DISTRIBUTIONS OF TWO CHICKEN BOMBESIN RECEPTORS, BOMBESIN RECEPTOR SUBTYPE-3.5 (chBRS-3.5) AND GASTRIN-RELEASING PEPTIDE RECEPTOR (chGRP-R) mRNAs IN THE CHICKEN TELENCEPHALON

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Abstract—Bombesin (BN)-like peptide receptors are known to be essential to the regulation of not only homeostasis, including feeding behavior, but also of emotional systems in mammal. Recently, two novel BN receptors, chicken BN-like peptide receptor subtype-3.5 (chBRS-3.5) and gastrin-releasing peptide receptor (chGRP-R), have been identified. Here, we report the localizations of these receptors’ mRNAs in the chick brain through development using in situ hybridization. First, chBRS-3.5 mRNA signals were found in the dorsal ventricular ridge at embryonic day (ED) 9. Strong signals were observed in the hyperpallium accessorium, nidopallium and nucleus basorostralis pallii, and moderate signals were found in the hyperpallium, nidopallium, considered to be similar to the mammalian cortex, as well as in the hippocampus, indicate participation of these molecules in the processing of sensory information, motor function, learning and memory. Telencephalic areas devoid of chBRS-3.5 signals were the entopallium, arcopallium anterius, globus pallidus, nucleus septalis lateralis, hypothalamic and thalamic areas. In contrast to chBRS-3.5, chGRP-R mRNA signals were found in the pallidum at ED5 and 9. At ED16, chGRP-R mRNA signals were localized in the medial striatum and hypothalamus. GRP-R expression in the hypothalamic region was phylogenetically conserved.

Thus, chBRS-3.5 mRNA signals were distributed in a broader region and were more intense than chGRP-R mRNA. Taken together, chGRP-R and chBRS-3.5 mRNA occurred in similar regions of mammals that express GRP-R. BN/GRP-immunoreactive neurons and varicosities were found mainly in the pallium, especially in the hyperpallium accessorium and nidopallium, and this distribution coincided with that of chBRS-3.5 mRNA. This result suggests that the endogenous ligands for chBRS-3.5 were likely BN-like peptides produced in the pallium.

Key words: avian brain, immunohistochemistry, in situ hybridization, hyperpallium, nidopallium.

Bombesin (BN) is a tetradecapeptide which was originally purified from the skin of the European frog Bombina bombina (Anastasi et al., 1971) and several BN-related peptides have been isolated from various amphibians (Erspamer, 1988; Spindel et al., 1993). Subsequently, two mammalian peptides, gastrin releasing peptide (GRP) and neuromedin B (NMB), which have structural similarities to BN have been purified from the porcine stomach and spinal cord, respectively (McDonald et al., 1978; Minamino et al., 1983). These two peptides are regarded as the only mammalian BN-related peptides cloned so far. These peptides are known to have important physiological effects such as endocrine, exocrine secretions and smooth muscle contraction (Lebacq-Verheyden et al., 1990; Ohki-Hamazaki, 2000). In addition to the gastrointestinal tract and secretory organs, NMB and GRP and their mRNAs are found in the CNS of the rat (Wada et al., 1990). The NMB mRNA is found most prominently in the external.
plexiform and mitral cell layers of the olfactory bulb and the polymorph layer of the dentate gyrus in the forebrain. High levels of GRP mRNA are observed in the isocortex, pre-subiculum, parasubiculum, subiculum, dentate gyrus (granular layer) and amygdala. Several lines of evidence demonstrated the important roles of these BN-related peptides in behaviors such as grooming (Johnston and Merali, 1988), scratching (Masui et al., 1993), feeding (Gibbs et al., 1979; Ladenheim et al., 1996) and emotional behavior (Moody et al., 1988; Merali et al., 1998), as well as the autonomic functions, such as regulation of body temperature (Brown et al., 1977) and circadian rhythm (McArthur et al., 2000).

Meanwhile, five types of BN-like peptide receptors have been cloned and characterized. Two receptors cloned at the beginning were designated as GRP receptor (GRP-R) and NMB receptor (NMB-R) because of their high affinities for each peptide in mammals (Spindel et al., 1990; Battey et al., 1991; Wada et al., 1991). In addition to these receptors, the third mammalian subtype of BN-like peptide receptor, named the BN receptor subtype-3 (BRS-3), which shows structural homology for other BN-like peptide receptors, but provides low affinity for both GRP and NMB, has been cloned (Fathi et al., 1993). The analysis of mice lacking GRP-R (Wada et al., 1997; Hampton et al., 1998), NMB-R (Ohki-Hamazaki et al., 1999) or BRS-3 (Ohki-Hamazaki et al., 1997b) revealed that these receptors mediated similar and/or different functions. For example, BRS-3 and GRP-R play a role in regulation of metabolism and adiposity (Ohki-Hamazaki et al., 1997b; Ladenheim et al., 2002), but NMB-R might not affect these regulations (Ohki-Hamazaki et al., 1999). The hypothermic effect is regulated by both GRP-R and NMB-R (Ohki-Hamazaki et al., 1999), but not by BRS-3 (Ohki-Hamazaki et al., 1997b). The contraction of smooth muscle in the stomach seems to be controlled exclusively by GRP-R (Wada et al., 1997; Ohki-Hamazaki et al., 1999). In addition to these mammalian subtypes, the BN receptor 4 (BB4) has been found in the frog brain and gastrointestinal tract (Nagalla et al., 1995), although its physiological functions remain uncertain. Recently, two subtypes of BN-like peptide receptor have been cloned from the chicken brain cDNA by reverse transcription (RT)-PCR using degenerate primers. These are chicken BRS-3.5 (chBRS-3.5) designated after the sequence similarities to both BRS-3 and BB-4, and chicken GRP-R (chGRP-R). Interestingly, pharmacological study has revealed that the binding profile of chBRS-3.5 was different from that of mammalian BRS-3, although chGRP-R had the same profile as mammalian GRP-R (Iwabuchi et al., 2003).

In this experiment, we investigated the distributions of chBRS-3.5 and chGRP-R mRNAs in the chicken telencephalon using radioisotope-in situ hybridization (ISH) during embryonic development and posthatch days (PD). In particular, the information about the distribution of chBRS-3.5 mRNA is expected to give us a useful clue in understanding which function is mediated by chBRS-3.5. Four types of BN-like peptide receptors mentioned previously vary greatly in terms of the mRNA localizations. Detailed ISH studies aimed at fine documentation of gene expression patterns of rat GRP-R and NMB-R revealed that both receptors were expressed moderately in the dentate gyrus and nucleus ambiguous, while in other brain regions their expressions were complementary (Wada et al., 1992). For example, GRP-R expression is prominent in the hypothalamic regions, whereas extensive NMB-R expression is observed in olfactory and thalamic regions. BRS-3 mRNA is localized mainly in the hypothalamus and lower brainstem (Ohki-Hamazaki et al., 1997a; Yamada et al., 1999; Liu et al., 2002), while BB4 mRNA is localized only in the forebrain and midbrain (Nagalla et al., 1995). Thus, to predict the function of chBRS-3.5, the comparison of its distribution pattern with those of other BN-like peptide receptors in mammals and amphibians is useful.

The satiety effect of BN was also demonstrated in Aves. As in mammals, when BN is injected intracerebroventricularly, food intake is suppressed in the chick and turkey (Savory and Hodgkiss, 1984; Denbow, 1989). Therefore, BN-like peptide receptor(s) may be present in the avian brain regions regulating food intake, especially in the hypothalamic area.

In addition to the ISH of chBRS-3.5 and chGRP-R mRNAs, we assessed the distribution of BN/GRP-immunoreactive (ir) cells in the forebrain and compared it with the distributions of chick BN-like peptide receptor mRNA signals. We have reported that chBRS-3.5 has an affinity for BN and the candidate for the unspecified endogenous ligand could be regarded as a BN-related peptide (Iwabuchi et al., 2003). The immunohistochemistry (IHC) for detecting BN/GRP provides important information via a vis unveiling the localization of intrinsic peptide(s) to react with both receptors.

EXPERIMENTAL PROCEDURES

Animals

To obtain chicken embryos and chicks at PD1, the fertilized eggs of the White Leghorn strain (Gallus domesticus) were obtained from a local supplier (Fukuyama Shukeijo, Hiroshima, Japan), and incubated from embryonic day (ED) zero at 37.5 °C. Adult hens were supplied from Suzuho Chicken Farm (Ayase, Tokyo, Japan). Sixteen chicks and nine embryos of each sex were used in this experiment: seven chicks at PD1 for RT-PCR, each three embryos at ED5, 9 and 16 for ISH, each three chicks at PD1 for either ISH or IHC and three hens at PD150 for ISH. After hatching, chicks were placed together in an incubator under a dim lamp. The Hamburger and Hamilton (HH) stages were also indicated. This experiment was approved by the Animal Care and Use Committee and has been properly carried out under the control of the Guide lines for Animal Experimentation of Tokyo Medical and Dental University. All efforts were made to minimize the number of animals used and their suffering.

RT-PCR

Chicks at PD1 were deeply anesthetized by diethyl ether and killed by decapitation. Their brains were dissected into four parts, namely the telencephalon, optic tectum (OT), cerebellum and brainstem containing the thalamus and hypothalamus under a dissecting microscope. Total RNAs from hatched chick brain tissues were prepared using TRIzol (Invitrogen, Carlsbad, CA, USA). Single-stranded cDNA was synthesized from 3 μg of total RNA with a Superscript II Reverse
Transcription Kit (Invitrogen). The cDNA was subjected to 35 cycles of PCR (94 °C 30 s, 58 °C 2 s, 74 °C 30 s) using 2.5 U of KOD dash (TOYOBO), 1× KOD buffer, 0.2 mM dNTPs mixture and 20 μM specific primers for either chGRP-R (GRP205: CAGTGAAGACGTC-CCCAATTTGT and GRP207: GAAAGGCGATAGGTGCAC or chBRS-3.5 (BRS412: CTTTCCAGGATTAGAAACTATGGTCGAC and BRS413: CAATCTGGTTCACTGAGGC). A control reaction was performed using two primers for the chick β-actin gene (forward: GTGATGAGACCAGCAGCAAG and reverse: AGGG-TCCGGATTTACTGACTC) under the same PCR conditions as for chGRP-R and chBRS-3.5. Ten microliters of each reaction mixture was resolved by 1.5% agarose gel electrophoresis and photographed. About 700 bp PCR products for putative fragments of chBRS-3.5 or chGRP-R were dissected, purified and sequenced directly. These PCR products were certified as correct fragments of chBRS-3.5 or chGRP-R cDNA, respectively.

ISH

For ISH, chick heads or brains were freshly obtained and frozen immediately with powdered dry ice. At ED5 and 9, the entire head was sectioned coronally. In other cases, brains were dissected from their heads prior to sectioning. Twenty micrometers of frozen coronal or sagittal sections were cut on a cryostat (CM1900; Leica, Nussloch, Germany) and mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Matsunami, Osaka, Japan). An oligonucleotide (5′-GCAAGGTCT-TCAGAGGAGCGTCTGACGCTGCAGTTGCTGCTGAC-3′) as an antisense probe for chBRS-3.5 mRNA, which corresponds to nucleotide residues 448–492 of chBRS-3.5 mRNA (GenBank accession number: AJ225967), was purchased from Prologi Japan Corp (Kyoto, Japan). Additionally, an antisense probe 5′-GCAAGTTTACAGGAACTGCTTGCAGCGCTGCTGCAGTTGCTGCTGAC-3′ and 5′-GAGATCGACCTGAAGTGGCTCAGCTGAGTGC-3′, which corresponds to nucleotide residues 460–504, and two sense probes complementary to each antisense probe were also used. For the GRP-R mRNA (GenBank accession number: AY225966), following two antisense oligonucleotides were purchased: 5′-ATAGAGGAAGCCTG-GTGAACACCTCTACGCTGAGCAGGAG-3′ and 5′-GAATGCACACTGAAGTGGCTCAGCTGAGTGC-3′, which correspond to nucleotide residues 85–129 and 865–909, respectively. Two sense probes were also used as controls. They were labeled with 33P-dATP using terminal deoxynucleotidyl transferase (BRL, Bethesda, MD, USA). Fresh-frozen sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 10 min at room temperature. All procedures before hybridization were performed at room temperature. Sections were immersed in 2 mg/ml of glycine in 0.05 M phosphate-buffered saline (PBS) for 10 min and 0.25% acetic anhydride in 0.1 M triethanolamine–HCl, pH 8.0, for 10 min and prehybridization buffer for 1 h. The prehybridization buffer was comprised of 50% formamide, 50 mM Tris–HCl, pH 7.5, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% SDS, 200 μg/ml of tRNA, 1 mM EDTA, and 10% dextran sulfate. Hybridization was performed at 42 °C for 10–14 h in the prehybridization buffer supplemented with 16,000 cpm/μl of 33P-labeled oligonucleotide probes. Slides were washed twice at 55 °C in 0.1× SSC containing 0.1% sarcosyl for 30 min. Slides were exposed to Hyperfilm− β-max (Amersham, Arlington Heights, IL, USA) for 3 weeks. Photographs were taken with a digital CCD camera (DSC-F505-V, Sony, Tokyo, Japan). Control experiments using sense oligonucleotides for chBRS-3.5 and chGRP-R were performed and we confirmed that no positive reaction appeared in any developmental stages (data not shown). In addition, we checked that two antisense nucleic acids for each mRNA detected the same mRNA signals in all developmental stages (data not shown).

IHC

A rabbit anti-BN/GRP polyclonal antibody raised against BN (7113; Peninsula) was used (anti-BN/GRP antibody, 1:20; RDI, Flanders, NJ, USA). Other reagents used were a mouse monoclonal antibody to the microtubule-associated protein-2, a protein markers of neurons (1:100; MAB3418; Chemicon, Temecula, CA, USA); a mouse monoclonal antibody to the glial fibrillary acidic protein (GFAP), a protein marker of glial cells (1:100; MAB3402; Chemicon); a mouse monoclonal antibody to vimentin, a protein marker of glial cells (1:100; 61013 Clone VIM3B4; Progen Biotechnik GmbH, Heidelberg). The EnVision+Mouse-HRP and Rabbit-HRP (K4000 and K4002, respectively) were purchased from DAKO Corporation (Glostrup, Denmark). DAB Liquid System (K3465; DAKO) and Vector SG Substrate Kit (SK-4700; Vector Laboratories, Inc., Burlingame, CA, USA) were used for peroxidase staining. FITC-conjugated donkey antirabbit IgG (1:200) and Cy3-conjugated donkey antimouse IgG (1:200) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA) for fluorescence detection.

Three chicks at PD0 were treated with 4 μl of colchicine solution (10 μg/ml in distilled water; 039–03851; Wako Pure Chemical Industries, Osaka, Japan) intracerebroventricularly using a stereotactic instrument (Model 900; David Kopf Instruments, Tujunga, CA, USA) with small bird adaptor under ether anesthesia. The beak bar was lowered 8 mm ventral from the ear bar. The position of the tip of the injector was 2 mm anterior from the ear bar, 3 mm ventral from the surface of the skull and 0.5 mm lateral from the midline. The injection was executed for 1 min using a multi-syringe infusion pump (KDS 230; kdScientific Inc., New Hope, PA, USA). Forty-eight hours after injection, each three-colchicine-injected and non-injected chick at PD2 was perfused transcardially with 50 ml 0.05 M PBS following 100 ml ice-cold Zamboni’s fixative containing 4% paraformaldehyde in 0.05 M PB, pH 7.5, and 15% saturated picric acid under deep pentobarbital anesthesia. Brains were removed, postfixed by the same fixative overnight at 4 °C, and cryoprotected with 30% sucrose in 0.05 M PB for 2–3 days at 4 °C. These brains were sectioned coronally at 50-μm thickness with a cryostat (CM1900; Leica). The localization of the target proteins was determined by either immunoperoxidase histochemistry or fluorescence IHC. All incubations were carried out at 4 °C and the sections were rinsed four times, for 15 min each time, with 0.05 M PBS between each step unless otherwise indicated.

For immunoperoxidase histochemistry, free-floating sections were (1) incubated with 0.05 M PBS containing 0.6% H2O2 at room temperature for 30 min to eliminate endogenous peroxidase activity, (2) incubated with 10% normal goat serum in 0.05 M PBS containing 0.05% Triton X-100 for 60 min at room temperature to block nonspecific binding of antibodies, (3) incubated for 2 days under constant shaking with primary antibodies diluted in the same solution used for blocking nonspecific binding of antibodies as described above, (4) incubated with EnVision+ solution for 30 min at room temperature with constant shaking. Sections were rinsed four times for 15 min each time in 0.1 M Tris–HCl buffer (pH 7.5). The immunoreactivities were visualized by the Dako DAB Liquid System. The positive reaction was recognized as brown in this case. To distinguish BN/GRP-ir cells from GFAP-ir cells, double IHC of peroxidase staining was performed. The DAB-stained sections using anti-GFAP were incubated in 0.1 M glycine–HCl buffer (pH 2.2) for 90 min to dissociate the antibody-antigen complex (Tsukahara and Yamanouchi, 2002). The buffer was replaced with a fresh one every 30 min. After repeating the procedure of peroxidase staining using anti-BN/GRP instead of anti-GFAP, the BN/GRP-ir cells were visualized with a Vector SG substrate kit. In this case, the reactions using anti-BN/GRP and anti-GFAP were recognized as blue and brown, respectively. Single-stained or
RESULTS

RT-PCR of chBRS-3.5 and chGRP-R in the chick brain at PD1

At first, we examined the expressions of both chBRS-3.5 and chGRP-R mRNAs in the PD1 chick brain using RT-PCR. ChBRS-3.5 mRNAs were detected in the telencephalon abundantly, but signals were barely detected in the cerebellum and optic tectum (OT) (Fig. 1). Weak signals were found in the brainstem/thalamus/hypothalamus. Concerning chGRP-R mRNA, the signals were detected in the telencephalon, OT and brainstem/thalamus/hypothalamus, but were very faint in the cerebellum (Fig. 1). The signals of β-actin mRNA were uniformly found in all samples.

ISH of chBRS-3.5 and chGRP-R mRNAs in the chicken brain through embryonic development

At 5 days of incubation (ED5, HH26), no chBRS-3.5 mRNA signal appeared in the forebrain (Fig. 2A-1). In contrast, we could detect chGRP-R mRNA signals in the pallium of the forebrain (Fig. 2A-2, arrowheads). At ED9 (HH31), chBRS-3.5 mRNA signals were found in the ventral region of the dorsal ventricular ridge (DVR; Fig. 2B-1 and 2, arrowheads), but no signals were detected in the dorsal half of DVR at this stage. In contrast to chBRS-3.5, a chGRP-R mRNA signal was found in the subpallium (Fig. 2B-1 and 2 right, arrowheads) and also in the hypothalamic region near the ventricle (Fig. 2B-3 and 4, arrowheads). At ED16, chBRS-3.5 mRNA signals were found diffusely in the hyperpallium accessorium (HA), nidopallium (N) and nucleus basorostralis palli (Bas) of the DVR (Fig. 3A-1-4). On the other hand, chGRP-R mRNA signals were found just ventral to the medial portion of the lamina pallio-subpallialis (PSP), which is the boundary between the N and striatum medialis (MS) of the telencephalon (Fig. 3B-1 and 2, black arrowheads). In addition, the hypothalamus and OT had chGRP-R mRNA signals (Fig. 3B-3 and 4). In the anterior hypothalamic, strong mRNA signals of chGRP-R were detected in the putative region of suprachiasmatic nucleus near the ventricle (Fig. 3B-2, black arrowheads), along with weak signals dispersed more dorsally (Fig. 3B-3, white arrowheads). In the posterior hypothalamic area, there were strong signals in the dorsal hypothalamic area (Fig. 3B-4, black arrowheads). Furthermore, weak signals were found diffusely in the MST (Fig. 3B-1 and 2, white arrowheads) and thalamic area (Fig. 3B-4, white arrowheads). Fig. 3B-3 and 4 show that chGRP-R mRNAs were expressed in the deep layer of OT (arrows). The strengths of the signals were not quantitatively analyzed in this experiment, but in general, the strength of chBRS-3.5 mRNA signals seemed to increase with development, although chGRP-R mRNA seemed to be constant throughout the embryonic period.

ISH of chBRS-3.5 and chGRP-R mRNAs in the chick brain at PD1 and 140

At PD1, chBRS-3.5 mRNAs were widely found in the chick telencephalon (Fig. 4A1-8,10-12), but none of the signals were detected in the thalamus, hypothalamus, OT, midbrain or cerebellum (Fig. 4A-9). Strong signals of chBRS-

Abbreviations used in the figures

| AI | arcopallium intermedium (archistriatum intermedium) |
| AP | arcopallium posterior (archistriatum posterior) |
| APH | area parahippocampus |
| BO | bulbus olfactorius |
| Bs | brainstem including thalamus and hypothalamus |
| Cb | cerebellum |
| L | lens |
| M | mesopallium (hyperstriatum ventrale) |
| NC | nidopallium caudale (neostriatum caudale) |
| NI | nidopallium intermedium (neostriatum intermedium) |
| OTu | tuberculum olfactorium |
| Sp | subpallium |
| T | telencephalon |
| V | ventricle |

Fig. 1. Representative photograph of the region-specific expressions of two chicken BN-like peptide receptors in the chick brain at PD1 analyzed by RT-PCR. Numbers above lanes are identification numbers assigned to each individual.
3.5 mRNA in the telencephalon were detected in the HA, N, Bas, N intermedium and N caudale of the pallium. Moderate signals were detected in the hippocampus (Hp), nucleus olfactorius anterior (OA), area temporo-parieto-occipitalis (TPO), area parahippocampus, hyperpallium intercalatum (HI), nucleus striae terminalis lateralis (BSTL), organum septi lateralis (LSO) and cortex piriformis (CPi). Weak mRNA signals were found in the bulbus olfactorius, mesopallium, hyperpallium densocellulare (HD), MST, nucleus taeniae (Tn), striatum laterale (LSt), posterior arcopallium and arcopallium intermedium. No signals were found in the globus pallidus (GP), tuberculum olfactorium, entopallium (E), nucleus intrapudendularis (INP), arcopallium anterius (AA), and nucleus septalis lateralis (SL). The localizations of chBRS-3.5 mRNA signals in the brain at PD140 were consistent with those at PD1 (Fig. 5). In

**Fig. 2.** Coronal sections showing chBRS-3.5 and chGRP-R mRNA expressions in the chick forebrain at ED5 (HH26, A) and 9 (HH31, B). The numbers (white on black background) in B were arranged in rostro-caudal order. In A-1, no positive signal of chBRS-3.5 mRNA was found at ED5. Arrowheads in A-2 indicate strong signals of chGRP-R mRNA in the pallidum at ED5. In B-1 and 2, arrowheads in the left figures indicate chBRS-3.5 mRNA signals in the ventral part of DVR at ED9. Arrowheads in the right figures of B-1 and 2 indicate chGRP-R mRNA signals in Sp. Arrowheads in B-3 and 4 indicate chGRP-R mRNA signals in hypothalamus. Scale bar=1 mm.
contrast, the expression of chGRP-R at PD1 was very limited. ChGRP-R mRNA signals were found at the medial part of PSL (Fig. 4B-1, arrowheads). In addition, weak signals were found in the dorsal hypothalamic area (Fig. 4B-2, arrowheads). Unfortunately, the chGRP-R mRNA signal was too weak to be detected at PD140 brain (data not shown).

IHC of BN/GRP

Fig. 6 demonstrates a montage image showing the distribution of BN/GRP-ir cells, varicosities and fibers in the chick brain at PD2. The BN/GRP-ir cells were found mainly in the pallium of the telencephalon. Especially, a lot of BN/GRP-ir cells were found in the HA (Fig. 6-1, brown) and lateral N (Fig. 6-2, brown). E and the subpallium were almost devoid of BN/GRP-ir cells. However, BN/GRP-containing fibers were found not only in the pallium, but also in the subpallium, especially in the MST and BSTL (Fig. 6-3, brown). At high magnification, immunoreactivities were observed in the soma and dendrite-like processes of BN/GRP-ir cells in the pallium (Fig. 7-1, arrowhead). In this region, it had been confirmed that MAP-2-ir cells were abundant by immunoperoxidase histochemistry (Fig. 7-2, brown). Therefore,
we tried to stain the BN/GRP-ir cells and MAP-2-ir cells simultaneously by dual IHC in order to define the nature of BN/GRP-ir cells. As a result, all BN/GRP-ir cells showed positive immunoreactivity to MAP-2 by fluorescence IHC (Fig. 7-3, arrowhead). Moreover, the dual IHC using peroxidase reaction (Fig. 7-4) revealed that BN/GRP-ir cells (blue, arrowheads) were not stained with anti-GFAP (brown, arrow). Separate localizations of these molecules were confirmed by fluorescence IHC (Fig. 7-5 and 6, arrowheads: BN/GRP, arrows: GFAP). In a similar region to Fig. 7-1–6, the vimentin-ir cells were only found in the surface of the brain (Fig. 7-7). In the MSt (Fig. 7-8), vimentin-ir fibers (red) were adjacent to BN/GRP-ir fibers (green), but these were apparently segregated. These results strongly suggest that these BN/GRP-ir cells in the telencephalon are most likely neurons. The distribution pattern of ir-cells was not altered after the treatment with colchicine, but the number of ir-cells in the same region seemed to increase after the treatment (data not shown). Thus, the figures of IHC in this paper are made from the brain sections of colchicine-treated animals.

**Fig. 4.** Coronal and sagittal sections showing chBRS-3.5 (A) and chGRP-R (B) mRNA expressions in the chick forebrain at PD1. The numbers (white on black background) in each figure were arranged in rostro-caudal order. In figure A-1-8, chBRS-3.5 mRNA signals were found widely in the coronal sections of the telencephalon. In figure A-9, no positive signals were detected in the hypothalamus (H), optic tectum (OT) or cerebellum (Cb). In figure A10-12, chBRS-3.5 mRNA signals were found in sagittal sections of telencephalon. In contrast to chBRS-3.5, chGRP-R mRNA signals were found in a limited region. In figure B, weak signals were found at the medial portion of the PSL (arrowheads in figure B-1) and in the dorsal H (arrowheads in figure B-2). Scale bar = 5 mm.
Coincidence of localizations of BRS-3.5 mRNA and BN/GRP-ir neurons and varicosities

Localizations of chBRS-3.5 mRNA detected by ISH and BN/GRP-ir neurons, varicosities and fibers revealed by IHC in the telencephalon at PD1 are summarized in Fig. 8 and Table 1. High numbers of BN/GRP-ir varicosities were found in the region where ir cell bodies were present. The regions with many BN/GRP-ir neurons and varicosities clustering around cell bodies, such as HA and N, showed moderate to strong BRS-3.5 mRNA signals. Few or no BN/GRP-ir neurons, along with a few varicosities, were found in the regions showing weak chBRS-3.5 mRNA signals. For example, the MSt contained BN/GRP-positive fibers and varicosities and weak chBRS-3.5 mRNA signals. The E possessed no BN/GRP immunoreactivity and was devoid of a chBRS-3.5 mRNA signal. In the hypothalamic area, BN/GRP-ir neurons, varicosities and fibers were observed at PD1. ChBRS-3.5 mRNA signal was absent at all stages examined in this area, but instead, chGRP-R mRNA signals were present throughout embryonic period.

DISCUSSION

The first chBRS-3.5 mRNA signals in the chick brain were detected at ED9 in the DVR, which is derived from the caudal portion of the brainstem and spinal cord (Nagalla et al., 1995). This localization is similar to that of chBRS-3.5. From the point of view of binding affinity, chBRS-3.5 binds to BN with high affinity, a characteristic shared with BB4, rather than BRS-3, which does not possess high affinity for BN (Wu et al., 1996; Liu et al., 2002; Iwabuchi et al., 2003). Taking these facts together, chBRS-3.5 has many characteristics in common with BB4, and chBRS-3.5 and BB4 may have similar functions in vivo.

ChBRS-3.5 is abundantly expressed in the pallium, especially in the HA, Hp and N throughout the posthatch period in the chicken. According to the cortex-layered hypothesis, proposed by Karten (1969) and then supported and modified by others (see http://www.avianbrain.org/ for details), HA and HD are considered comparable to the mammalian dorsal neocortex, and N was considered to be similar to mammalian cortical layers II/III. In the rat cortex, GRP-R is expressed in layers II/III. In contrast, cortex layer IV is devoid of any BN-like peptide receptors in the rat (Wada et al., 1992). The avian E, where no expressions of chBRS-3.5 or chGRP-R have been detected, is assumed to be part of this layer. Several substantial lines of evidence support the view that avian Hp is an anatomical and functional homologue of mammalian Hp (Colombo and Broadbent, 2000). In rat hippocampal formation, the entorhinal area, presubiculum, parasubiculum, subiculum, CA1/CA3 and dentate gyrus are positive for both GRP-R and NMB-R mRNAs (Wada et al., 1992). From these results, it can be concluded that chBRS-3.5 does not simply correspond to a subtype of the mammalian BN-like peptide receptor as mentioned above, but its expression pattern in the pallium overlaps with GRP-R expression in the rat cortex and hippocampal formation. These pallial regions are also shown to be rich in BN/GRP-ir cells and varicosities (Figs. 6 and 8) as has been shown in the pigeon brain (Dubbeldam and den Boer-Visser, 2000). In the rat brain, the highest levels of GRP mRNA are observed in the isocortex and hippocampal formation (Wada et al., 1990). These facts also suggest the phylogenetically conserved function of BN and its related receptors in Aves and mammals. The dorsomedial part of the avian pallium is termed the wulst and contains HA, IHA (nucleus interstitialis hyperpallii accessorii), HI and HD. The avian wulst is likely to be homologous to the primary visual area and the primary somatosensory–somatomotor area of the superior neocor-
Therefore, BN-like peptides and their receptors may be important in the modulation of these functions. The presence of BN/GRP and receptors in Hp in both Aves and mammals suggests that BN-like peptides regulate learning and memory. In fact, BN has been reported to facilitate the learning process in mammals (Medina and Reiner, 2000).
Fig. 7. Photomicrographs showing the colocalizations of BN/GRP-immunoreactivity with specific cell-type markers. 1) A BN/GRP-ir cell (brown, arrow) in the N. 2) MAP-2-ir cells (brown) in a similar region as 1). 3) Colocalization (yellow, arrowhead) of MAP-2-ir (red) and BN/GRP-ir (green) cells in a similar region as 1). 4) Differential morphologies and localizations between GFAP-ir (brown, arrow) and BN/GRP-ir (blue, arrowheads) cells revealed by immunoperoxidase histochemistry. 5) A BN/GRP-ir cell (green, arrowhead) near the surface of the brain that is positive for GFAP immunoreactivities (red, arrow). BN/GRP-ir fibers (green) are also shown in this image. 6) GFAP immunoreactivities (red, arrow) adjacent to blood vessel (v) were not colocalized with BN/GRP immunoreactivities (green, arrowhead). BN/GRP-ir fibers (green) are also shown in this image. 7) Vimentin-immunoreactivity (brown) was not detected except the brain surface in a similar region as 1). 8) BN/GRP-ir fibers (green) were adjacent to vimentin-ir fibers (red) of ependymal cells in MS1, but these are clearly segregated. Scale bar = 70 μm.
process in rodents (Flood and Morley, 1988; Bowers et al., 1990; Williams and McGaugh, 1994).

In contrast to chBRS-3.5, chGRP-R showed more restricted expression. A positive signal was detected in the dorsal hypothalamic region at PD1. Thus, it can be concluded that the expression of GRP-R in the hypothalamus is one of the well-conserved features among mammals, birds and amphibians (Wada et al., 1992; Nagalla et al., 1995). It may be related to the feeding suppression elicited by BN or GRP injection. Indeed, the anorectic effect of BN has been reported in Aves (Savory and Hodgkiss, 1984; Denbow, 1989).

Recently, an important role of GRP-R in fear memory has been presented (Shumyatsky et al., 2002). In the mouse, GRP-R is expressed by the inhibitory neurons in the lateral nucleus of the amygdala, and upon stimulation with GRP, these neurons inhibit the activity of principal neurons and this results in decreased long-term potentiation. Thus, GRP-R is important for weakening long-term fear memory. Avian arcopallium and Tn are structures that have many features in common with the mammalian amygdala (http://www.avianbrain.org/). However, chGRP-R-positive cells were not observed in these structures in posthatch chicks. Instead, chBRS-3.5 is expressed in these regions. This suggests that a certain divergence of receptors occurred during evolution and three types of mammalian BN-like peptide receptors may have evolved in order to carefully split minutely the functions; thus, mam-

Fig. 8. Schematic diagram illustrating the expressions of both chBRS-3.5 mRNA and BN/GRP in a day-old chick brain. The regions expressing chBRS-3.5 mRNA are painted gray and the localization of BN/GRP-ir cell bodies, varicosities and fibers are indicated by filled orange dots, open orange dots and orange lines, respectively.

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malian GRP-R acquired new roles that had not been assigned to chGRP-R.

Using IHC, BN/GRP-ir neurons, varicosities and fibers were observed in the chick brain at PD1. Our results regarding the distribution of BN/GRP immunoreactivity in the telencephalon was comparable to a report using the adult collared dove (Dubbeldam and den Boer-Visser, 2000). In the present study, we identified many BN/GRP-ir neurons and varicosities in HA and N, which are regions showing strong chBRS-3.5 mRNA signals (Fig. 8). From this coincidence, we assume that the endogenous ligand(s) for chBRS-3.5 is likely BN/GRP-like peptide(s) stained by our IHC. One of the target substances of our anti-BN/GRP antibody is expected to be chicken GRP, which has already been identified (McDonald et al., 1980; Campbell et al., 1990). However, a previous report indicated that chBRS-3.5 possesses limited affinity for mammalian GRP (Iwabuchi et al., 2003). Taking these facts into consideration, we conclude that an endogenous ligand for chBRS-3.5 is similar to amphibian BN.

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