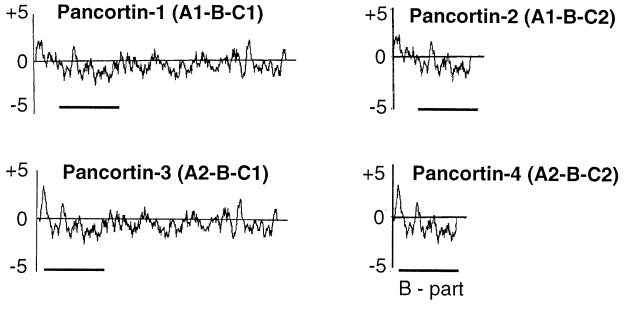

Fig. 3. Hydrophobicity profiles of Pancortins. Hydrophobicity profiles are calculated based on Kyte and Dolittle [8]. Regions corresponding to B part are underlined.

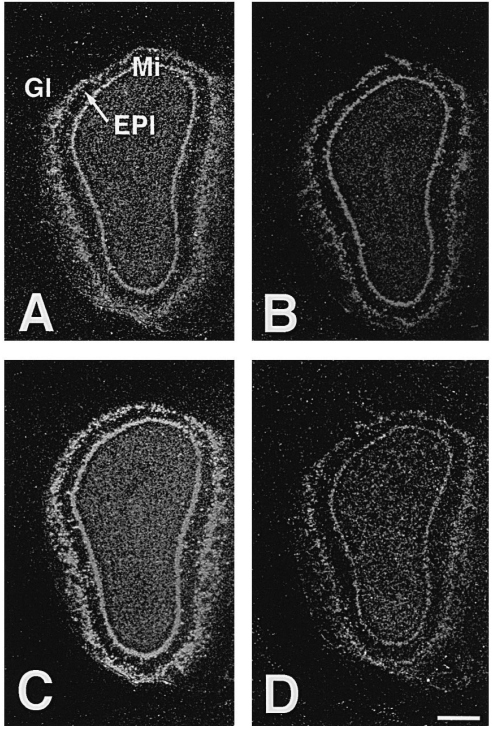

Fig. 4. Part-specific expression of pancortin mRNAs in the mouse olfactory bulb. Frontal sections: micro-autoradiogram. A: expression of A1 part of pancortin. The remaining panels are as follows: (B) A2 part, (C) C1 part, (D) C2 part. Prominent hybridization signals of A1 and C1 are recognized in cells in the mitral cell layer (Mi) and the glomerular layer (Gl). Few A1- and C1-positive cells are scattered in the external plexiform layer (EPl). Scale bar: 0.5 mm.

3.3. cDNA cloning of pancortin family

As there are many gene modification techniques applicable to the mouse, we screened the mouse cDNA library instead of the rat cDNA library. Screening of the newborn mouse brain cDNA library revealed the #103 fragment could hybridize with different types of cDNA clones. The clones obtained were categorized into four different types of cDNAs, designated as pancortin-1 to -4. Two alternative sequences exist at both 5′- and 3′-ends (A1 and A2, and C1 and C2), respectively, having a common sequence (B) between them. Fig. 2 is a schematic drawing of cDNA clones obtained from the mouse cDNA library and the composition of the *pancortin* family based on their deduced amino acid sequences.

3.4. Characterization of Pancortins

3.4.1. Hydrophobicity profiles of Pancortins

Hydrophobicity profiles are calculated based on their deduced amino acid sequences following Kyte and Doolit-

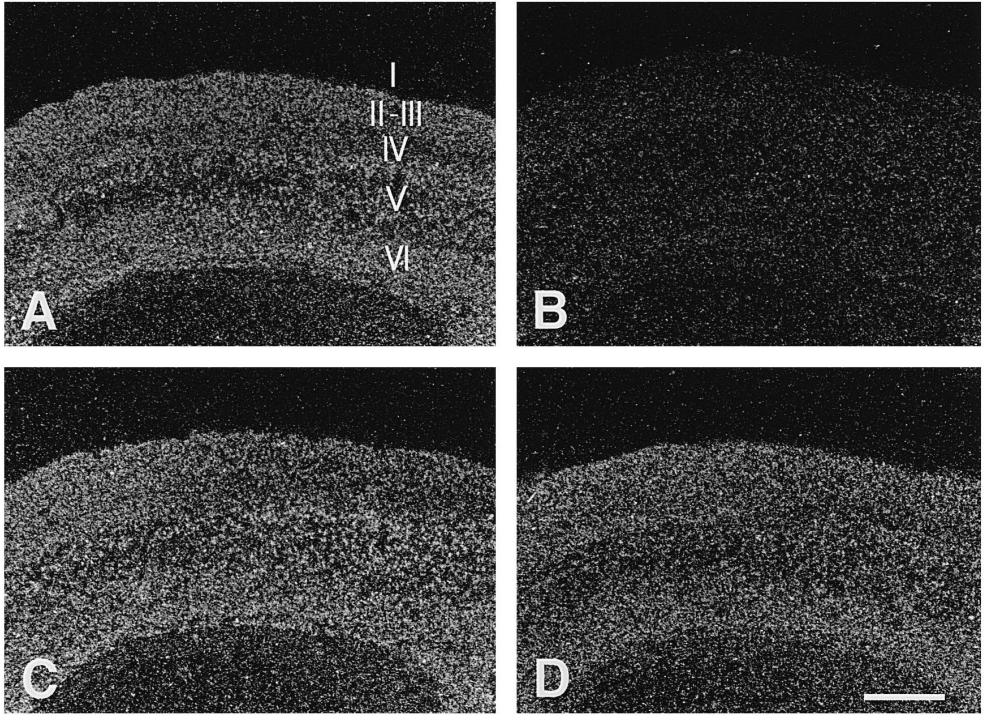

Fig. 5. Part-specific expression of pancortin mRNAs in the mouse cerebral cortex. Frontal sections: micro-autoradiogram. A: expression of A1 part of pancortin. The remaining panels are as follows: (B) A2 part, (C) C1 part, (D) C2 part. Expression of A1, C1 and C2 signals are corroborated in the cortex of layers II through VI. Only faint expression of A2 signals could be verified. No area specificity is noticed. Scale bar: 0.5 mm.

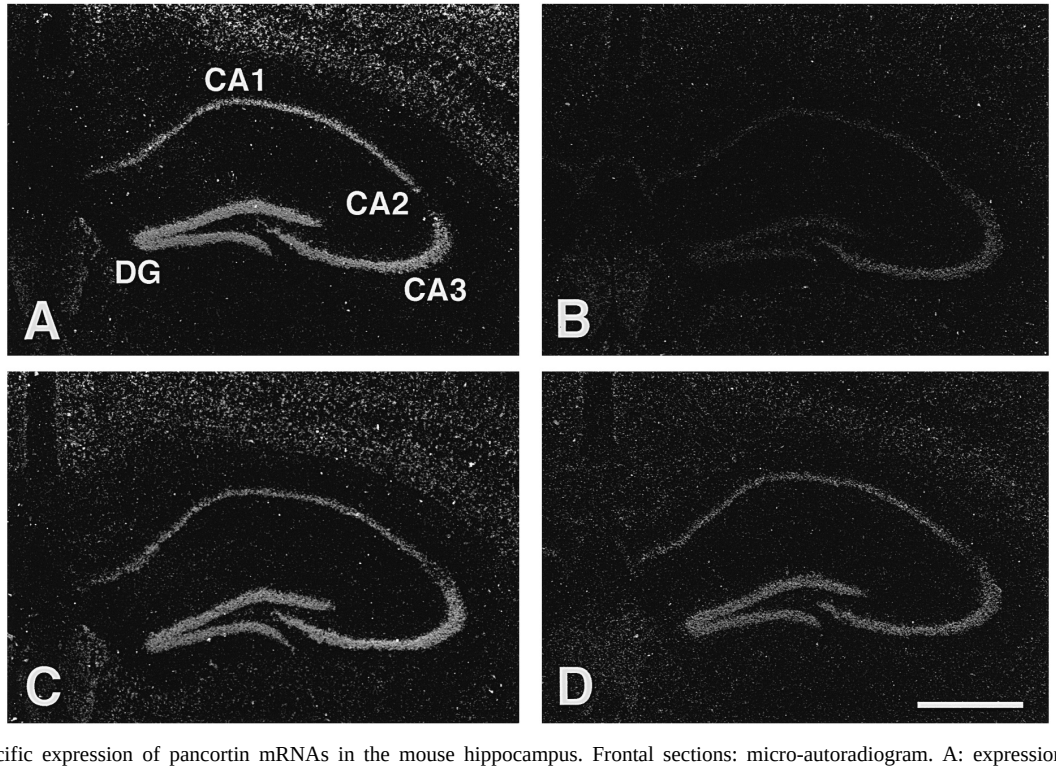

Fig. 6. Part-specific expression of pancortin mRNAs in the mouse hippocampus. Frontal sections: micro-autoradiogram. A: expression of A1 part of pancortin. The remaining panels are as follows: (B) A2 part, (C) C1 part, (D) C2 part. Strong A1, C1 and C2 signals and weak A2 signals are observed in the dentate gyrus (DG) and the Ammon's horn of CA1 and CA3. In the Ammon's horn of CA2, weak signals of A1, C1 and C2 and fairly strong signals of A2 are confirmed. Scale bar: 0.5 mm.

tle [8]. Each Pancortin has a hydrophobic region in its N-terminal. No possible trans-membrane region is found (Fig. 3).

3.4.2. ISHH using part-specific oligonucleotide probes

#103 DNA fragment contains some portion of the pancortin B part, which is common to all the four pancortin cDNAs (Fig. 2). Therefore, #103 fragment-derived probe could hybridize with any pancortin mRNAs. To investigate the regional expression pattern of individual pancortin mRNA, ISHH was carried out using four different synthetic oligonucleotide probes complementary to each part of the pancortin cDNA (Fig. 2; these probes are referred to as A1, A2, C1 and C2 probe, respectively.). Signals detected by A1, A2, C1 and C2 probes are referred to as A1, A2, C1 and C2 signals in this text.

Regional expression patterns of each pancortin mRNAs were examined mainly in the prosencephalon. Although pancortin mRNAs were expressed in various regions in the brain, strong accumulations of silver grains were significant in the olfactory bulb, cortex and the hippocampus. Positive signals of pancortin mRNA were detected in some neurons, although no distinct positive signals were observed in the glial cells.

3.4.2.1. Telencephalon–olfactory bulb. Prominent hybridization signals of A1 and C1 were recognized in cells in the mitral cell layer and the glomerular layer (Figs. 4 and 7). Few A1- and C1-positive cells were scattered in the external plexiform layer. Although A2 and C2 signals were also recognized in the mitral cell layer and the glomerular layer, signal intensity was weaker than those of A1 and C1. In addition, while A1- and C1-positive cells were widely distributed in the glomerular layer, A2- and

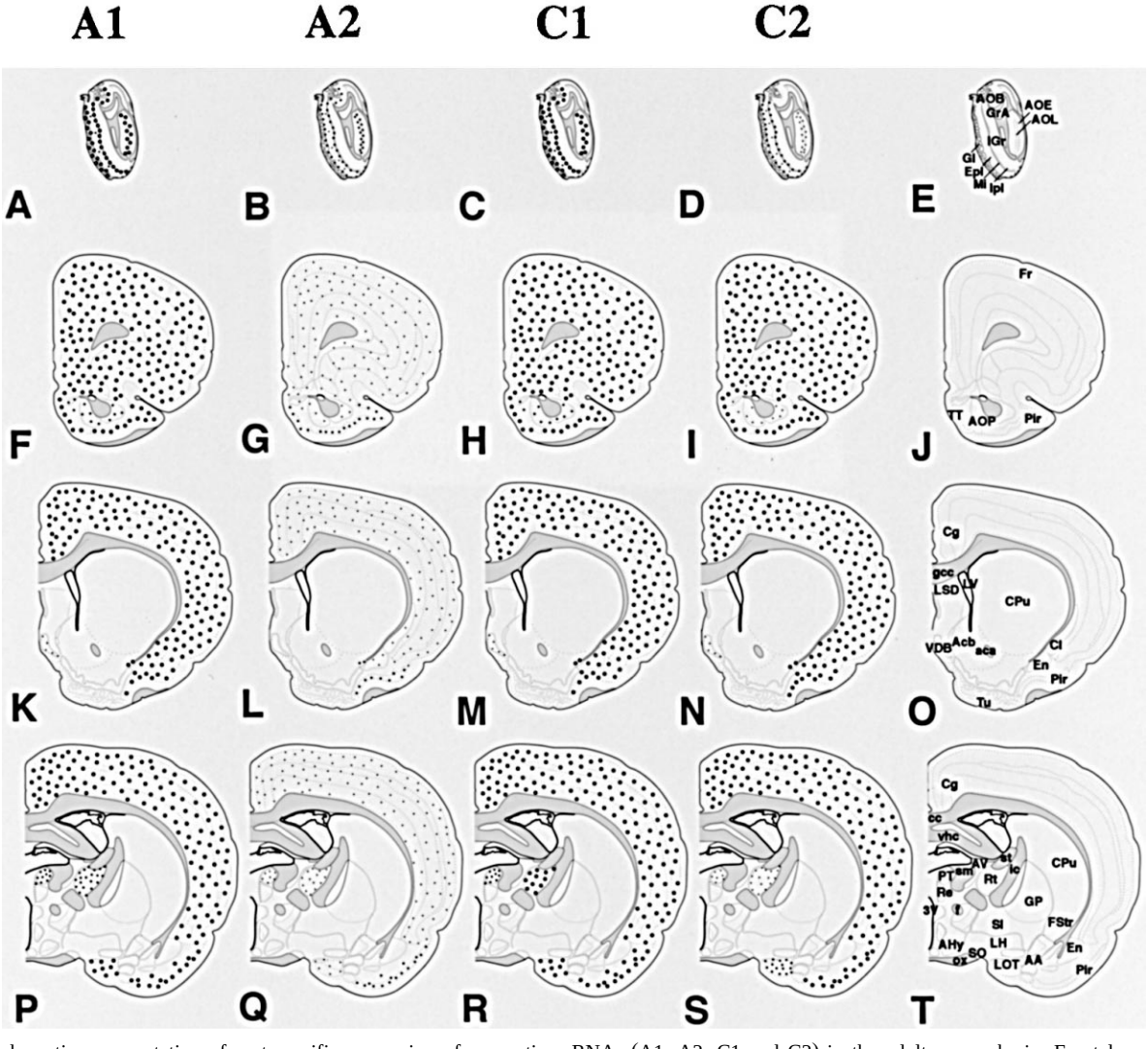

Fig. 7. Schematic representation of part-specific expression of pancortin mRNAs (A1, A2, C1 and C2) in the adult mouse brain. Frontal sections are arranged rostrocaudally and the location of cells with positive signals are represented by close circles. Pancortin mRNA positive neurons are represented by dots. Large dots indicate neurons labeled strongly, medium-size dots indicate neurons labeled weakly, while small dots indicate neurons labeled very faintly.

C2-positive cells were observed mainly in the region close to the external plexiform layer. Distinct positive A1 and C1 signals and weak A2 signals existed in the accessory olfactory bulb and the anterior olfactory nuclei. Very faint C2 signals were confirmed in the anterior olfactory nuclei, yet no distinct signals were confirmed in the accessory olfactory bulb.

3.4.2.2. Telencephalon–cortex. Robust expression of A1, C1 and C2 signals were corroborated in the cortex (neocortex, cingulate cortex and retrosplenial cortex) of layer II through layer VI (Figs. 5, 7 and 8), in the piriform cortex (especially of layer II) and the entorhinal cortex. Faint expression of A2 signals could be verified. No area specificity was noticed. Similar tendencies were noticed in the amygdalopiriform transition.

3.4.2.3. Telencephalon–hippocampal formation. Strong A1, C1 and C2 signals and weak A2 signals were observed in the dentate gyrus and in the Ammon's horn of CA1 and CA3. In the Ammon's horn of CA2, weak signals of C1 and C2 and fairly strong signals of A2 were confirmed (Fig. 6). In the areas related to hippocampal formation, strong signals of A1 and C1 were observed in the triangular septal nucleus, while weak signals of A2 and C2 were noticed there.

3.4.2.4. Telencephalon–other areas (Figs. 7 and 8). Strong accumulation of grains were observed with A1, C1 and C2 probes in the tenia tecta, while A2 signals were weak in that area. In the diagonal band, faint signals of A1, C1 and C2 were noticed. In the amygdaloid complex, distinct A1, C1 signals, weak C2 signals and very faint A2 signals

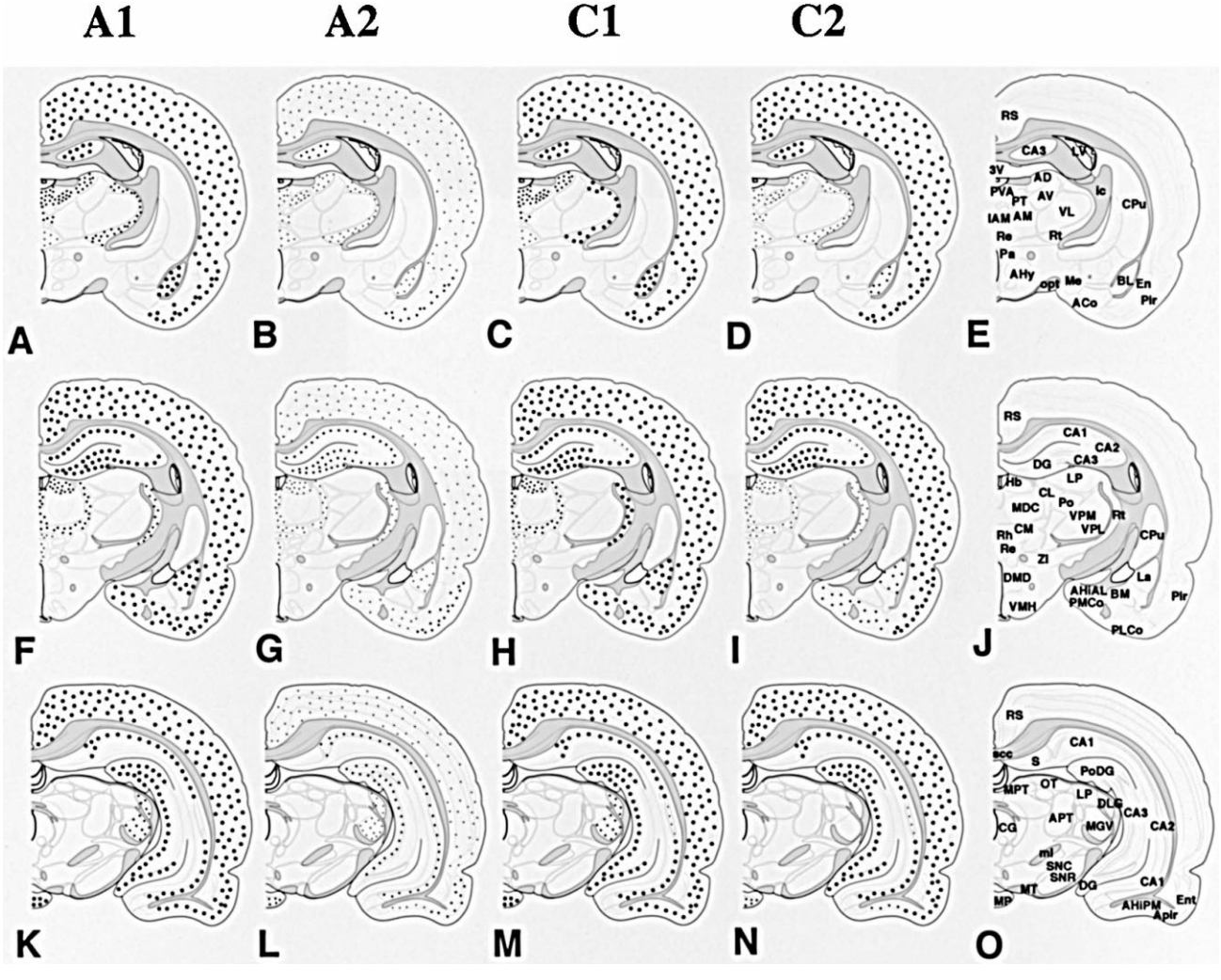

Fig. 8. Schematic representation of part-specific expression of pancortin mRNAs (A1, A2, C1 and C2) in the adult mouse brain. Frontal sections are arranged rostrocaudally, and the location of cells with positive signals are represented by close circles. Pancortin mRNA positive neurons are represented by dots. Large dots indicate neurons labeled strongly, medium-size dots indicate neurons labeled weakly, while small dots indicate neurons labeled very faintly.

were recognized in the lateral amygdaloid nucleus, basolateral amygdaloid nucleus, amygdalohippocampal area, posterolateral and posteromedial cortical amygdaloid nucleus. In the nucleus of the lateral olfactory tract, strong A1, C1 and weak C2 signals were observed, while faint but positive signals of A2 could be recognized.

3.4.2.5. Diencephalon (Figs. 7 and 8). In the thalamus, pancortin mRNAs were detected in the habenular nucleus. In addition, the anterodorsal thalamic nucleus, paratenial thalamic nucleus, paraventricular thalamic nucleus, central medial thalamic nucleus, interanteromedial thalamic nucleus, rhomboid thalamic nucleus, reuniens thalamic nucleus, centrolateral thalamic nucleus and the reticular thalamic nucleus were positive for pancortin mRNAs. Generally, A1 and C1 signals were strong, while A2 signals were weaker and C2 signals were very faint in the thalamus. Similar tendencies were noticed in the lateral and medial geniculate nuclei yet no distinct C2 signals were confirmed there.

3.4.2.6. Representative areas where pancortin mRNA expression is robust in the mesencephalon and the rhombencephalon. In the mesencephalon, A2 and C1 signals were observed in the occulomotor nucleus. Strong signals of A1 and C1 and weak signals of A2 and C2 were detected in the medial mammillary nucleus and the interpeduncular nucleus. In the rhombencephalon, noticeable signals of pancortin mRNAs were observed in the pontine nucleus, the interpeduncular nucleus and granular layer of the cerebellum.

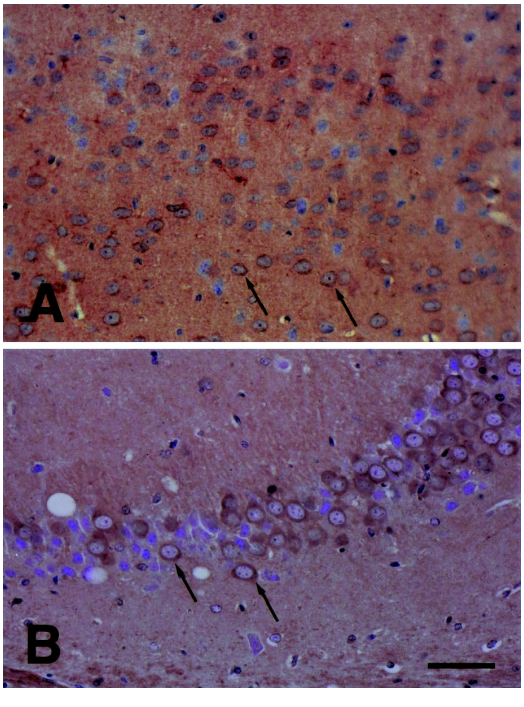

Fig. 9. Immunohistochemical demonstration of Pancortins in the mouse brain. Frontal sections: (A) cortex (layer 4 and 5), (B) hippocampus (CA3). Positive cells are shown by arrows. Pancortin-like immunoreactivity (brown) is localized mainly in the cell body, counterstained with thionin. Scale bar: 0.1 mm.

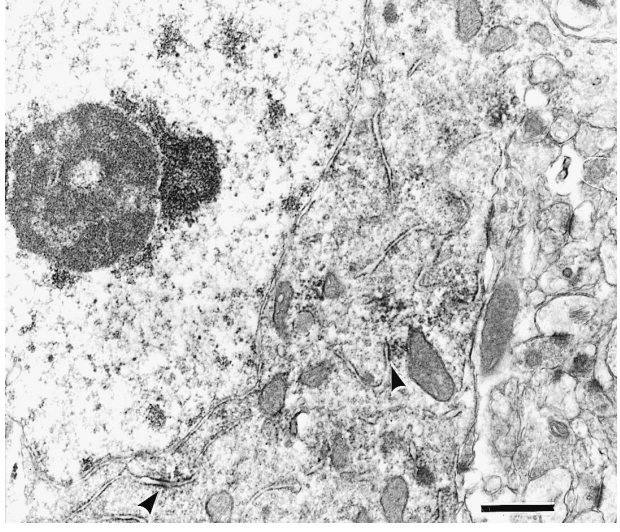

Fig. 10. Immuno-electron microscopic image of Pancortin-like structure in a cell. Pyramidal cells in the mouse hippocampus. Immunopositive structures, which are mainly localized with endoplasmic reticula, are shown with arrowheads. Scale bar: 500 nm.

3.4.3. Immunohistochemical analysis of pancortins

Monoclonal antibodies against all possible Pancortins were raised and the specificity was confirmed by Western blot analysis (data not shown). Then the localization of Pancortin-like immunoreactivity was studied. Pancortin-like immunoreactivity was examined in the cortex and the hippocampal formation (Fig. 9). Immuno-electron microscopic results showed that Pancortin-like immunoreactivity was localized mainly in the endoplasmic reticulum (Fig. 10). No obvious immunoreactivity was observed in the Golgi apparatus.

4. Discussion

Pancortins consist of four independent members (designated Pancortin-1 to -4) sharing a common part in the center of their sequences.

4.1. Structural features of pancortin

Hydrophobicity profiles of the deduced amino acid sequence of Pancortins indicate that all four Pancortins have putative signal peptide-like sequences but no transmembrane region. This fact suggests that Pancortins are secreted from a cell. However, no obvious immunoreactivity was detected in the Golgi apparatus, although immunoreactive deposits of Pancortins were observed in the endoplasmic reticulum. This indicates that Pancortins are

not released from the cell but are localized in the endoplasmic reticulum. In addition, the C-terminal sequence of the C1 part is similar to that of the endoplasmic reticulum anchor sequence of *Plasmodium falciparum* [7,12,16,17], implying that Pancortin-1 and -3 may localize in the lumen of endoplasmic reticulum.

The amino acid sequence of pancortin C1 part is 33% homologous with olfactomedin over 178 amino acids, an extracellular matrix (ECM) component specific to olfactory mucosa [25]. Some of the cysteine residues in the olfactomedin amino acid sequence, which seem important to the formation of its molecular structure, are not conserved in the C1 part [25]. Since the involvement of olfactomedin is expected in maintenance, growth and differentiation of olfactory neuronal processes [25], it is possible that Pancortin-1 and -3 play a similar role.

4.2. Regional distribution of pancortins

Pancortin mRNAs are localized in neurons in a region-specific manner in the adult prosencehpalon. Most of the positive regions are classified as cortical tissues, including the paleocortex and the allocortex.

It is difficult to observe the tissue expression pattern of each pancortin mRNA separately by the ISHH with a cRNA probe because the cRNA probes for any pancortin mRNA may bind to the common part that is shared by all. Therefore we utilized synthetic oligonucleotide probes corresponding to individual pancortin parts (A1, A2, C1 and C2) then examined the part-specific expression of the pancortin mRNAs, yet the sensitivity of oligonucleotide probes is not high enough to detect a small amount of mRNAs than that of cRNA probe, it is possible that areas where a small amount of pancortin mRNAs are expressed were not recognized.

Throughout the prosencephalon, the expression of A1, C1 and C2 parts is stronger than A2, indicating that Pancortin-1 and -2 are dominant. Danielson et al. [2] has reported the cloning of olfactomedin-related genes (an apparent rat homologue of Pancortins) and that they are coded in a single chromosome. Their results indicate that the A1 and A2 parts of Pancortins are derived by a differential promoter utilization and that C1 and C2 parts are derived by an alternative splicing. In addition, they have discussed that all four transcripts (Pancortin-1 to -4) exist in an approximately equal concentration throughout brain. On the contrary, we have observed that the A1 and A2 are differentially expressed in many regions of the brain; generally A1 is stronger than A2, but in the Ammon's horn of CA2 in the hippocampus, A2 is expressed while A1 is not detected. This observation raises the possibility that two (or more) promoters exist for A1 and A2.

There are several regions where Pancortins are considered to be co-localized. In the Ammon's horn of CA1 and CA3 in the hippocampus, nearly all pyramidal cells appear to be positive for both C1 and C2 probes. Therefore, it is likely that Pancortin-1 and -2 co-exist there. If they are co-localized in a single cell, it is possible that Pancortins exist in a dimer (homodimer or heterodimer) or in a polymerized form. The olfactomedin, which is a partially analogous to Pancortin, is supposed to exist in a polymerizing form.

4.3. Possible predominant Pancortins in the brain

4.3.1. Olfactory bulb

The mitral cells and tufted cells give distinct hybridization signals with both A1 and C1 part-specific probes. Weak positive signals were detected with A2- and C2-specific probes. This suggests that in the olfactory bulb, Pancortin-1 is dominant.

4.3.2. Cerebral cortex and hippocampus

In these areas, both the C1 and C2 parts are dominant, in addition to the A1 part, Pancortin-1 and -2 are dominant types of Pancortins in the cerebral cortex and hippocampus.

Conversely, in other regions in the paleocortex, it is likely that both Pancortin-2 and -4 are significantly expressed since the C2 probe also gives positive signals there. Moreover, both the A2 and C2 probes give strong hybridization signals in the piriform cortex. This indicates that a large quantity of Pancortin-3 and/or -4 are localized there.

In the allocortex and neocortex, A1, C1 and C2 probes give positive signals suggesting that Pancortin-1 and -2 are dominant. Furthermore, no obvious difference in the signal density was observed between the C1 and C2 signals.

4.3.3. Other regions

In the diencephalon, expression of A1 and C1 parts are strongest, indicating that Pancortin-1 is the dominant type.

4.4. Possible roles of pancortin in the brain

The nucleotide sequence of each pancortin transcript contains in its 3′-untranslated region a mRNA destabilizing motif ($5' > AUUUA < 3'$), which can be correlated with accelerated turnover of the transcript [14,19]. This indicates that Pancortins are types of molecules in which expression is precisely regulated in response to other stimuli.

In the neocortex, pancortin mRNA is distributed widely. Most neurons, not only the long projecting neurons but also the local circuit neurons, appear to be Pancortin-positive. This fact indicates that Pancortin is important to most neurons, not specific to some subgroups of neurons of specific function, implying that the biological function of Pancortins is related to non-specific events in the cell. In addition, since the Pancortins are related to the endoplasmic reticulum, it is possible that the role of Pancortins is

similar to that of molecules anchored to the endoplasmic reticulum, e.g. a chaperon molecule, yet this concept is still open at present.

The #103 gene fragment was obtained as a gene which is expressed predominantly in the pons but not in the cortex in a newborn pup. This fact indicates that expression of *pancortin* gene is changed developmentally in the cortex, since robust expression of pancortin mRNAs are observed in the adult cortex. Our preliminary study supports this indication; pancortin mRNAs are gradually expressed in the cortex during ontogenesis. This fact suggests that at least one of Pancortins is related to the cortical development, yet more studies on the expression pattern of pancortin mRNAs during the development and functions of Pancortins are required to confirm this assumption.

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