Regional Characteristics of [125 I]Cholecystokinin Octapeptide Specific Binding to Rat Brain Membranes Following 6-Hydroxydopamine Treatment

Yasuhiro KIMURA,* Kenji KIHARA, Katsushi MIYAKE, Teruaki KITAURA, and Hiroshi FUKUCHI

Department of Pharmaceutical Services, Hiroshima University Hospital, 1–2–3, Kasumi, Minami-ku, Hiroshima 734, Japan. Received September 30, 1994; accepted November 7, 1994

The relative changes in the amount of specific [125 I]cholecystokinin octapeptide (CCK8) bound to regional brain membrane preparations after 6-hydroxydopamine (6-OHDA) treatment were examined. The specific binding in the frontal cortex and striatum decreased and reached a minimum on the 3rd day after 6-OHDA treatment. Thereafter, the specific binding recovered to 60% and 65% of control values in the frontal cortex and striatum respectively on the 28th day. On the other hand, in the nucleus accumbens, where CCK8 co-exists in the dopamine neuron, the specific binding decreased gradually, and its recovery was delayed compared with that of the frontal cortex and striatum. In the hippocampus, 6-OHDA treatment had no effect on the specific binding throughout the experimental period. The decrease of [125 I]CCK8-specific binding could be caused by enhanced release of CCK8 in the frontal cortex, striatum, and nucleus accumbens, and the recovery of specific binding could be induced by depletion of CCK8 in nerve terminals. Particularly in the nucleus accumbens, the delayed recovery of specific binding suggests the loss of the pre-synaptic binding site, which exists in the CCK8/DA co-existing neuron, together with a change in CCK8 release.

Key words cholecystokinin octapeptide (CCK8); [125 I]CCK8-specific binding; 6-hydroxydopamine (6-OHDA); dopamine (DA); CCK8/DA co-existing neuron

Cholecystokinin (CCK) is known as a brain-gut peptide which produces gall bladder contraction and stimulates pancreatic enzyme secretion.^{1,2)} Recently, a high concentration of CCK-like immunoreactivity in cortical or limbic areas was found in several vertebrates.^{3,4)} In each species studied, the major CCK entity was identified to be the sulfated carboxyl terminal octapeptide (CCK8). Several investigations have supported its role as a neurotransmitter or neuromodulator in the central nervous system including changes in CCK8-like immunoreactivity (CCK8LI) tissue levels and CCK8 release following dopaminergic manipulations.^{5,6)} In addition, CCK8 is often co-localized with dopamine (DA) in synaptic terminal regions and neuronal cell bodies of the ventral tegmental area (VTA).⁷⁾ This experimental result suggests that CCK8 may exert important physiological roles by co-existing with DA. Although its pharmacological mechanism in the brain and the functional significance of this co-existence is uncertain, it is frequently assumed that CCK8 may play a role as a regulating neurotransmitter or neuromodulator at synapses *via* interactions with pre- or post-synaptic receptors.

We have previously reported drastic changes in CCK8LI levels in rat brain after treatment with 6-hydroxydopamine (6-OHDA), a compound which has frequently been used for selective denervation of DA neuron.⁸⁾ The CCK8LI levels in several brain regions increased on the 1st day after 6-OHDA treatment. This increase was transient and the levels then decreased to become subnormal on the 7th day after treatment. The subnormal levels were maintained until the 28th day. These reports indicate a significant interaction between CCK8 and DA. This time-course profile of CCK8LI tissue levels is similar to that of the extracellular DA levels after 6-OHDA treatment previously

reported.⁹⁾ Therefore, we speculated that the CCK8LI levels would depend on the extracellular DA level, with perhaps DA acting as an inhibitor of CCK8 release from nerve terminals. This hypothesis is supported by several investigations showing that addition of exogenous DA elevates the CCK8LI levels in several brain regions¹⁰⁾ and CCK8 release is suppressed by a DA₂ agonist¹¹⁾ and enhanced by extracellular DA depletion.¹²⁾

Several investigators have also described the characteristics of CCK8 binding to brain membranes,^{13–17)} the regional distribution of CCK receptors in the rat brain,^{18,19)} and the effects of dopaminergic drugs on CCK receptors.²⁰⁾ However, there are few reports that demonstrate a change in CCK8 binding to the receptor site after denervation of the dopaminergic neuron, such as after 6-OHDA treatment. Moreover, binding studies of CCK8 to membrane preparations will be required to understand CCK8 release, since it is well known that alteration of this release affects binding capacity, perhaps by up- or down-regulation of the receptor.

In the present study, in order to clarify the mechanism of the change in CCK8LI levels following 6-OHDA treatment, we examined the relative changes in the amount of [125 I]CCK8 bound to regional membrane preparations of rat brain. According to our hypothesis, the amount of [125 I]CCK8 bound to membrane preparations will be decreased by the enhanced release of CCK8 and increased by the depletion of CCK8 in nerve terminals.

MATERIALS AND METHODS

Materials 6-Hydroxydopamine hydrobromide (6-OHDA) was obtained from Aldrich Chemical Co. (Milwaukee, U.S.A.). Pargyline hydrochloride, bacitracin

* To whom correspondence should be addressed.

and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Desmethylinipramine hydrochloride was kindly supplied by Ciba-Geigy (Tokyo, Japan). Synthetic sulfated CCK8 was from Peptide Institute Inc. (Osaka, Japan) and [125 I]CCK8 labelled with Bolton Hunter reagent was obtained from Amersham International (London, England). All the other reagents were of analytical grade and used without further purification.

Animal Treatment Male Wistar rats, weighing 260–280 g, were housed in a temperature controlled room with a standard dark–light schedule (12/12 h). Rats were anesthetized with 15 mg/kg pentobarbital sodium. A solution of 6-OHDA (250 μ g as the free base in 10 μ l saline containing 0.1% ascorbic acid) or vehicle (10 μ l saline containing 0.1% ascorbic acid) was injected over 5 min with a Hamilton microsyringe into the lateral cerebral ventricle using stereotactic techniques. The needle was removed carefully 10 min after the end of injection. The coordinates with the bregma as the reference were P 0.8 mm, L 1.5 mm, and V 3.6 mm, according to the atlas of Paxinos and Watson.²¹ Pargyline hydrochloride (50 mg/kg) and desmethylinipramine hydrochloride (25 mg/kg) were administered intraperitoneally to all the rats 60 min before intracerebroventricular injections of 6-OHDA or its vehicle. Pargyline inhibits monoamine oxidase and, thereby, enhances the toxicity of 6-OHDA while desmethylinipramine protects the central noradrenergic neurons against the effects of the toxin.^{22,23}

Preparation of Regional Brain Membranes Saline- or 6-OHDA-treated rats were decapitated on the 1st, 3rd, 7th, 14th and 28th day after treatment between 10:00 and 11:00 a.m., the brains were immediately removed and chilled in saline (4 °C) before regional dissection. The various regions of the brain (frontal cortex, striatum, hippocampus, and nucleus accumbens) were dissected according to the method of Glowinski and Iversen.²⁴ Membranes were prepared as previously described.¹⁷ Briefly, tissues were homogenized in 40 vol. ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 5 mM MgCl₂. The homogenate was centrifuged at 50000 $\times g$ for 20 min at 4 °C. The pellet was resuspended in the same volume of ice-cold 50 mM Tris–HCl buffer (pH 7.4), containing 5 mM MgCl₂, and recentrifuged twice for 20 min periods at 50000 $\times g$. The final membrane pellets were frozen at –80 °C until used in the binding assay. No difference in binding parameters was found between tissues used immediately and tissues stored for 1 month at this temperature.

Binding Assay The [125 I]CCK8 receptor-binding study was performed according to the method of Takeda *et al.* with only minor modification.¹⁷ Briefly, the membrane preparations from various regions of the brain were resuspended in 80 vol. incubation medium [50 mM Tris–HCl buffer (pH 7.4) containing 5 mM MgCl₂, 1 mM ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.02% bacitracin, and 0.2% bovine serum albumin] in small glass test-tubes. For the binding assay, 25 μ l assay buffer (for total binding) or 25 μ l of 1 μ M unlabelled CCK8 in assay buffer (for non-specific binding) and 25 μ l [125 I]CCK8 (2000 Ci/mmol) to give a final

concentration of 0.1–0.2 nM were added to 450 μ l membrane suspension after preincubation for 15 min at 25 °C. Incubation was carried out in a shaking water-bath at 25 °C for times ranging from 0 to 120 min. The incubation was terminated by adding 2 ml ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 5 mM MgCl₂ and 0.2% bovine serum albumin and then the mixture was filtrated rapidly through a Whatman glass filter (GF/F). The filter was washed three times with 3 ml of the same ice-cold buffer and the radioactivity on the filter was counted in an automatic gamma counter (Aloka, ARC-600). Specific binding was calculated as the difference between the radioactivities bound in the presence and absence of 1 μ M unlabelled CCK8. The blank was obtained by incubation with membrane-free assay buffer and radioligand. The radioactivities of total and non-specific binding were corrected for the blank radioactivity.

Protein Assay Protein was measured by the method of Lowry *et al.*²⁵

Statistical Analysis Student's *t*-test was carried out on the difference between the specific binding of vehicle- and 6-OHDA-treated rat brain membrane preparations. A significant difference was defined as $p < 0.05$.

RESULTS

The effect of the frontal cortex membrane protein on radioligand binding at 25 °C is shown in Fig. 1. Both total and non-specific binding were linear functions of membrane protein up to 2 mg/assay tube, whereas at a higher concentration (2.4 mg/assay tube), there was a significant decline in the amount of radioligand bound. The time course of [125 I]CCK8 binding to the cerebral cortical membrane was rapid at 25 °C, and maximum binding was reached in 60 min (Fig. 2). At a lower temperature (4 °C), the [125 I]CCK8 binding to membrane was markedly inhibited and at a higher temperature (37 °C), the binding rate was less than at 25 °C (data not shown). Therefore, the following incubations were carried out at membrane protein concentration of <2 mg/assay tube (4 mg/ml), at 25 °C, and for 60 min.

Fig. 1. Effect of Membrane Protein Concentration on [125 I]CCK8 Binding to Normal Rat Cortical Membranes

Increasing amounts of membrane protein (0.4–2.4 mg/assay tube) were incubated with radioligand in the absence (total binding, ■) and presence (non-specific binding, ●) of 1 μ M CCK8 at 25 °C for 60 min. Data are expressed as the percentage radioligand bound. Each point is the average of triplicate determinations in two separate experiments.

Fig. 2. Time-Course Profile of [125 I]CCK8 Binding to Normal Rat Cortical Membranes

A membrane preparation of rat frontal cortex was incubated with radioligand in the absence (total binding, ■) and presence (non-specific binding, ●) of $1 \mu\text{M}$ CCK8 at 25°C . At indicated times, the incubation medium was treated as described in the text. Each point shows the mean of triplicate determinations in two separate experiments.

Fig. 3. Total, Specific, and Non-specific Binding of [125 I] CCK8 to Regional Brain Membrane Preparations from Normal Rat

Data are expressed as counts of bound [125 I]CCK8 per mg membrane protein and each bar shows the mean $\pm$ S.E.M. of 6 rats. ■, total binding; ▨, specific binding; □, non-specific binding.

The [125 I]CCK8 binding to regional brain membranes is shown in Fig. 3. There was no significant difference in non-specific binding among the regional membrane preparations examined. The specific binding was 69%, 65%, and 65% of the total binding in the frontal cortex, striatum, and nucleus accumbens, respectively. However, the specific binding in the hippocampus was only 24% of the total binding.

The changes in [125 I]CCK8 binding to the rat cerebral cortex membranes at various intervals after 6-OHDA treatment are shown in Fig. 4. The total binding changed markedly, whereas no significant change was observed in the non-specific binding throughout the experimental period.

Figure 5 shows the changes in the specific binding of the regional brain membranes. Vehicle treatment had no effect on the specific binding throughout the experimental period in any of brain regions examined. In the 6-OHDA-treated rats, the specific binding changed markedly in the frontal cortex, striatum, and nucleus accumbens but not in the hippocampus. In the frontal cortex (Fig. 5A) and striatum (Fig. 5B), the specific binding fell respectively to 21% and 39% that of the vehicle treated rats on the 3rd day

Fig. 4. Effects of 6-OHDA Intracerebroventricular Administration on Total, Specific, and Non-specific Binding of [125 I]CCK8 to Rat Frontal Cortex Membrane Preparation at Various Intervals Following 6-OHDA Treatment

Rats were decapitated at several intervals after 6-OHDA treatment and the binding assay was carried out as described in the text. Data are expressed as counts of bound [125 I]CCK8 per mg membrane protein and each bar shows the mean $\pm$ S.E.M. of 6 rats. ■, total binding; ▨, specific binding; □, non-specific binding.

after treatment. Thereafter, the specific binding recovered to 60% and 65% in the frontal cortex and striatum respectively, on the 28th day after treatment. In the hippocampus (Fig. 5C), no significant change in the specific binding was observed throughout the experimental period. On the other hand, in the nucleus accumbens (Fig. 5D), the specific binding gradually decreased and delayed recovery of specific binding was observed compared with that of the frontal cortex and striatum. The minimum value (47% of the controls) of specific binding was found on the 7th day after treatment. The specific binding in the nucleus accumbens recovered to 70% that of the vehicle-treated rats on the 28th day, whereas recovery of the specific binding was seen on the 7th day in the frontal cortex and striatum.

DISCUSSION

In the present study, the relative changes in the amount of [125 I]CCK8 specific binding to rat regional brain membranes after 6-OHDA treatment were studied. The binding assay in the present study was used under the following conditions: the amount of membrane protein was less than 2 mg/assay tube, and incubations was carried out at 25°C and for 60 min. At a higher temperature (37°C), the binding rate was less than at 25°C , suggesting a deleterious effect of the higher temperature on radioligand and/or membrane-binding sites. The specific binding for CCK8 in the rat brain membranes satisfied some criteria used to define interactions between a peptide hormone and receptor. Radioligand binding to the cortical membrane was rapid at 25°C , it was also temperature- and protein concentration-dependent, and saturable. These findings are in good agreement with previously reported data.^{13,15,16)}

In normal rats, regional differences in specific binding were observed, and the non-specific binding was similar in all the brain regions examined. The specific binding in the frontal cortex, striatum, and nucleus accumbens was 69%, 65%, and 65% of the total binding, respectively,

Fig. 5. Changes in Specific Binding of [125 I]CCK8 Binding to the Regional Brain Membranes from the Frontal Cortex (A), Striatum (B), Hippocampus (C), and Nucleus Accumbens (D) at Various Intervals Following 6-OHDA Treatment

Binding assay was carried out on the 1st, 3rd, 7th, 14th, and 28th day after 6-OHDA treatment. Data are expressed as counts of bound [125 I]CCK8 per mg membrane protein and each point shows the mean $\pm$ S.E.M. of 6 rats. ■, saline-treated; ●, 6-OHDA-treated. a) $p < 0.001$, b) $p < 0.01$, c) $p < 0.05$, significantly different compared with the saline-treated group at each interval (Student's t -test).

whereas it was only 24% of the total binding in the hippocampus. Saito *et al.* have investigated the distribution of CCK8 receptors in the rat brain,¹⁸⁾ and their findings are in good agreement with our results in the present study showing that the specific binding in the frontal cortex, striatum, and nucleus accumbens was relatively high, while in the hippocampus it was relatively low. In our previous reports, CCK8LI tissue levels in the hippocampus were unaffected by intracerebroventricular injection of 6-OHDA⁸⁾ and intraperitoneal L-DOPA administration.¹⁰⁾ It has also been reported that neuromorphological differences in CCK immunoreactive neurons exist in several structures, such as long ascending projections arising in the substantia nigra and ventral tegmental area, and innervating the striatal, limbic and cortical structures. On the other hand, local circuit neurons have been identified in the hippocampus.^{26,27)} These neuromorphological differences may closely relate to the regional differences in the specific binding of CCK8. 6-OHDA treatment affected total but not non-specific binding, suggesting that the changes in total binding are attributable to changes in the specific binding. These changes were observed in the frontal cortex, striatum and nucleus accumbens where CCK8 neurons may be closely associated with DA neurons. On the other hand, no significant change in the specific binding was observed in the hippocampus where CCK8 neurons were identified as local circuit neurons. It is suggested that these regional differences in the time-course profile of the specific binding may be related to the neuromorphological differences in each

region.

Fukumauchi *et al.* have demonstrated the effect of the administration of a D_2 agonist and antagonist on the binding of CCK8 to rat cortical membranes.²⁰⁾ Scatchard analysis indicated that dopaminergic drugs affected the CCK8 binding capacity but not binding affinity. Moreover, several investigators have also reported that the changes in CCK8 release induced by pharmacological manipulation of the dopaminergic system affected only the binding capacity of CCK8.¹⁴⁾ Thus, the relative changes in the specific binding observed in the present study are due to the change in the CCK8 binding capacity of the brain membranes. Furthermore, two CCK receptors have been identified. The CCK_A receptor is found in the periphery²⁸⁾ and in several discrete brain regions,²⁹⁾ while the CCK_B receptor has a widespread distribution in the central nervous system.^{13,30)} It has also been reported that the CCK receptor has a single binding site in the pancreatic acini and various brain regions.¹⁷⁾ These reports indicate that almost all of the CCK receptors in the central nervous systems can be considered as CCK_B receptors. Therefore, we speculate that the change in the specific binding in this study would correspond to a change in CCK_B receptor.

We have previously reported regional changes in CCK8LI tissue levels after 6-OHDA treatment as stated in our introduction. Zigmond *et al.* have also reported that 6-OHDA treatment induces compensatory responses in the striatum, the first of which is a transient increase in DA release. This DA release is enhanced and the striatal DA content increases for 24 h after 6-OHDA treatment

but the striatal DA content drops sharply thereafter to subnormal levels. It ultimately reaches low levels 2 to 3 d after treatment.⁹⁾ Therefore, we speculate that the elevations in CCK8LI tissue levels may be due to the inhibition of CCK8 release induced by elevated extracellular DA on the 1st day after 6-OHDA treatment, and the subsequent decrease in CCK8LI tissue levels may be due to the enhanced release of CCK8 due to depletion of extracellular DA. In the present study, the decrease in CCK8 specific binding in the frontal cortex and striatum after 6-OHDA treatment can be reasonably explained by increased release of CCK8. Additionally, the subsequent recovery of the CCK8 specific binding would be attributable to the depletion of available CCK8 in nerve terminals. On the other hand, the changes in the CCK8LI tissue levels in the nucleus accumbens showed a similar time-course profile to the frontal cortex and striatum in a previous report, while the time-course profile of the specific binding in the nucleus accumbens was quite different from that in the frontal cortex and striatum. The co-existence of CCK8 and DA has been found in the mesolimbic structure, and these neurons have been reported to be denervated by treatment with 6-OHDA, in a manner similar to DA intrinsic neurons.⁷⁾ The delayed recovery of the specific binding observed in the nucleus accumbens suggests the loss of binding sites in the pre-synaptic region together with a change in binding capacity. Consequently, the changes in CCK8LI tissue levels in the frontal cortex and striatum would be caused by changes in CCK8 release from nerve terminals, while in the nucleus accumbens the change in CCK8LI tissue levels would be caused by loss of CCK8LI in the CCK/DA co-existing neurons together with the change in CCK8 release.

In our previous report,⁸⁾ we suggested that the changes in the CCK8LI tissue levels would be attributable to changes in CCK8 release, depending on the extracellular DA level. Although the CCK8 tissue levels increased transiently in some regions on the 1st day after 6-OHDA treatment, the specific binding showed a decrease rather than an increase. The specific binding showed a minimum value in the frontal cortex and striatum on the 3rd day, while the recoveries reached only 65% and 60% of controls in the frontal cortex and striatum, respectively, even on the 28th day after 6-OHDA treatment. These observations indicate that the decrease in the specific binding seems to have occurred more rapidly than the recovery in the specific binding.

In conclusion, 6-OHDA treatment affected the specific binding of CCK8 in the area where CCK8 neurons may be closely associated with DA neurons. The decrease and

recovery of the specific binding correspond to the enhancement of CCK8 release and the depletion of CCK8 in nerve terminals, respectively. Additionally, there is a great difference in the time-course profile of the specific binding among the brain regions examined, depending on their neuromorphological structures.

REFERENCES

- 1) A. C. Ivy, E. Oldberg, *Am. J. Physiol.*, **85**, 381 (1928).
- 2) V. Mutt, J. E. Jorpes, *Eur. J. Biochem.*, **6**, 156 (1968).
- 3) G. J. Dockray, *Nature* (London), **264**, 568 (1976).
- 4) J. E. Muller, E. Straus, R. Yalow, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 3035 (1977).
- 5) P. Fray, *Eur. J. Pharmacol.*, **95**, 87 (1983).
- 6) J. B. Hutchinson, J. Strupish, S. R. Nahorski, *Brain Res.*, **370**, 310 (1986).
- 7) T. Hökfelt, J. F. Rehfeld, L. Skirboll, B. Ivemark, M. Goldstein, K. Markey, *Nature* (London), **285**, 476 (1980).
- 8) Y. Kimura, K. Miyake, T. Kitaura, K. Kihira, H. Fukuchi, *Biol. Pharm. Bull.*, **17**, 1210 (1994).
- 9) M. Z. Zigmond, T. W. Berger, A. A. Grace, E. M. Stricker, *Mol. Chem. Neuropathol.*, **10**, 185 (1989).
- 10) Y. Kimura, K. Kihira, K. Miyake, T. Kitaura, H. Fukuchi, *Biol. Pharm. Bull.*, **18**, 13 (1995).
- 11) U. Conzelmann, A. Holland, D. Meyer, *Eur. J. Pharmacol.*, **101**, 119 (1984).
- 12) S. P. Butcher, A. Varro, J. S. Kelly, G. J. Dockray, *Brain Res.*, **505**, 119 (1989).
- 13) A. Saito, I. D. Goldfine, J. A. Williams, *J. Neurochem.*, **37**, 483 (1981).
- 14) R. S. L. Chang, V. J. Lotti, G. E. Martin, T. B. Chen, *Life Sci.*, **32**, 871 (1983).
- 15) M. Praissman, P. A. Martinez, C. F. Saladio, J. M. Berkowitz, A. W. Steggle, J. A. Finkelstein, *J. Neurochem.*, **40**, 1406 (1983).
- 16) C. W. Lin, T. Miller, *J. Pharmacol. Exp. Ther.*, **232**, 775 (1985).
- 17) Y. Takeda, M. Hoshino, N. Yanaihara, C. Yanaihara, J. Isobe, N. Sugiura, K. Kashimoto, Y. Takano, H. Kamiya, *Jpn. J. Pharmacol.*, **49**, 471 (1989).
- 18) A. Saito, H. Sankaran, I. D. Goldfine, J. A. Williams, *Science*, **208**, 1155 (1980).
- 19) R. Sekiguchi, T. Moroji, *Brain Res.*, **399**, 271 (1986).
- 20) F. Fukumauchi, T. Yoshikawa, S. Kaneno, H. Shibuya, R. Takahashi, *Neuropeptides*, **10**, 221 (1987).
- 21) G. Paxinos, C. Watson, "The Rat Brain in Stereotaxic Coordinates," 2nd ed., Academic Press, New York, 1986.
- 22) G. R. Breeze, T. D. Traylor, *Br. J. Pharmacol.*, **42**, 88 (1971).
- 23) G. Jonson, *Ann. Rev. Neurosci.*, **3**, 169 (1980).
- 24) J. Glowinski, L. L. Iversen, *J. Neurochem.*, **13**, 655 (1966).
- 25) O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biochem.*, **193**, 265 (1951).
- 26) J. H. Fallon, K. M. Serroogy, *Ann. N. Y. Acad. Sci.*, **448**, 121 (1985).
- 27) D. K. Meyer, Z. Protopapas, *Ann. N. Y. Acad. Sci.*, **448**, 133 (1985).
- 28) H. Sankaran, I. D. Goldfine, C. W. Deveney, K. Y. Wong, J. A. Williams, *J. Biol. Chem.*, **254**, 9349 (1980).
- 29) T. H. Moran, P. H. Robinson, M. S. Goldrich, P. R. McHugh, *Brain Res.*, **362**, 175 (1986).
- 30) P. Gaudreau, S. St.-Pierre, C. Pert, R. Quirion, *Ann. N. Y. Acad. Sci.*, **448**, 198 (1985).