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Ontogeny of cholecystokinin-8 and glutamic acid decarboxylase in cerebral neocortex of macaque monkey

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Summary. Concentration of cholecystokinin-8 and the activity of glutamic acid decarboxylase were determined in the various cerebral cortical subdivisions of Japanese monkey (*Macaca fuscata fuscata*) at three different ages (embryonic 4 months, full-term and adult). The CCK-8 immunoreactive material extracted with 90% methanol from the cerebral cortex of the adult and foetal monkey were shown to be identical with synthetic cholecystokinin-8 by the criterion of co-elution on gel filtration chromatography (Sephadex G-50). The peptide concentration increased dramatically by about 30–80 fold (in terms of protein) and 17–28 fold (in terms of wet weight) between embryonic 4-month-old and full-term monkeys, while the level decreased 1/6–1/16 (protein) and 1/4–1/10 (wet weight) between full-term and adult monkeys. In adults, the highest levels of the peptide was observed in the association cortex, orbital prefrontal cortex and posterior parietal cortex. Glutamic acid decarboxylase activity, on the other hand, gradually increased about 4–10 fold (protein) between embryonic 4-month-old and adult animals and there was little variation in the increase rate among the cerebral subdivisions. In contrast to cholecystokinin-8, no reduction in the enzyme activity occurred between full-term and adult animals. The high level of cholecystokinin-8 in the embryonic period suggests that the peptide may participate in the regulation of the development of primate cerebral cortex.

Key words: Cholecystokinin-8 – Glutamic acid decarboxylase – Cerebral cortex – Primate – Ontogeny

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Introduction

Cholecystokinin-8 (CCK-8) immunoreactivity has been demonstrated in various vertebrate cerebral cortex by radioimmunoassay (Beinfeld et al. 1981, 1983; Dockray 1976; Eng et al. 1983; Geola et al. 1981; Rehfeld 1978; Straus and Yallow 1978) and immunohistochemical methods (Innis et al. 1979; Mcdonald et al. 1982; Straus et al. 1977; Vanderhaeghen et al. 1980). The immunohistochemical studies showed that the peptide is present in non-pyramidal neurons such as bipolar and fusiform neurons mainly in layers I–III in the cerebral cortex. The peptide showed both inhibitory (MacVicar et al. 1987) and excitatory effects (Chiodo and Bunney 1983; Dodd and Kelly 1981) on neurons of the central nervous system. The peptide is present in the synaptosome fraction and released by depolarization (Emson et al. 1980; Pinget et al. 1979), indicating that it acts as a neurotransmitter or a neuromodulator. Moreover, peptide-containing neurons have nonsynaptic interaction with blood vessels (Hendry et al. 1983), suggesting that the peptide may play a role in the maintenance of cerebral blood flow.

A recent immunohistochemical study (Hendry et al. 1984) indicates that the vast majority of CCK-8 positive neurons in monkey cerebral cortex are also glutamic acid decarboxylase (GAD) positive. Furthermore other neuropeptides, somatostatin and neuropeptide Y, are also reported to be colocalized with GAD, indicating that GABAergic neurons can be subdivided based on the presence of neuropeptides. The colocalization of neuropeptides with GAD in monkey cortical neurons raised the question of how these are expressed during development of primate neocortex.

The present study was performed to clarify the ontogenesis of CCK-8 and GAD in various cortical subdivisions of macaque monkey. A preliminary

study has been reported elsewhere (Hayashi et al. 1988).

Material and methods

Four adult Japanese monkeys (4–7 years old, 5.8–8.5 kg), four full term foetal Japanese monkeys (embryonic 5.5 months old) and four Japanese monkeys of embryonic 4 months were obtained from our Institute. Monkeys were deeply anesthetized with ketamine hydrochloride (10 mg/kg, i.m.) and pentobarbital sodium (25 mg/kg, i.p.) and sacrificed by bloodletting from the artery. The skulls were opened and dissection of cerebral cortex performed on crushed ice as described previously (Hayashi and Oshima 1986). The cerebral tissue was stored at –80° C until use. The regions which were dissected from the cerebral cortex are indicated in Fig. 1.

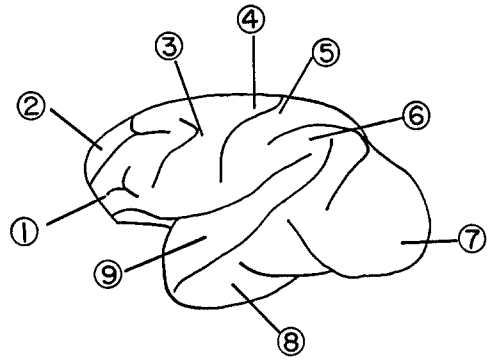

Fig. 1. Cortical subdivisions dissected for CCK-8 analysis. 1, orbital prefrontal cortex; 2, dorsolateral prefrontal cortex; 3, premotor cortex; 4, precentral gyrus; 5, postcentral gyrus; 6, posterior parietal cortex; 7, occipital cortex; 8, inferior temporal gyrus, anterior; 9, superior temporal gyrus

Antisera production

Antisera against CCK-8 were raised against synthetic sheep CCK-8 sulfated form (Peptide Institute, Inc, Osaka, Japan), conjugated bovine thyroglobulin in a molar ratio of 40 : 1 as previously described (Hayashi et al. 1983). The immunization schedule employed multiple intradermal injections of the peptide conjugates emulsified in Freund's complete adjuvant (IATRON) supplemented with tubercule bacilli (DIFCO), together with subcutaneous administration of pertussis vaccine (TAKEDA). Three rabbits were injected every 2 weeks for the fourth injection. The ability of serial dilutions of antisera to bind I^{125}-labeled human gastrin I was determined to detect antibodies.

Labeled peptide

Human gastrin I was labeled by the chloramine T method. One mCi of NaI^{125} (New England Nuclear) was added to 0.95 nmol peptide dissolved in 25 µl H_2O, followed by 50 µl of 0.25 M sodium phosphate buffer, pH 7.5 and 10 µl of chloramine T (2 mg/ml). After 15 s, 20 µl of sodium metabisulfate (5 mg/ml) was added. The mixture was then applied to a Sephadex G-10 column (1 × 10 cm) and eluted with 0.05 M imidazole buffer, pH 7.5. One milliliter fractions were collected and the fractions comprising the first peak of radioactivity were collected. The pooled fractions from Sephadex G-10 column were applied to DEAE-Sephadex A-25 (1 × 15 cm) equilibrated with 0.05 M imidazole buffer, pH 7.5. Elution was accomplished using a linear sodium chloride gradient (0–1.0 M). Fractions mono [I^{125}] iodo human gastrin I peak were pooled and stored at –20° C.

CCK-8 radioimmunoassay

The brain tissue was placed in siliconized glass tubes (5 × 40 mm) and weighed. Ten volumes of 90% methanol were added and homogenized with a Teflon pestle. A portion of homogenate was taken for protein determination and the remainder was centrifuged (10,000 rpm, 15 min) at 4° C in a microfuge (KS 15000). The supernatant was freeze-dried. Radioimmunoassay buffer was added to the freeze-dried sample. The CCK-8 radioimmunoassay was performed as follows.

300 µl of a standard CCK-8 solution and sample was added to 100 µl diluted rabbit antiserum in the assay buffer of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% (w/v) gelatin and 0.02% (w/v) sodium azide. After incubation for 1 day at 4° C, 100 µl of I^{125}-labeled human gastrin I (about 5000 cpm) was added and the incubation was continued for 1 day at 4° C. The final dilution of the antiserum was 1 : 50,000. Separation of bound tracer was performed adding 1 ml dextran-coated charcoal suspension to the tube (5 mg/ml Norit A suspended in 50 mM sodium phosphate buffer, pH 7.5, containing 0.25 mg/ml dextran T-70, supplied by Pharmacia). After 10 min, the tubes were centrifuged at 1,000 g for 15 min and the supernatants decanted from the charcoal sediments. They were counted for 2 min in an Aloka JDC-752 gamma counter with a counting efficiency of 70%. All values were corrected for the recoveries of authentic CCK-8 (200 fmol) to the tissue homogenate of monkey brain at embryonic 4 months, embryonic 5.5 months and adult. The recoveries were 81%, 85% and 89%, respectively.

GAD activity

The monkey brain tissue was homogenized with ten volumes of 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM β-mercaptoethanol, 0.2% triton X-100 and 0.5 mM pyridoxal phosphate in a glass homogenizer (5 × 40 mm). An aliquot of homogenate was taken for protein determination. GAD activity was measured by the method of Wilson et al. (1972) using 5 mM [1-^{14}C]-L-glutamic acid (New England Nuclear). $^{14}CO_2$ liberated was captured by a strip of Whatman 3MM chromatographic paper containing 20 µl of 1 M hyamin hydroxide. The paper was entered in univer gel (Nakarai) and radioactivity was counted in a liquid scintillation spectrometer (Aloka LSC-700).

Gel filtration

Freeze-dried tissue sample from embryonic 4-month-old, full-term and adult monkeys were chromatographed on the Sephadex G-50 Superfine column (0.9 × 100 cm, Pharmacia) eluted with 0.05 M phosphate buffer, pH 7.5. Fractions of 1 ml were collected at a flow rate of 10 ml/h. The void volume of the column was detected by blue dextran 2000 (Pharmacia) and the salt region was detected by $COCl_2$.

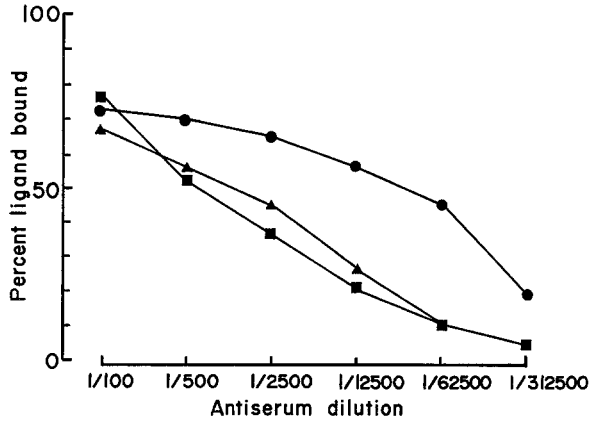

Fig. 2. Binding of ^{125}I-labeled human gastrin I by antisera. The antisera at 10 days after fourth injection of the conjugate were about 5000 cpm ^{125}I-labeled human gastrin I. ■, no. 1 antiserum; ▲, no. 2 antiserum; ●, no. 3 antiserum. Determination of bound and free peptide conducted as described under Material and methods

Protein assay

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (fraction V, Armour Pharmaceuticals, Chicago, USA) as the standard.

Results

Production and characterization of antisera

All three rabbits produced antibodies against CCK-8 conjugate after the fourth injection of the conjugate. Figure 2 shows antisera dilution curves for I^{125}-labeled human gastrin I. Anti-CCK-8 antiserum (no. 3) displaced highest titer against I^{125}-labeled human gastrin I.

When tested against various peptides (Table 1), no. 3 antiserum displaced partial reactivity with C-terminal fragment (CCK-4) although its crossreactivity diminished greatly with the N-terminal octapeptide, indicating that the antiserum is directed to the C-terminal portion of the molecule. Human gastrin I and CCK-8 (nonsulfated form) showed 50–60% inhibition. 100 ng of the following peptides had no effect on the assay: substance P, substance K, somatostatin-14, vasoactive intestinal polypeptide, bradykinin, angiotensin II, methionine enkephalin, leucine enkephalin, thyrotropin-releasing hormone and luteinising hormone-releasing hormone. The standard displacement curve shows that the sensitivity was 2 fmol CCK-8/tube and the amount of CCK-8 needed to inhibit binding of label by 50% was 40 fmol/tube (Fig. 3).

Table 1. Cross-reactivity of various peptides in CCK-8 radioimmunoassay

Compound	Cross-reactivity
CCK-8	1
CCK-8 (non-sulfated form)	0.58
Human gastrin I	0.55
CCK-4	0.01
Asp-Tyr(SO_3H)-Met-Gly	10^{-4}

The immunological cross-reactivity is expressed as the reciprocal of the concentration ratio of peptide to tracer which displaced 50% of the counts bound by the activity in the CCK-8 radioimmunoassay

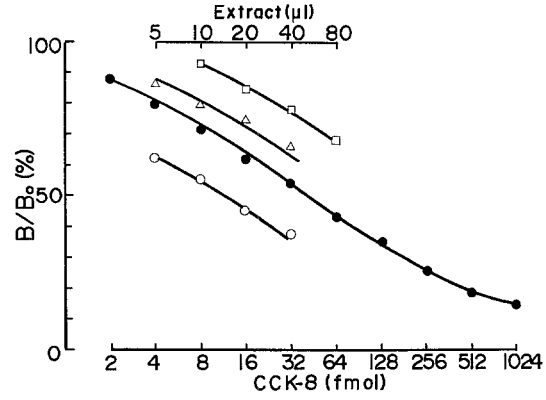

Fig. 3. Inhibition curves for immunoreactive materials in monkey brain extract in CCK-8 radioimmunoassay. ●, synthetic sheep CCK-8; △, extracts of cerebral cortex from 4-month-old embryo; ○, extracts of cerebral cortex from 5.5-month-old embryo; □, extracts of cerebral cortex from adult. The ordinate B/B_0 is the ratio of labeled peptide bound to the antibodies in the presence (B) and absence (B_0) of sample

Ontogeny of CCK-8

The ontogenic development of CCK-8 concentration in the various cortical subdivisions is shown in Fig. 4. At embryonic 4 months, the peptide was already detectable among the cortical subdivisions. The levels of the peptide were 15.3–47.1 pmol/g protein and 2.3–7.6 pmol/g tissue. The peptide concentration dramatically increased about 30–80-fold (in terms of protein) and 17–28-fold (in terms of wet weight) between embryonic 4 months and full-term animals. Within the cortex, induction of the peptide on a per-gram protein basis was remarkable in association cortex such as dorsolateral prefrontal cortex (62-fold), premotor cortex (66-fold) and posterior parietal cortex (62-fold). The induction was relatively low in the precentral gyrus (31-fold), postcentral gyrus (31-fold), occipital cortex (55-fold) and superior temporal gyrus (36-fold). Between full-term and adult, the peptide level decreased. Reduction in

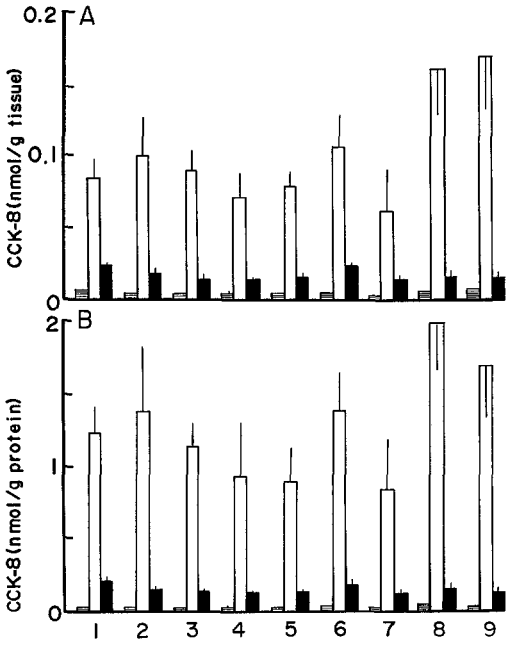

Fig. 4A, B. Specific concentrations of CCK-8 in cerebral subdivisions in 4-month-old embryo (▤), 5.5-month-old embryo (□) and adult (■). The values are expressed per g tissue (**A**) and per g protein (**B**). The number of samples was 4 at each stage. The bars indicate S.E.M. The numbers on the horizontal axis show cortical regions dissected

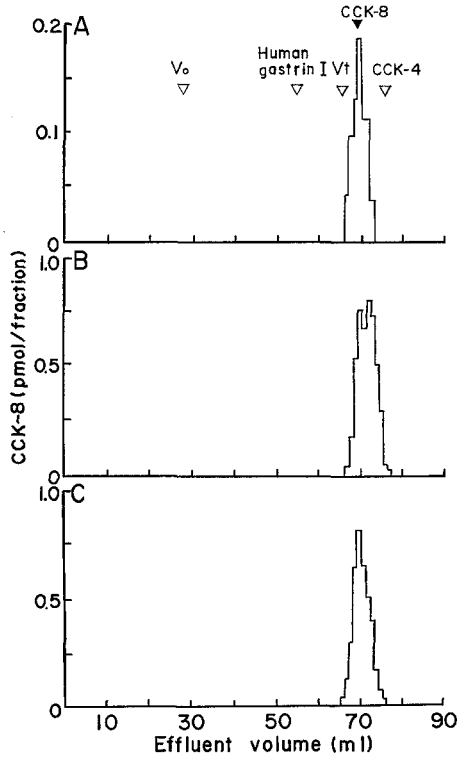

Fig. 5A–C. Sephadex G-50 superfine gel filtration chromatography of monkey extracts assayed for CCK-8. The elution positions of Vo and Vt were determined by blue dextran 2000 and $COCl_2$. **A** Extracts of cerebral cortex from 4-month-old embryo. **B** Extracts of cerebral cortex from 5.5-month-old embryo. **C** Extracts of cerebral cortex from adult. The recoveries of CCK-8 immunoreactivity from the column were 69% for **A**, 87% for **B**, and 77% for **C**

peptide level was also significant in the following association areas: dorsolateral prefrontal cortex (1/9), premotor cortex (1/8), anterior inferotemporal gyrus (1/12), and superior temporal gyrus (1/13). In other areas, the reductions were 1/6–1/8.

In adult cerebral cortex, high levels were noted in the association cortex such as orbital prefrontal cortex (212.7 ± 22.7 pmol/g protein, 24.3 ± 1.3 pmol/g tissue) and posterior parietal cortex (184.2 ± 29.3 pmol/g protein, 22.3 ± 3.8 pmol/g tissue). Relatively low levels were found in premotor cortex, precentral gyrus, postcentral gyrus and occipital cortex (126.9–138.4 pmol/g protein, 13.7–15.4 pmol/g tissue).

Characterization of peptides in monkey cerebral cortex

To characterize the peptides present in the cerebral tissue at embryonic 4 months, full-term and adult stages, the displacement curves between the cerebral extracts and the standard CCK-8 were compared. As shown in Fig. 3, the standard CCK-8 paralleled the cerebral extracts, indicating that the peptide present in the cerebral tissue at the three different stages are CCK-8-like molecules. As a criterion for identifying the immunoreactive material with synthetic CCK-8, Sephadex G-50 gel filtration chromatography was used to establish whether standard CCK-8 co-migrated with the immunoreactivities extracted from the monkey cerebral tissue. The immunoreactivity extracted from monkey cerebral cortex at three different stages chromatographed as a single peak of activity at the position of synthetic CCK-8 in Sephadex G-50 gel filtration (Fig. 5).

Ontogeny of GAD activity

The ontogenic development of GABAergic marker enzyme GAD are indicated in Fig. 6. At embryonic 4 months, GAD activity was already detectable among the various cortical subdivisions. Between embryonic 4 months and full-term, the enzyme increased 1.9-fold (orbital prefrontal cortex) to 3.6-fold (anterior inferotemporal gyrus). Between full-term and adult, the highest increase was observed in the occipital cortex (4.1-fold). In other areas, the induction of the

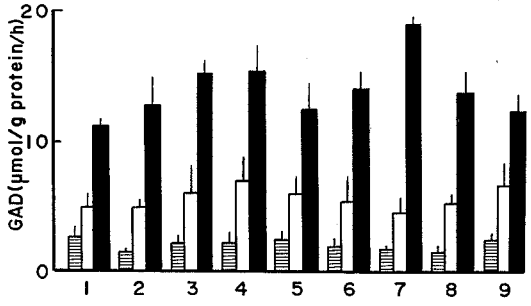

Fig. 6. Specific enzyme activity of GAD (μmol/g protein/h) in cerebral subdivisions in 4-month-old embryo (▤), 5.5-month-old embryo (□) and adult (■). The number of samples was 4 at each stage. The bars indicate S.E.M. The numbers on the horizontal axis show cortical regions dissected

enzyme was 1.9-fold (superior temporal gyrus) to 2.7-fold (anterior inferotemporal gyrus). In adult cerebral cortex, the highest enzyme level occurred in the occipital cortex (18.1 ± 1.3 μmol/g protein/h) and the lowest was found in the orbital prefrontal cortex (11.2 ± 0.4 μmol/g protein/h). In other areas, the level of activity ranged between 12.2 μmol/g protein/h and 15.5 μmol/g protein/h.

Discussion

Radioimmunoassay and characterization of the peptide

The CCK-8 antiserum which was used in the present study reacted to the C-terminal residue of CCK-8 (Table 1) as expected; the rabbits were immunized with a conjugate of CCK-8 coupled via its N-terminal amino group to thyroglobulin, leaving the C-terminal as the antigenic site. The sensitivity of the radioimmunoassay system is similar to those reported previously (Beinfeld et al. 1981; Rehfeld 1978). The concentration of the peptide in monkey cerebral tissue was comparable with that found in various mammalian species (Dockray 1976; Geola 1981; Straus and Yallow 1978). However, higher levels of the peptide have been reported in other studies (Beinfeld et al. 1981, 1983; Rehfeld 1978). The reason for this variation in levels of the peptide in mammalian cerebral tissue is not clear at present. The difference may be due to the antisera used or the radioimmunoassay system.

The present study showed that only one immunoreactivity which co-eluted with synthetic CCK-8 molecule is detected by gel filtration method regardless of the age of the tissue. This is consistent with the finding that 90% methanol extracts essentially only CCK-8 and not many other molecular forms of CCK (Eng et al. 1983; Marley and Rehfeld 1984).

Ontogeny of CCK-8 and GAD

The cortical neurons in the rhesus visual cortex are known to generate between embryonic days 40 and 100 (Rakic 1974). In the present study, CCK-8 immunoreactivity was already detectable at embryonic 4 months. This is similar to the finding reported in the cerebral cortex of precocial species such as guinea pig and chicken (Goldman et al. 1985), while in the rat, an altricial species, the peptide is known to develop mainly after birth (Beinfeld et al. 1983; Mcdonald et al. 1982; Schneider et al. 1979).

We observed remarkable increases in peptide concentration until full-term. However, between full-term and adult stages, the peptide level declined. The recovery of synthetic CCK-8 from monkey brain tissue did not change significantly with age, indicating that the low CCK-8 level at adulthood was not due to an incomplete extraction procedure. In a previous study (Hayashi and Oshima 1986), we observed the reduction of other neuropeptides such as somatostatin, vasoactive intestinal polypeptide and substance P in monkey cerebral cortex between full-term and adult stages, indicating that similar mechanisms may operate in the ontogenesis of peptidergic neurons in primate neocortex. At adult stage, the concentration of CCK-8 was high in association cortex such as orbital prefrontal cortex and posterior parietal cortex. These results are consistent with the results previously described in rhesus monkey (Beinfeld et al. 1983).

GAD activity, on the other hand, increased by degrees until adulthood. Moreover, we found that the increase rate in the GAD activity was relatively homogeneous among various cerebral subdivisions. In addition, at the adult stage, the enzyme activity was distributed uniformly among the cerebral subdivisions. These results coincide with the finding that almost the same proportion of GABAergic neurons (25% of the total neuronal population) are present in monkey cerebral subdivisions (Hendry and Kennedy 1986).

The vast majority of neuropeptide-containing neurons in the monkey cerebral neocortex have been reported to co-localize with GAD (Hendry et al. 1984). The high concentration of CCK-8 and other neuropeptides at full-term period indicates that the numbers of GABAergic neurons which express peptide phenotype may be greater than at adulthood. Mutability in the expression of neurotransmitters

during ontogeny has been demonstrated in autonomic neurons (Patterson 1978). A similar plasticity may operate in the expression of GAD and neuropeptides in the monkey cerebral cortical neurons. Although the factors which regulate the expression of neurotransmitters are not clear at present, it is becoming apparent that the neuronal activity regulates the expression of neurotransmitters and neurotransmitter-related enzymes. In rat sympathetic neurons, activation of neuronal impulses induces tyrosine hydroxylase molecules (Thoenen et al. 1979), whereas it depresses the expression of neuropeptide transmitters such as substance P and somatostatin (Black et al. 1982). A similar plasticity in the expression of transmitter-related substances has also been reported in the central nervous system. Early visual deafferentation resulted in an increase of somatostatin labeled neurons in the rat visual cortex (Jeffery and Parnavelas 1987). In the monkey visual cortex, GABA-immunoreactivity decreased after monocular deprivation (Hendry and Jones 1986), while that of the calmodulin-dependent protein kinase increased (Hendry and Kennedy 1986). The reciprocal ontogenic patterns in CCK-8 and GAD between full-term stage and adulthood in the present study may be due to the neuronal activity in the monkey cortical neurons.

Recently it has been clarified that synaptic density in macaque monkey cerebral cortex increases at a rapid rate during the last 2 months of gestation (Rakic et al. 1986). After postnatal 2–4 months, the synaptic density declined until adulthood. The similar developmental patterns of CCK-8 in the present study suggest that the peptide may play a role in the development, e.g. synaptogenesis, of the primate neocortex.

Furthermore, CCK immunoreactive neurons have been reported to have their synapses on the somata and proximal dendrites of pyramidal neurons in the adult monkey cerebral cortex (Jones et al. 1986). Our preliminary histochemical study showed that the number of CCK immunoreactive neurons in the monkey cerebral cortex is much larger at embryonic 140 days and newborn stages than at adult stage (unpublished observations). Although we do not know whether peptide is released at the synapses on the pyramidal neurons during the embryonic stages, the high concentration of CCK-8 and the larger number of CCK immunoreactive neurons suggest that peptide may participate in the development of the pyramidal neurons. Further immunohistochemical study is required to demonstrate CCK immunoreactivity at the synapses on the dendrites of pyramidal neurons in the developing monkey cerebral cortex.

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