Title	発達に伴い脳内に一過性に発現するニューロテンシン（NT），ニューロメジンN （NN） の意義について
Author(s)	佐藤, 真
Citation	大阪大学, 1991, 博士論文
Version Type	VoR
URL	https://doi.org/10.11501/2964378
rights	
Note	

Developmental Brain Research, 54 (1990) 249–255
Elsevier

BRESD 51106

Different ontogenetic profiles of cells expressing prepro-neurotensin/neuromedin N mRNA in the rat posterior cingulate cortex and the hippocampal formation

Makoto Sato[1], Yasuhide Lee[1], Jian Hua Zhang[1], Sadao Shiosaka[2], Koichi Noguchi[1], Yasuhiro Morita[3] and Masaya Tohyama[1]

[1]*Department of Anatomy and* [2]*Department of Neuroanatomy Biomedical Research Center School of Medicine, Osaka University, Osaka (Japan) and* [3]*Department of Anatomy, School of Medicine, Kagoshima University, Kagoshima (Japan)*

(Accepted 6 March 1990)

Key words. Neurotensin, Neuromedin N, Hippocampal formation; Posterior cingulate cortex, Hybridization histochemistry, in situ, Ontogeny

The ontogeny of the expression of prepro-neurotensin/neuromedin N messenger RNA (prepro-NT/NN mRNA) in the rat posterior cingulate cortex (retrosplenial cortex) and the hippocampal formation was investigated using in situ hybridization histochemistry In the primordium of the posterior cingulate cortex and the hippocampal formation, prepro-NT/NN mRNA was first expressed on embryonic day 17, and was found in the subiculum, layers II–III in areas 29a and 29b, and layer VI in the posterior cingulate cortex at birth Expression was also observed in the CA1 field In the adult rat, the expression of prepro-NT/NN mRNA was reduced in the posterior cingulate cortex, and only a few positive cells were seen here. However, the CA1 field and the subiculum still contained numerous positive cells

INTRODUCTION

Neurotensin (NT) is a hypotensive tridecapeptide[7], that is distributed unevenly in the central nervous system[8,17,19,20,22,25,27,34–38]. This peptide has many biological activities affecting hemodynamics[7,33], pain sensation[4,12,21], smooth muscles[7], the neuroendocrine system[14,16,17,26,39,40], thermoregulation[5,9,21,29], and gastric secretion[32]. In addition, NT mediates the release of other neurotransmitters, such as dopamine, through a presynaptic mechanism[31].

In the brain of the adult rat, NT-containing cells have been detected by immunohistochemistry in many regions, such as the paraventricularis or periaqueductal grey[17,20,35,38], but they were rarely present in the hippocampal formation or the posterior cingulate cortex[20,25,37]. On the other hand, we found, in very young rats, a large number of NT-like immunoreactive (NT-LI) cells in the hippocampal formation and the posterior cingulate cortex[18]. Recently the complementary DNA (cDNA) for the NT/neuromedin N[30] precursor (pro-NT/NN) was cloned[13,23] and nucleotide sequence analysis allowed us to study the expression of prepro-NT mRNAs. In this study, we investigated ontogenetic changes of prepro-NT/NN mRNA expression in the posterior cingulate cortex and the hippocampal formation by in situ hybridization histochemistry.

MATERIALS AND METHODS

Preparation of the oligonucleotide probe

The 39-mer oligonucleotide probe was synthesized with an Applied Biosystems 381A DNA synthesizer (Foster City, CA), and purified by HPLC (Hitachi, Japan) It was labeled with [α-^{35}S]dATP (for in situ hybridization) and [α-^{32}P]dCTP (for Northern blot analysis) by a 3′ end-labeling method, giving a specific activity of 1 0–1 5 × 10^9 dpm/μg (16–25 MBq/μg); thus, by computation, about 6 nucleotides were incorporated onto the 3′ end This probe was complementary to the mRNA sequence coding for NT of the rat NT/NN precursor gene (nucleotides 701–739)[23]

Animals

Wistar rats were used 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 the lights on from 08.00 to 20 00 h After mating, the first day of the presence of sperm in the vaginal smear was designated as embryonic day (E) 0, and the day of birth was postnatal day (P) 0 The number of rats used at each stage was as follows: 2(E16), 3(E17), 6(P0), 6(P2), 6(P5), 9(P7), 6(P10), 6(P14), 3(P21), and 6(adult) Rats were anesthetized with sodium pentobarbital (Nembutal, 60 mg/kg, i.p.) and then decapitated In the case of embryos, mother rats were anesthetized with sodium pentobarbital (30 mg/kg, i p), embryos were removed by caesarean section, anesthetized with sodium pentobarbital, and decapitated The brains were removed, frozen in dry-ice, and stored at −80 °C

Correspondence M Sato, Department of Anatomy II, Osaka University Medical School, 4-3-57 Nakanoshima, Kita-ku, Osaka 530, Japan

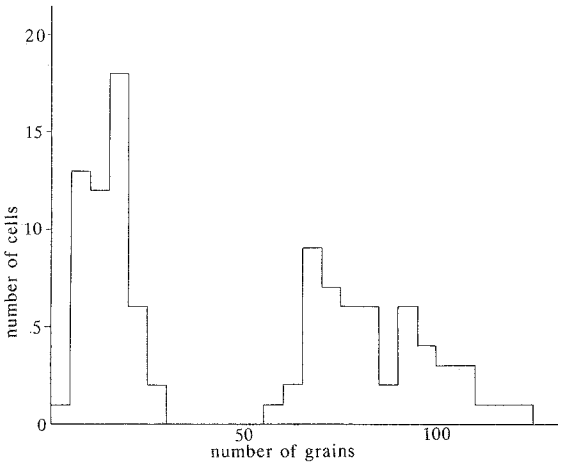


Fig. 1. Histogram showing the frequency distribution in the hippocampal formation and the posterior cingulate cortex neurons of the number of grains per cell in 7-day-old rats

Procedure for Northern blot analysis

Total cellular RNA was extracted from the rat brain by ultracentrifugation with guanidium isothiocyanate/caesium chloride[28] Poly(A)$^+$ RNA was obtained using oligotex-dT 30 (Japan Roche), denatured and separated by electrophoresis on agarose–formaldehyde gels The RNA was transferred to a nylon membrane and hybridized with ^{32}P-labeled probes Filters were washed in 1 × SSC (0 15 M NaCl, 0 015 M sodium citrate) and 1% SDS at 60 °C for 1 h before being processed for autoradiography

Procedure for in situ hybridization

Sections (16-μm) were cut on a cryostat and thaw-mounted on subbed poly-L-lysine-coated glass slides, and then stored at −80 °C until being processed for in situ hybridization The sections were fixed in 4% paraformaldehyde for 30 min After being rinsed twice in 0 1 M phosphate-buffered saline for 5 min each time, sections were dehydrated in alcohol, treated in chloroform and dehydrated again Brain sections were hybridized with the oligonucleotide probe (5 × 10^5 dpm or 8 3 kBq per slide) in hybridization solution at 40 °C for 12 h, and then washed 4 times for 15 min each in 1 × SSC (0 15 M NaCl, 0 015 M sodium citrate) at 55 °C The hybridization solution consisted of 50% deionized formamide, 4 × SSC, 1 × Denhart's solution (0 02% bovine serum albumin, 0.02% polyvinyl pyrollidone, 0 02% Ficoll), 0.12 M sodium phosphate (pH

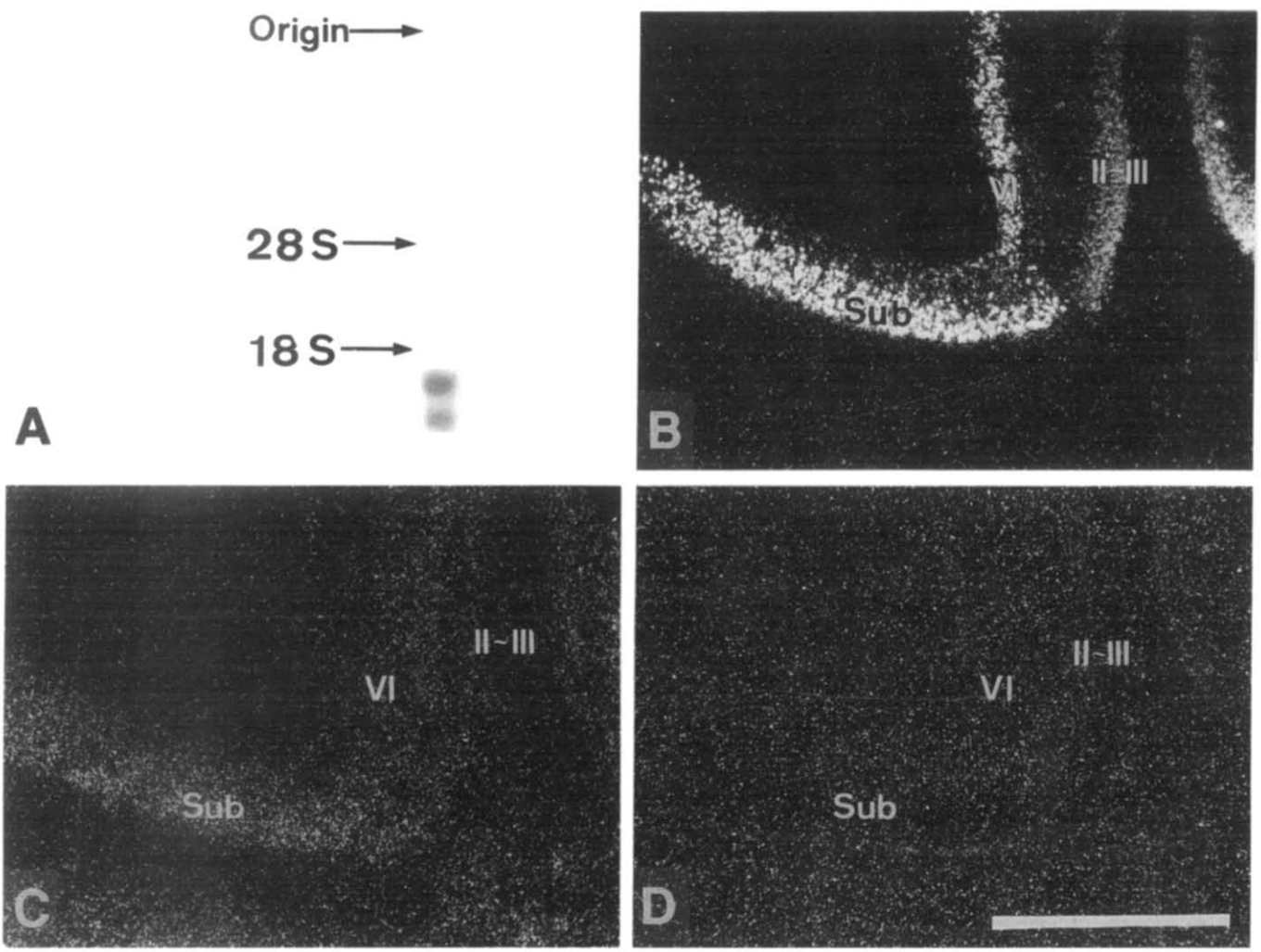


Fig. 2 Northern blot hybridization of the NT probe to poly (A)$^+$ RNA extracted from the brain of 7-day-old rat (A) Photomicrographs showing the serially sectioned tissues (B–D) Prepro-NT/NN mRNAs in the subiculum (Sub), layers II–III (II–III) in areas 29a and 29b, and the layer VI (VI) in the posterior cingulate cortex (B) Sections pretreated with RNase (C) and hybridized with an excess amount of non-labeled NT probe (D) Bar = 1 mm

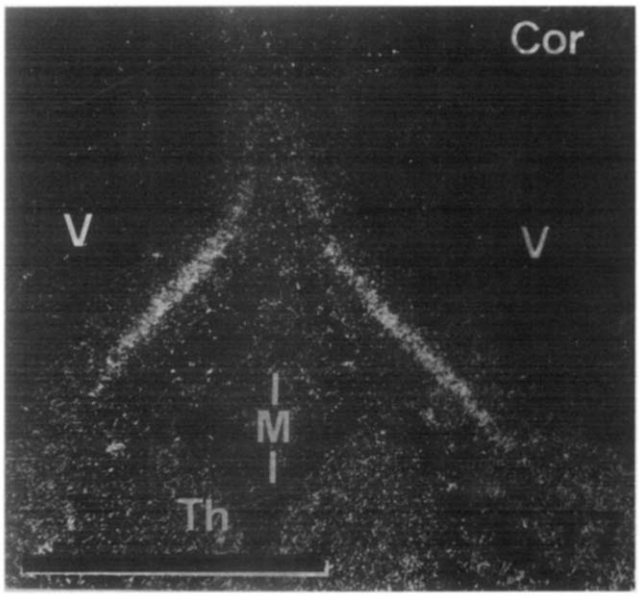


Fig 3. Photomicrographs showing the appearance of prepro-NT/NN mRNAs in the primordium of the rat hippocampal formation on embryonic day 17 Th, thalamus, Cor, cortex, V, ventricle, M, midline Bar = 1 mm

7 2), 0.025% tRNA, 50 mM dithiothreitol and 10% dextran sulfate For autoradiography sections were dipped in K-5 emulsion (Ilford), stored in black boxes and exposed at 4 °C for 20 days. They were developed with K-19 (Kodak) and fixed with Fuji-fix (Fuji-film), taking 5 min for each process After washing in running water, the sections were stained with thionine (Merck, F.R G), dehydrated, and coverslipped for observation Generally, an aggregate of silver grains observed on each cell corresponds to the hybridization signals

Counting of cell numbers and silver grains

This analysis was carried out on 100 cells in the cortex or the hippocampal formation which were selected from 7-day-old rats by random sampling. The number of silver grains on each cell was counted using high magnification with a light microscope, and only cells having nuclei were counted. The number of grains on each cell was then used to construct a histogram (Fig 1). The frequency distribution of the cells showed two populations. In the present study, cells belonging to the group with a large number of grains was taken as positive The average number of grains on the cells belonging to the group with few grains was taken as the background level (noise) This process was repeated for the rats at other ages In the subiculum and layer VI of the posterior cingulate cortex, each cell was clearly able to be distinguished, and the number of labeled cells and number of grains on each cell were counted Positive cells were counted in all sections selected from every 6 brain slices 16-μm in thickness We calculated 'number of positive grains' of each cell by subtracting the noise value from the actual grain number (Table

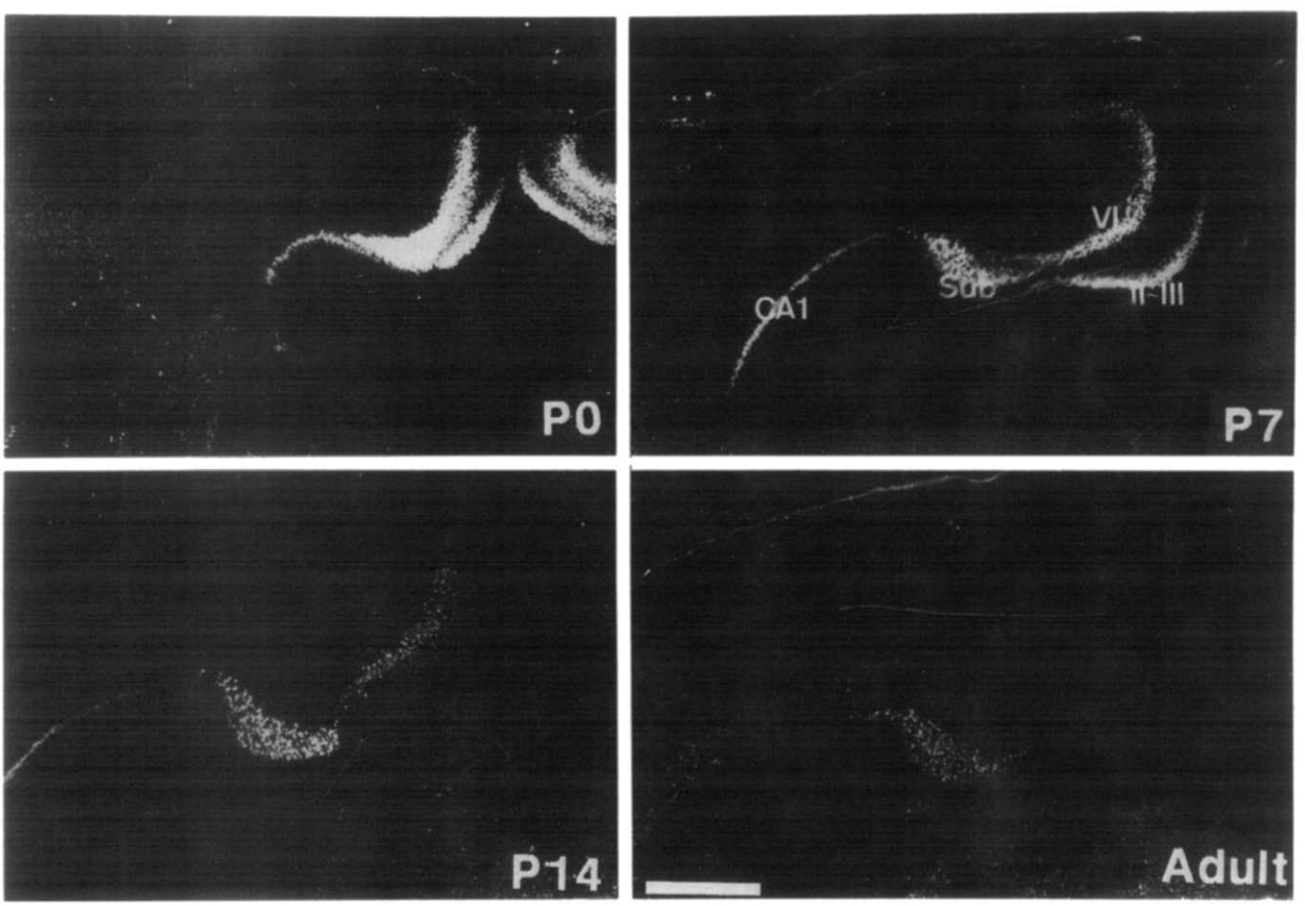


Fig. 4. Photomicrographs showing ontogenetic changes in the distribution of prepro-NT/NN mRNAs prepro-NT/NN mRNAs are distributed in the subiculum (Sub), CA1 field (CA1), layers II–III of areas 29a and 29b (II–III), and layer VI (VI) of the posterior cingulate cortex on postnatal 0 day (P0) and 7 day (P7) Note the disappearance of signals in layers II–III on postnatal day 14, and in layer VI in the adult Bar = 1 mm

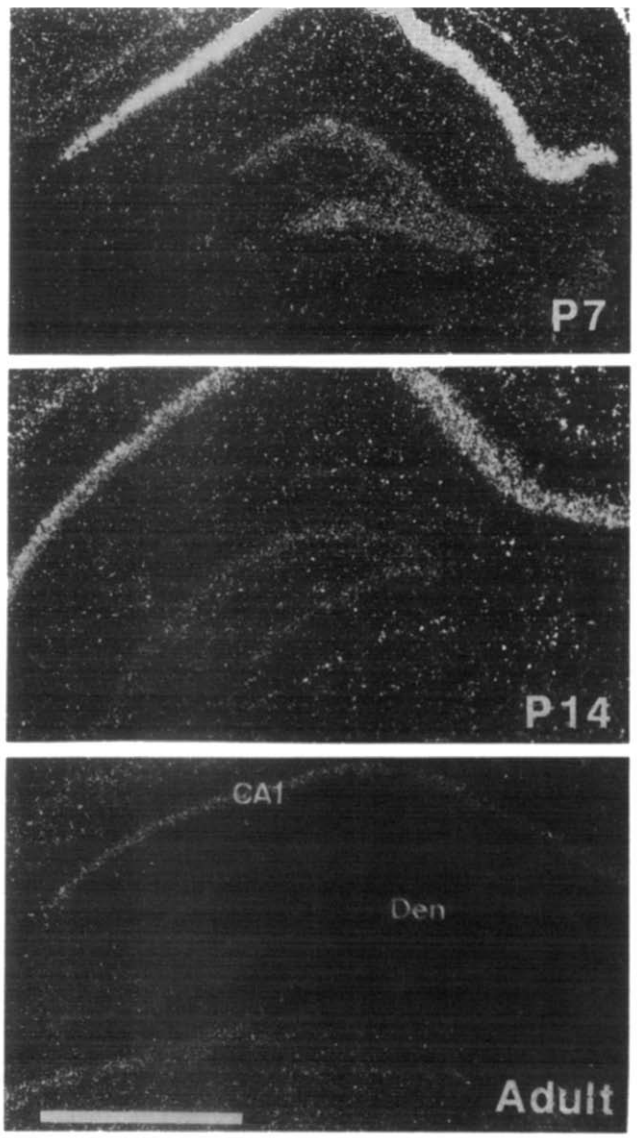


Fig. 5. Photomicrographs showing the localization of prepro-NT/NN mRNAs in the CA1 field (CA1) and transient appearance in the dentate gyrus (Den). The intensity of expression in the dentate gyrus was less than that in the CA1 field and was slightly more diffuse These sections were exposed for a longer period (32 days) than the others to detect weak signals. Bar = 1 mm

I) However, in other areas (CA1 field, the dentate gyrus, and layers II–III of the posterior cingulate cortex), the compact cellular arrangement made it difficult to distinguish single cells. Accordingly, in these areas, the grain number per 320 μm^2 (40 × 80 μm) was counted, and divided by the grain number per 320 μm^2 in other regions. This ratio was taken as the grain/noise ratio When the density of grains was measured, areas with a grain/noise ratio of more than 2 were regarded as positive (Table II)

Control experiments

Northern blot analysis and competition experiments were carried out to evaluate the specificity of the hybridization signals Poly(A)$^+$ RNA from rat brain hybridized with the NT probe appeared in two bands (Fig. 2A). The prepro-NT/NN gene transcribes the types of mRNA, which differ only in poly(A)$^+$ length[23]. The two prepro-NT/NN mRNAs we detected were about 1 0 kb and 1 5 kb in length, which was consistent with an earlier report[23] Fig 2B shows

TABLE I

The 'number of positive grains' per cell (mean value ± S.D)

	Subiculum	*Layer VI*
P7	64 ± 13	70 ± 14
adult	54 ± 19	*

* Approximately 0.

expression of the prepro-NT/NN mRNA in the subiculum and the posterior cingulate cortex. When sections were treated with ribonuclease (RNase; 0.1 mg/ml) in 0 1 M Tris-HCl (pH 7.4) at 37 °C for 60 min before prehybridization, signals were virtually absent (Fig. 2C). In the competition experiment, sections were hybridized with an excess of non-labeled NT probe (38 ng of probe per slide), and hybridization signals were also found to be absent (Fig. 2D). These control experiments were performed with serial sections from one rat. They were hybridized with the same amount of labeled probe (8.3 kBq) at the same time and exposed for the same period (20 days)

Terminology

The nomenclature used follows that of Vogt and Peters[41], Vogt and Miller[42], and Bayer[3]

RESULTS

No positive cells were seen in the primordium of the posterior cingulate cortex or hippocampal formation until E17. Between E17 and birth, positive cells increased in number and intensity, and were observed in layers II–III of areas 29a and 29b, layer VI in the posterior cingulate cortex, the dorsal and ventral subiculum, and the CA1 field in the hippocampus at birth (Figs. 3, 4). After the first postnatal week, positive cells in the posterior cingulate cortex, decreased remarkably in number with age and only a few were seen in adult rats (Fig. 4 and Tables I, II). The decrease was more prominent in layers II–III of areas 29a and 29b than in layer VI. On postnatal day 14 no labeled cells were detected in layers II–III of areas 29a and 29b, while those in layer VI still remained. Aggregates of silver grains were also observed in the granular cell layer of the dentate gyrus in the hippocampus during the first postnatal week (Fig. 5). After postnatal day 7, they began to decrease in intensity.

In contrast, the number of the labeled cells in the subiculum of the adult animals did not decrease so

TABLE II

The 'grain/noise ratio' in each region (mean value ± S.D.)

	Layers II-III	*CA1*	*Dentate gyrus*
P7	3 5 ± 0.3	3.1 ± 1.1	2 0 ± 0.3
adult	*	2 3 ± 0.5	*

* Approximately 1

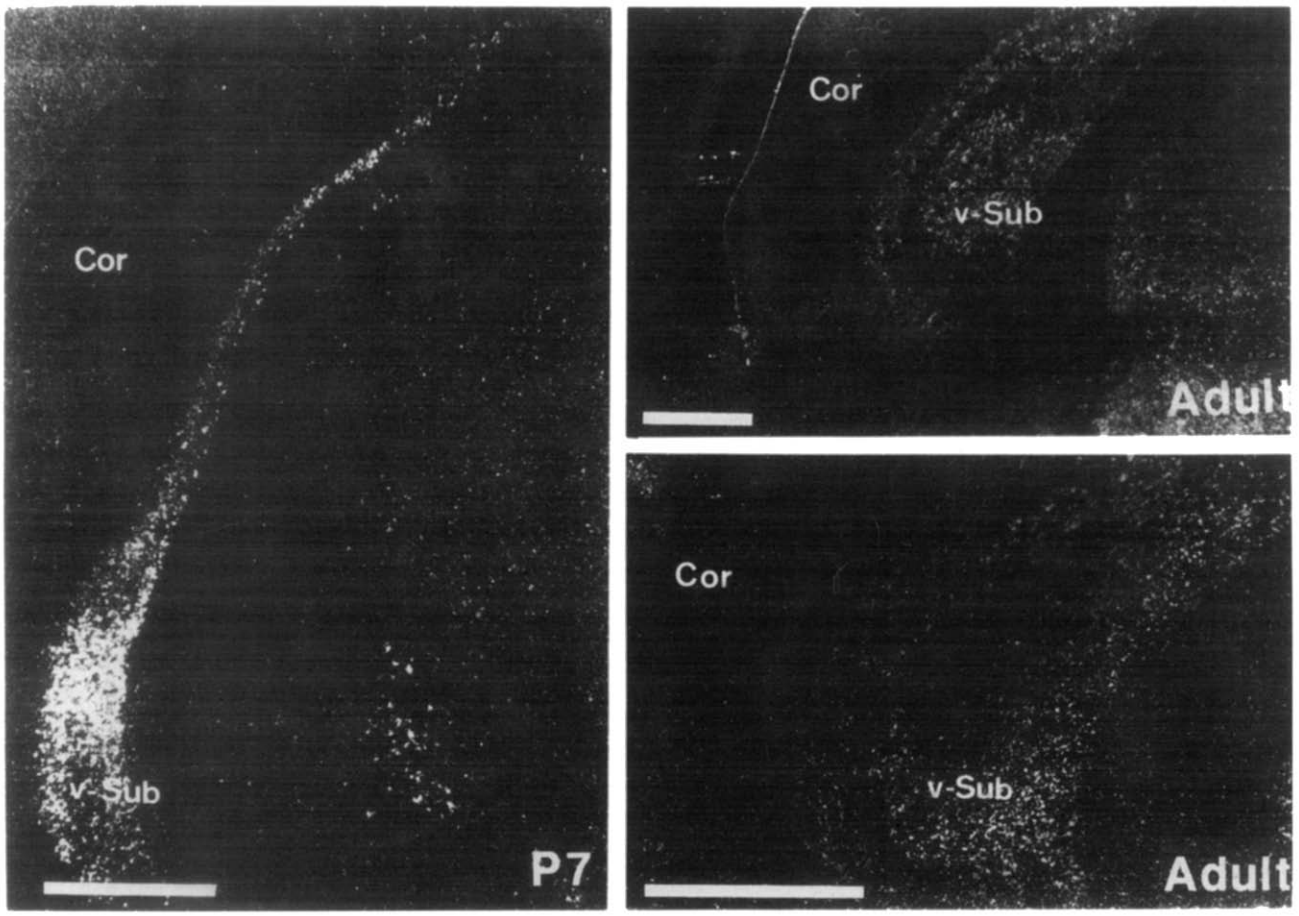
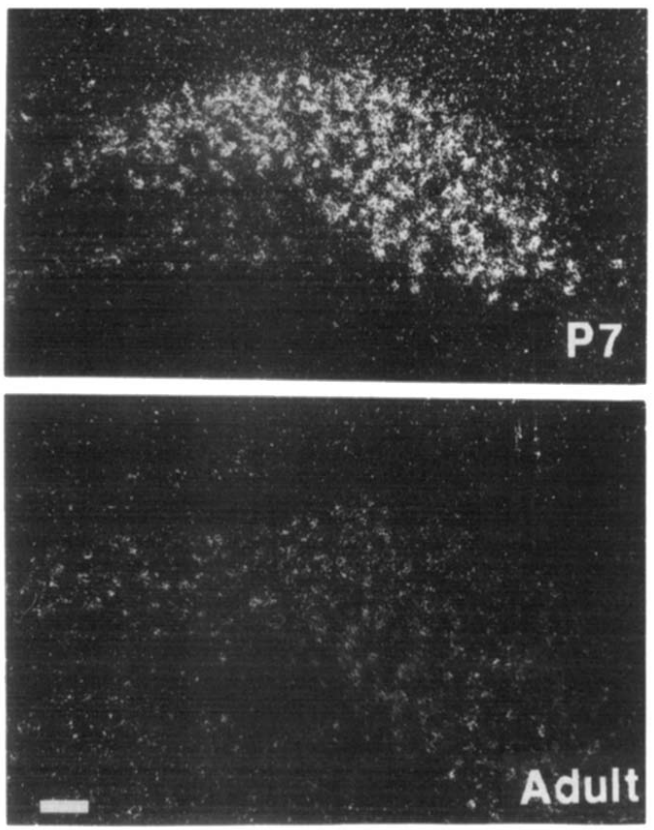


Fig 6. Photomicrographs showing prepro-NT/NN mRNAs in the ventral subiculum (v-Sub) during development In the adult, signals could also be observed in the ventral subiculum The upper panel (Adult) shows a more caudal region than the lower Cor, cortex Bar = 1 mm



prominently compared with the perinatal stage (Figs. 6, 7). The average number of positive *cells* in the subiculum on postnatal day 7 (n = 3) was 3124, while that of adult rats (n = 3) was 2971. Though the positive cells did not decrease so much in number in these areas during development, the amount of prepro-NT/NN mRNA expressed per cell decreased (Table I). In the CA1 field, prepro-NT/NN mRNA is still expressed in adult rats (Fig. 5, Table II).

DISCUSSION

Bayer reported that cells in the subiculum first appear on E16 or E17[2]. Our study has revealed that prepro-NT/NN mRNA is first expressed in the posterior cingulate cortex and the hippocampal formation on E17. Thus, the neurons in these areas begin to produce NT just after neurogenesis when their dendrites and axons are not well matured.

←

Fig 7 Photomicrographs showing the persistence of prepro-NT/NN mRNAs in the dorsal subiculum The number of prepro-NT/NN mRNA containing cells in the adult is almost the same as that in the 7-day-old rat Bar = 100 μm

A recent study using in situ hybridization histochemistry revealed that in adult rats the CA1 field and the dorsal and ventral subiculum had neurons containing prepro-NT/NN mRNA[1]. Our study confirmed these findings, and in addition showed that very young rats had a number of cells containing prepro-NT/NN mRNA not only in the subiculum or CA1 field but also in the posterior cingulate cortex. As to the CA1 field, probes often bind to this area non-specifically. However, Alexander et al. found an abundance of authentic prepro-NT/NN mRNA in the CA1 field using S1 protection assay[1]. We previously found a number of NT-LI neurons in the posterior cingulate cortex and dorsal subiculum with no or only a few immunoreactive cells in the CA1 field in young rats[18]. Only speculative considerations about the discrepancy between immunohistochemistry and in situ hybridization histochemistry findings are possible at present. Generally, mRNA exists in the cell bodies, whereas peptides are mainly present in nerve termini. However, it is also possible that the prepro-NT/NN mRNA is not translated to NT peptide in the areas which have a number of cells containing prepro-NT/NN mRNA but lack immunoreactive cells. Perhaps prepro-NT/NN mRNA is differentially processed and the amount of peptide is not sufficient for visualization by immunohistochemistry. The possibility that only neuromedin N is processed neuron-specifically also cannot be excluded. Similar discrepancies between immunocytochemical and in situ hybridization histochemical findings have been reported for the cholecystokinin (CCK) distribution in the rat thalamus[6,43]

One explanation for transient appearance of NT-LI neurons in the dorsal subiculum and the posterior cingulate cortex is neuronal cell death, which occurs during early embryonic stages[11]. However, for the subiculum, the possibility can be excluded because numerous prepro-NT/NN mRNA-containing cells were still present in adult rats. Also, in the terminal field of these neurons, the mamillary body, a NT fiber plexus, is found in adult rats[18,24] Accordingly, it is possible that NT is translated from prepro-NT/NN mRNAs even in adult rats and then transported rapidly from the cell soma to the terminal field. However, in the posterior cingulate cortex, prepro-NT/NN mRNA-containing cells appeared transiently in addition to NT-LI cells. In very young rats, NT neurons in the posterior cingulate cortex project to the anterior ventral thalamic nucleus[15], while in adult rats, no or only a few immunoreactive fibers are seen in this nucleus[20,25,37]. These findings suggest that NT cells either die during ontogenesis or stop producing NT during maturation.

Acknowledgement This study was supported in part by Grants-in-Aid for scientific research from the Ministry of Education, Science and Culture of Japan

REFERENCES

1 Alexander, M.J , Miller, M A , Dorsa, D M , Bullock, B P , Melloni, Jr , R.H., Dobner, P.R and Leeman, S E , Distribution of neurotensin/neuromedin N mRNA in rat forebrain unexpected abundance in hippocampus and subiculum, *Proc. Natl Acad Sci. U S A* , 86 (1989) 5202–5206

2 Bayer, S.A , Development of the hippocampal region in the rat I Neurogenesis examined with ^{3}H-thymidine autoradiography, *J Comp. Neurol* , 190 (1980) 87–114

3 Bayer, S A , Hippocampal region In G. Paxinos (Ed), *The Rat Nervous System,* Academic, Sydney, 1985, pp 335–374

4 Behbehani, M M and Pert, A , A mechanism of the analgesic effect of neurotensin as revealed by behavioral and electrophysiological techniques, *Brain Res* , 324 (1984) 35–42

5 Bissette, G , Nemeroff, C B , Loosen, P T , Prange, A J , Jr and Lipton, M A , Hypothermia and intolerance to cold induced by intracisternal administration of the hypothalamic peptide neurotensin, *Nature (Lond)*, 262 (1976) 607–609

6 Burgunder, J -M and Young, III, S , The distribution of the thalamic projection on neurons containing cholecystokinin messenger RNA, using in situ hybridization histochemistry and retrograde labeling, *Mol Brain Res* , 4 (1988) 179–189

7 Carraway, R and Leeman, S E , The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami, *J Biol Chem* , 248 (1973) 6854–6861

8 Carraway, R and Leeman, S E , Characterization of radioimmunoassayable neurotensin in the rat, *J Biol Chem* , 251 (1976) 7045–7052

9 Chandra, A., Chov, H C , Chang, C and Lin, M T., Effects of intraventricular administration of neurotensin and somatostatin on thermoregulation in the rat, *Neuropharmacology,* 20 (1981) 715–718

10 Checler, F , Vincent, J.-P. and Kitabgi, P , Neuromedin N high affinity interaction with brain neurotensin receptors and rapid inactivation by brain synaptic peptidases, *Eur J Pharmacol.,* 126 (1986) 239–244.

11 Chun, J J M , Nakamura, M.J. and Shatz, C.J , Transient cells of the developing mammalian telencephalon are peptide-immunoreactive neurons, *Nature (Lond),* 325 (1987) 617–620.

12 Clineschmidt, B.V. and McGuffin, J C , Neurotensin administered intracisternally inhibits responsiveness of mice to noxious stimuli, *Eur. J. Pharmacol* , 46 (1977) 395–396.

13 Dobner, P.R., Barber, D L , Villa-Komaroff, L and McKierman, C , Cloning and sequence analysis of cDNA for the canine neurotensin/neuromedin N precursor, *Proc. Natl. Acad. Sci. U S.A.,* 84 (1987) 3516–3520

14 Enjalbert, A , Arancibia, S., Priam, M., Bluet-Pajot, M.T. and Kordon, C , Neurotensin stimulation of prolactin secretion in vitro, *Neuroendocrinology,* 34 (1982) 95–98

15 Fukami, K., Kiyama, H , Shiotani, Y. and Tohyama, M., Neurotensin-containing projections from the retrosplenial cortex to the anterior ventral thalamic nucleus in the rat, *Neuroscience,* 26 (1988) 819–826

16 Goedert, M , Lightman, S.L , Nagy, J I , Marley, P.D and Emson, P C., Neurotensin in the rat anterior pituitary gland, *Nature (Lond.),* 298 (1982) 163–165.

17 Goedert, M., Lightman, S.L., Mantyh, P.W., Hunt, S P and Emson, P C , Neurotensin-like immunoreactivity and neurotensin receptors in the rat hypothalamus and in the neurointermediate lobe of the pituitary gland, *Brain Res.,* 358 (1985) 59–69.

18 Hara, Y., Shiosaka, S., Senba, E., Sakanaka, M., Inagaki, S., Takagi, H., Kawai, Y , Takatsuki, K., Matsuzaki, T and Tohyama, M , Ontogeny of the neurotensin-containing neuron system of the rat immunohistochemical analysis I. Forebrain and diencephalon, *J Comp Neurol* , 208 (1982) 177–195

19 Hu, H , Rao, J K., Prasad, C and Jayaraman, A , Distribution pattern of cell bodies and fibers with neurotensin-like immunoreactivity in the cat hypothalamus, *J Comp Neurol* , 272 (1988) 269–279
20 Jennes, L , Stumpf, W. and Kalivas, P W , Neurotensin: topographical distribution in rat brain by immunohistochemistry, *J Comp. Neurol* , 210 (1982) 211–224
21 Kalivas, P W , Jennes, L , Nemeroff, C B and Prange, A J , Jr , Neurotensin. topographical distribution of brain sites involved in hypothermia and antinociception, *J Comp Neurol* , 210 (1982) 225–238
22 Kataoka, K , Mizuno, N. and Frohman, L A , Regional distribution of immunoreactive neurotensin in monkey brain, *Brain Res Bull* , 4 (1979) 57–60
23 Kislauskis, E D , Bullock, B , McNeil, S and Dobner, P R , The rat gene encoding neurotensin and neuromedin N, *J Biol Chem* , 263 (1988) 4963–4968
24 Kiyama, H , Shiosaka, S , Sakamoto, N , Michel, J -P , Pearson, J and Tohyama, M , A neurotensin-immunoreactive pathway from the subiculum to the mammillary body in the rat, *Brain Res* , 375 (1986) 357–359
25 Kobayashi, R.D , Brown, M and Vale, W , Regional distribution of neurotensin and somatostatin in rat brain, *Brain Res* , 126 (1977) 584–588
26 Maeda, K and Frohman, L A , Dissociation of systemic and central effects of neurotensin on the secretion of growth hormone, prolactin, and thyrotropin, *Endocrinology*, 103 (1978) 1903–1908
27 Mai, J K , Triepel, J. and Metz, J , Neurotensin in the human brain, *Neuroscience,* 22 (1987) 499–524
28 Maniatis, T , Fritsch, E F and Sambrook, J., *Molecular Cloning A Laboratory Manual,* Cold Spring Harbor Laboratory, New York, 1982, p 196
29 Martin, G E , Bacino, C B and Papp, N.L , Hypothermia elicited by the intracerebral microinjection of neurotensin, *Peptides,* 1 (1980) 333–339.
30 Minamino, N , Kangawa, K and Matsuo, H , Neuromedin N a novel neurotensin-like peptide identified in porcine spinal cord, *Biochem Biophys Res Commun* , 122 (1984) 542–549
31 Myers, R D and Lee, T F , In vivo release of dopamine during perfusion of neurotensin in substantia nigra of the unrestrained rat, *Peptides,* 4 (1983) 955–961
32 Nemeroff, C G and Prange, A.J , Jr , Eds Neurotensin a brain and gastrointestinal peptide, *Ann N Y Acad Sci* , 400 (1982) 1–368
33 Rioux, F., Quirion, R , St -Pierre, S , Regoli, D , Johicoeur, F B , Belanger, F and Barbeau, A., The hypotensive effect of centrally administered neurotensin in rats, *Eur J Pharmacol* , 69 (1981) 241–247
34 Sakamoto, N , Michel, J -P , Kopp, N and Pearson, J , Neurotensin immunoreactive neurons in the human infant diencephalon, *Brain Res* , 403 (1987) 31–42
35 Shipley, M T., McLean, J H and Behbehan, M M , Heterogeneous distribution of neurotensin-like immunoreactive neurons and fibers in the midbrain periaqueductal gray of the rat, *J Neurosci* , 7 (1987) 2025–2034
36 Uhl, G and Snyder, S M , Regional and subcellular distributions of brain neurotensin, *Life Sci* , 19 (1976) 1827–1832
37 Uhl, G , Kuhar, M and Snyder, S M , Neurotensin immunohistochemical localization in rat central nervous system, *Proc Natl Acad Sci U S A* , 74 (1977) 4059–4063
38 Uhl, G , Goodman, R R and Snyder, S H , Neurotensin-containing cell bodies, fibers and nerve terminals in the brain stem of the rat immunohistochemical mapping, *Brain Res* , 167 (1979) 77–91
39 Vijayan, E and McCann, S M , In vivo and in vitro effects of substance P and neurotensin on gonadotropin and prolactin release, *Endocrinology*, 105 (1979) 64–68
40 Vijayan, E and McCann, S M , Effect of substance P and neurotensin on growth hormone and thyrotropin release in vivo and in vitro, *Life Sci* , 26 (1980) 321–327
41 Vogt, B A and Peters, A , Form and distribution of neurons in rat cingulate cortex: areas 32, 24, and 29, *J Comp Neurol* , 195 (1981) 603–625
42 Vogt, B A and Miller, M W , Cortical connections between rat cingulate cortex and visual, motor, and postsubicular cortices, *J. Comp Neurol* , 216 (1983) 192–210
43 Voigt, M M and Uhl, G R , Preprocholecystokinin mRNA in rat brain regional expression includes thalamus, *Mol Brain Res* , 4 (1988) 247–253

Developmental Brain Research, 58 (1991) 97–103
Elsevier

BRESD 51209

Neurotensin and neuromedin N elevate the cytosolic calcium concentration via transiently appearing neurotensin binding sites in cultured rat cortex cells

Makoto Sato[1], Sadao Shiosaka[2] and Masaya Tohyama[1]

[1]*Department of Anatomy II, Osaka University Medical School and* [2]*Department of Neuroanatomy, Biomedical Research Center, Osaka University Medical School, Osaka (Japan)*

(Accepted 2 October 1990)

Key words: Neurotensin; Neuromedin N; Fura-2; Calcium; Receptor

Through assessment of the changes in the intracellular free-calcium concentration ($[Ca^{2+}]_i$), which was measured using the calcium sensitive dye, fura-2, the character of the neurotensin (NT) binding sites which appeared transiently during the early ontogenetic stage in the rat cerebral cortex was analyzed in primary cultures of cerebral cortex cells from neonatal rats. NT (1–1000 nM) elevated $[Ca^{2+}]_i$ of the cells even when extracellular calcium was chelated with 1 mM ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA). These findings suggest that the transiently appearing NT-binding sites in the cortex are receptors for NT and that some of the NT-induced increase in $[Ca^{2+}]_i$ is due to mobilization from the intracellular calcium store. Further application of NT after 10 min washing caused an increase in $[Ca^{2+}]_i$ again. This is in contrast to the findings for cortical slices from adult rats and mRNA-injected oocytes; desensitization due to NT was of long duration and further application of NT failed to activate the neurons which had responded the first time to NT. These facts suggest that the character of the NT-binding sites in the cerebral cortex differs between neonatal and adult rats. In addition, we showed that neuromedin N had a similar property to NT as to mobilization of $[Ca^{2+}]_i$ and acted only on NT-responsive cells, suggesting the interaction between NT and neuromedin N at the postsynaptic level via the same receptor.

INTRODUCTION

On the basis of ontogeny, previous studies involving in vitro autoradiography revealed that there are two kinds of neurotensin (NT)[4] binding sites: one is that seen in the cerebral cortex and the other that seen in other brain regions such as the ventral tegmental area and substantia nigra pars compacta[13,17]. The former kind of NT binding sites transiently appears during the early ontogenetic stage. Namely, it increases during the early ontogenetic stage, the maximum level being reached on postnatal day 10. Thereafter, it decreases markedly, a very low level of NT binding sites being found in the adult rat. On the other hand, the latter kind of NT binding sites increases progressively during development after birth, the plateau level being reached on postnatal day 15. No conspicuous decrease can be seen thereafter.

In support of these observations, several lines of biochemical and pharmacological evidence have suggested the presence of two types of NT binding sites[14,15,19]. For example, using the histamine antagonist, levocabastine[23], Schotte et al.[19,20] confirmed the presence of two different binding affinity sites: NT high affinity (levocabastine insensitive) and low affinity (levocabastine sensitive) sites. From an ontogenetic viewpoint, high affinity sites are abundant at birth and reach the maximum level on postnatal day 10, after which they decrease markedly to reach a low plateau level, while low affinity sites are very few at birth, increase in number and reach a plateau level on postnatal day 15. From that time, no conspicuous decrease in them can be detected[20]. Thus, judging from the developmental pattern of the NT binding sites reported by Kiyama et al.[13] and Palacios et al.[17], and that by Schotte et al.[19,20], it is likely that the high affinity sites correspond to NT binding sites which appear transiently during the early ontogenetic stage in the cerebral cortex ('transient NT binding sites'). Little is known about the character of the 'transient NT binding sites'. In addition, it is not clear whether the two distinct profiles of NT binding sites are due to originally different structures or to different structures originating from the same structure.

In this study, using a primary culture of cerebral cortex cells of neonatal rats, in which all the NT-binding sites

Correspondence: M. Sato, Department of Anatomy II, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan.

are 'transient NT binding sites'[13,20], we attempted to characterize the 'transient NT-binding sites', assessing the changes in the intracellular free calcium (Ca^{2+}) concentration, $[Ca^{2+}]_i$, evoked by NT. In addition, in the same culture system, we examined the effect of neuromedin N (NN)[16] which is coded tandemly in the same precursor of NT[6,11] in relation to NT, aiming to determine whether or not NN acted on the same immature cortical cells as those affected by NT and, if this was so, to examine the possibility that NT and NN express their roles via the same receptor or binding sites.

MATERIALS AND METHODS

The methods used for culturing cells and measuring $[Ca^{2+}]_i$ were described in detail elsewhere[25]. Briefly, the cortices were removed from newborn Wistar rats, incubated with papain (Worthington Biochemical Corp., U.S.A.), triturated and then seeded into 0.28 cm^2 wells with Flexiperm covers (Heraus, F.R.G.), on polyethylenimine-coated glass cover-slips. The cells were cultured for 1–6 days in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal bovine serum (FBS; Sigma, U.S.A.) and antibiotics under an atmosphere of 95% air and 5% CO_2 (v/v) at 37 °C. $[Ca^{2+}]_i$ was measured by use of the calcium-sensitive fluorescent dye, fura-2 acetoxymethylester (fura-2/AM; Dojin, Japan)[7,26]. The cultured cells were loaded with fura-2/AM at the concentration of 5 μM in modified Eagle's minimum essential medium (MEM) without phenol red (Auto-Pow MEM; Flow Laboratories, U.S.A.) for 30 min at 37 °C. The fura-2-loaded cells were washed twice with the same medium containing 15 mM Hepes (pH 7.3) without sodium bicarbonate. The cultured cells were then viewed, through the coverslip, under an inverted microscope (Nikon, Japan). Images were taken every 3.33 or 8.3 s with a video camera (C-2400; Hamamatsu Photonics, Japan) equipped with an Argus-100/CA (Hamamatsu Photonics, Japan), which controlled the image acquisition and display. $[Ca^{2+}]_i$ was subsequently computed and determined from images at 340 and 380 nm by use of the ratio method. We calibrated the fura-2 signals by the method recommended by Hamamatsu Photonics. However, in this experiment, note that transient elevation of $[Ca^{2+}]_i$ is more important than the absolute value of $[Ca^{2+}]_i$.

Changes in $[Ca^{2+}]_i$ of cultured cells were examined under the following conditions: (i) application of NT alone, (ii) that of NT at various concentrations followed by application of 100 nM NT, (iii) that of 100 nM NT analogues followed by 100 nM NT application, (iv) that of 100 nM NT in the continuous presence of ethylene glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) to chelate the extracellular calcium, (v) that of 100 nM NT followed by washing and 100 nM NT re-application, (vi) that of NN instead of NT in the same way as described above, (vii) that of 100 nM NT followed by washing and application of 100 nM NN, or that of NN and then NT, and (viii) that of 100 nM NT followed by application of 100 nM NN, washing and 100 nM NN re-application, or that of NN followed by NT application, washing and NT reapplication. For these applications, NT, NN, NT analogues and EGTA were dissolved in modified Eagle's MEM (containing 15 mM Hepes, pH 7.3), and the wells were continuously perfused with each solution. Before the application of each solution, in some cases, modified Eagle's MEM was applied to the application site for each solution to exclude cells which would show a shock response to the application. For washing, cells were continuously perfused with modified Eagle's MEM (2.5 ml/min). The wells, the equipment, and the solutions were thermostatically maintained at 35–37 °C throughout the measurements. All fluorescence records were similar to at least 6 others.

TABLE I

Ability of NT and neuromedin N to elevate cytosolic Ca^{2+}

Peptide	*Concentration (nM)*	*Ca^{2+} response*
NT	0.1	(±)*
	1	(+)
	10	(+)
Neuromedin N	1	(–)
	10	(+)

Positive response (+); inactive response (–).
* 20% of 100 nM NT-responsive cells showed a positive response.

RESULTS

$[Ca^{2+}]_i$ elevation by NT

Effect of a single application of NT. Various concentrations of NT (1–1000 nM) induced an increase in $[Ca^{2+}]_i$ of primary cultured cortical cells (Table I). The response curve pattern of $[Ca^{2+}]_i$ as to NT application was similar throughout the examination, though the extent of mobilization of Ca^{2+} ions in the cortical cells reactive as to NT application varied from 179 to 514 nM. Fig. 1A shows an example of mobilization of free Ca^{2+} ions in cells in response to NT (100 nM). A drastic

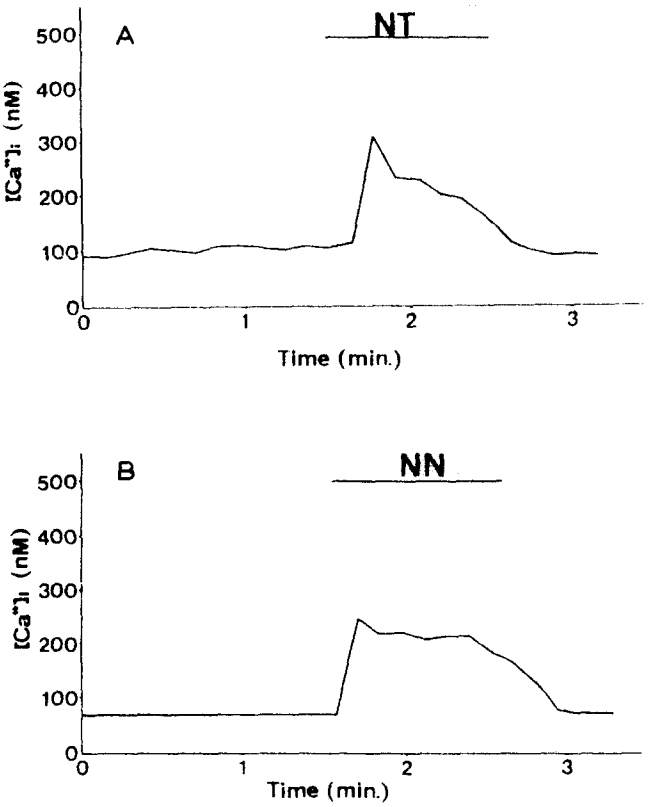


Fig. 1. A: 100 nM neurotensin (NT) caused a transient increase in $[Ca^{2+}]_i$ in cultured cortex cells. B: 100 nM neuromedin N (NN) caused a transient increase in $[Ca^{2+}]_i$ in cultured cortex cells.

TABLE II

Ability of NT analogues (100 nM) to elevate cytosolic Ca^{2+}

Peptide	*Ca^{2+} response*
NT	(+)
NT(1–8)	(–)
NT(8–13)	(+)
[D-Trp^{11}]NT	(–)
Neuromedin N	(+)

Positive response (+); inactive response (–).

1 8 11
NT: pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
Neuromedin N: Lys-Ile-Pro-Tyr-Ile-Leu

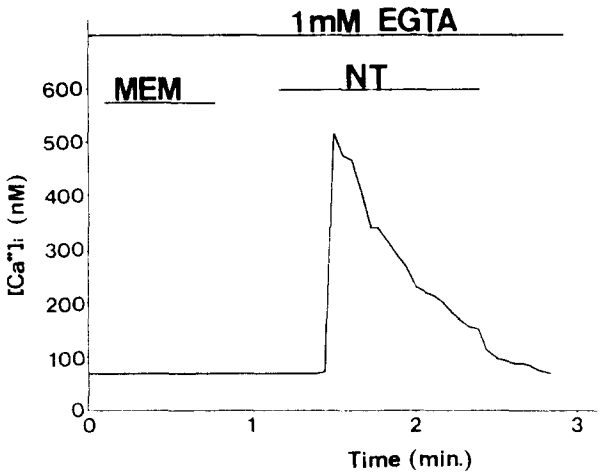


Fig. 2. 100 nM NT caused a transient increase in $[Ca^{2+}]_i$ in cultured cortex cells in the continuous presence of 1 mM EGTA. Modified Eagle's minimum essential medium containing 15 mM Hepes (pH 7.3) (MEM) was applied to the same injection site as for NT before the NT application to exclude cells which would show a shock response to the application.

increase in $[Ca^{2+}]_i$ occurred in the cortical cells immediately after an application of NT. This increase was transient: after reaching a peak (306 nM $[Ca^{2+}]_i$ in this case) within 20 s, the elevated $[Ca^{2+}]_i$ level began to decrease progressively, the basic level being reached at about 2 min after addition of NT to the medium. This effect of NT could not be detected in all cases in which 0.1 nM NT was applied (Table I). Since there was a possibility that the primary culture cells lacked cells containing NT binding sites, we added 100 nM NT to the medium after washing the wells. Two cells showed an increase in $[Ca^{2+}]_i$ with 0.1 nM NT, whereas 8 cells showed an increase in $[Ca^{2+}]_i$ only with 100 nM NT, indicating that cells containing NT binding sites show an increase in $[Ca^{2+}]_i$ with a higher concentration of NT

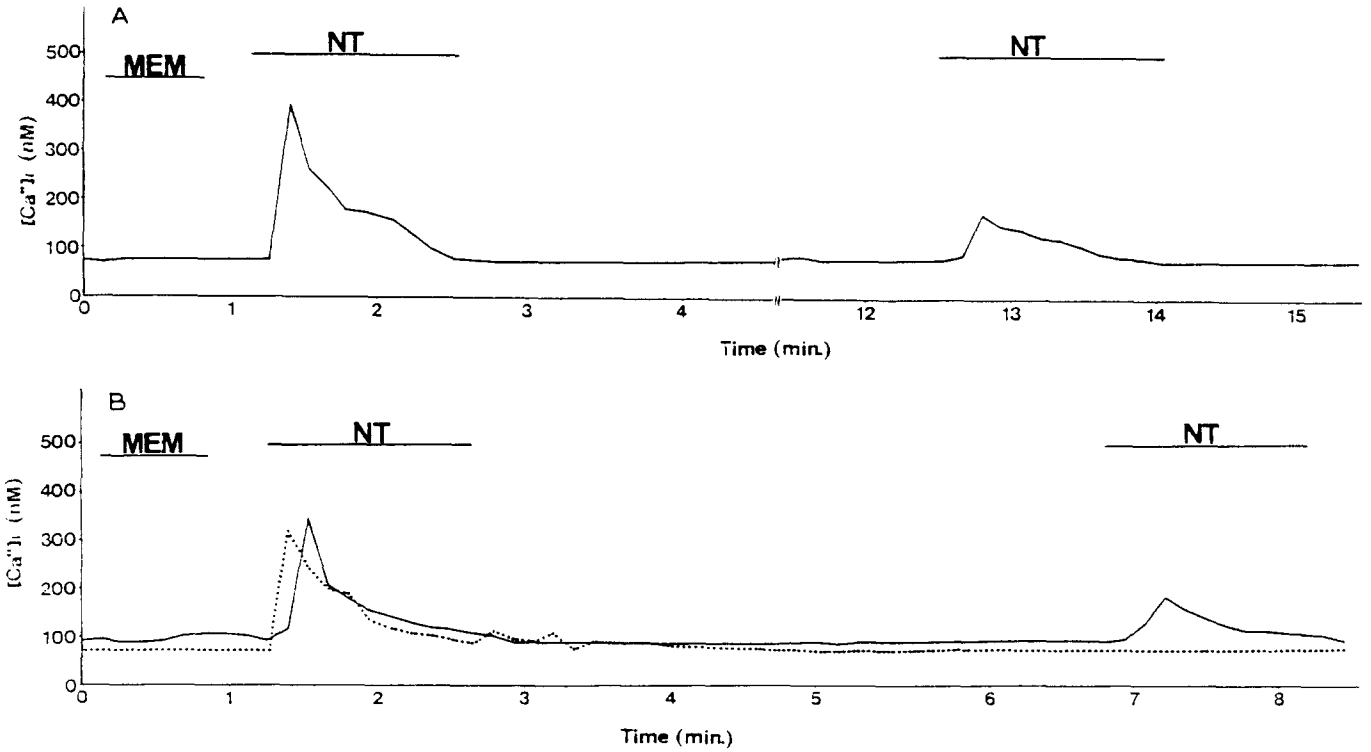


Fig. 3. A: re-application of 100 nM NT caused a transient increase in $[Ca^{2+}]_i$ in cultured cortex cells after a first application of 100 nM NT and washing for 10 min. B: re-application of 100 nM NT after washing for 4 min caused a transient increase in $[Ca^{2+}]_i$ in cells (solid line). Some cells did not respond to the re-application (dotted line). Modified Eagle's minimum essential medium containing 15 mM Hepes (pH 7.3) (MEM) was applied to the same injection site as for NT before the application of NT to exclude cells which would show a shock response to the application.

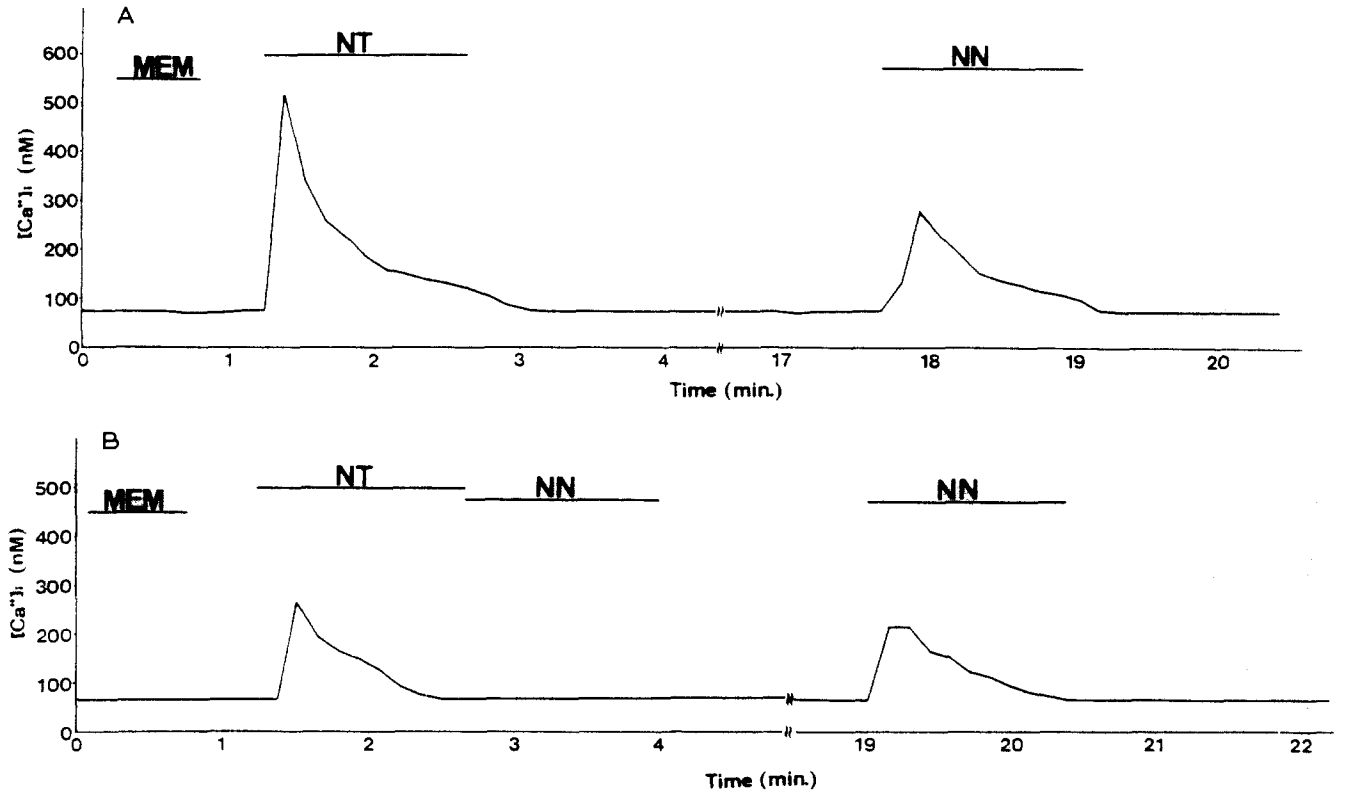


Fig. 4. A: application of 100 nM neuromedin N (NN) induced a transient increase in $[Ca^{2+}]_i$ in cultured cortex cells after 100 nM NT application and washing for 15 min. No cells were observed which only responded to NN. B: first 100 nM NT was applied and then 100 nM NN was applied. Re-application of 100 nM NN after washing for 15 min induced a transient increase in $[Ca^{2+}]_i$, though the first application of NN did not. Modified Eagle's minimum essential medium containing 15 mM Hepes (MEM) was applied to the same injection site as for NN or NT to exclude cells which would show a shock response to the application.

than 0.1 nM. When 1 nM NT was applied, no cells were observed which only responded to subsequent application of 100 nM NT.

Determination of the active part of NT in the increase in $[Ca^{2+}]_i$. One-hundred nM C-terminal octapeptide of NT, NT(8–13), caused a similar increase in $[Ca^{2+}]_i$ in cortical cells to that caused by NT, while the same concentration of N-terminal octapeptide NT, NT(1–8), failed to cause an increase in $[Ca^{2+}]_i$ in the cortical cells (Table II), showing that the C-terminal octapeptide played a role in the increase in $[Ca^{2+}]_i$ in cortical cells caused by NT. Furthermore, an NT analogue, [D-Trp^{11}]NT, in which the 11th residue of NT is replaced by D-Trp, did not elevate $[Ca^{2+}]_i$ at the concentration of 100 nM (Table II).

Determination of the source of mobilized Ca^{2+} ions with an application of NT. In the continuous presence of 1 mM EGTA, an application of 100 nM NT resulted in a similar increase in $[Ca^{2+}]_i$ in cultured cortical cells, suggesting that mobilization of Ca^{2+} ions from the intracellular Ca^{2+} store is an important factor in the increase in $[Ca^{2+}]_i$ caused by an NT application (Fig. 2).

Determination of whether desensitization of NT receptors occurs or not in cortical neurons of neonatal rats. Fig. 3A and B (solid line) show the changes in $[Ca^{2+}]_i$ after further addition of 100 nM NT to the medium. As described above, NT caused transient elevation of $[Ca^{2+}]_i$ in the cells. After the return to the basic level, the wells were washed for 10 min and then 100 nM NT was again added to the medium. An increase in $[Ca^{2+}]_i$ was again detected, though the amount of Ca^{2+} ions which were mobilized in the cells with this further application of NT (179 nM of $[Ca^{2+}]_i$) was less than the initial one (394 nM $[Ca^{2+}]_i$) (Fig. 3A). So far as we observed, every NT responsive cell was reactive to the second application of NT after washing for 10–20 min (n = 11), whereas when the wells were washed for 4 min, about 33% of the cells in which $[Ca^{2+}]_i$ was elevated by NT was also reactive to a further application of NT, while the remaining cells were reactive to the first application of NT but not to the second one, as shown in Fig. 3B by a dotted line.

$[Ca^{2+}]_i$ elevation by NN

An application of NN caused similar transient elevation of $[Ca^{2+}]_i$ to that seen in the cases in which NT was added (Fig. 1A and B, and Table II). However, the minimum concentration which causes an elevation of $[Ca^{2+}]_i$ of NN was higher (10 nM) than that of NT (1 nM)

(Table I). The continuous presence of EGTA in the medium (1 mM) did not inhibit the increase in $[Ca^{2+}]_i$, as seen for NT.

Determination of whether or not NT and NN act on the same cells

Fig. 4A shows an example of the cases in which 100 nM NT was first applied, followed by an application of 100 nM NN. The pattern of the elevation of $[Ca^{2+}]_i$ in the cells obtained in this case was similar to that seen in the cases in which NT was repeatedly applied (Fig. 3A). An application of NT caused transient elevation of $[Ca^{2+}]_i$ (514 nM in this case). After 15 min washing, NN added also caused transient elevation of $[Ca^{2+}]_i$ in the same cells, though the amount of mobilized Ca^{2+} ions with NN was less (278 nM in this case) than that with NT. Every cell which was reactive to NT was also reactive to NN (n = 15). On the other hand, there were no cells which were only reactive to NN. Moreover, when we first added 100 nM NN, followed by washing for 15 min, and then applied 100 nM NT, the same results as mentioned above were obtained: every cell responsive to NN also responded to subsequently applied NT (n = 9), though the mobilization of Ca^{2+} ions by NN was greater than that by subsequently applied NT. No cells were observed which were only responsive to NT. These findings showed that NT and NN act on the same cortical cells.

Next, we examined the possibility that NT and NN bind to the same receptor. An example is shown in Fig. 4B. NN (100 nM) was applied immediately after transient elevation of $[Ca^{2+}]_i$ caused by NT (100 nM). As shown in Fig. 4B, subsequent NN application failed to elevate the $[Ca^{2+}]_i$, while NN application at the same concentration after 15 min washing caused elevation of $[Ca^{2+}]_i$ in the same cells (Fig. 4B). When NN and NT were exchanged for the experimental processes mentioned above, the same observations were made: NT application immediately after transient elevation of $[Ca^{2+}]_i$ caused by NN failed to elevate the amount of Ca^{2+} ions, while the addition of NT to the medium subsequent to 15 min washing after the first NT application caused elevation of $[Ca^{2+}]_i$.

DISCUSSION

Our study revealed that NT causes transient elevation of $[Ca^{2+}]_i$ in cultured cortical cells from neonatal rats, even in the continuous presence of 1 mM EGTA to chelate the extracellular Ca^{2+}. These findings show that mobilization of Ca^{2+} from the intracellular Ca^{2+} store plays an important role in the elevation of $[Ca^{2+}]_i$ caused by NT, and the 'transient NT binding site' is considered to be the receptor involved because the intracellular events only occurred with NT. Several binding studies have shown that the binding ability of NT(1–8) or [D-Trp[11]]NT is very weak or nonexistent, while NT(8–13) has as strong binding ability as NT[10,12]. Furthermore, our study revealed that NT and NT(8–13) cause a transient elevation of $[Ca^{2+}]_i$, while NT(1–8) does not. These findings show that NT(8–13) plays an important role in the binding of NT to the 'transient binding sites' and in expression of their functions.

It has been reported that NT causes activation of inositol phospholipid metabolism in a carcinoma cell line[1], and that the NT response is inhibited by the pertussis toxin and mimicked by intracellularly injected inositol 1,4,5-triphosphate (IP_3) in oocytes injected with mRNA from rat brain[9]. As the pertussis toxin is known to abolish the functions of guanine nucleotide-binding proteins[22] (G-proteins) through adenosine 5′-diphosphate ribosylation of the proteins[27], it is likely that NT receptors belong to the G-protein linked receptor family and that activation of G-proteins by NT is related to elevation of $[Ca^{2+}]_i$ via activation of phospholipase C[3]. Our finding of Ca^{2+} mobilization from the intracellular Ca^{2+} store due to NT does not conflict with these reported characteristics of NT receptors.

An electrophysiological study involving cortical slices from adult rats has indicated that cortical neurons activated by NT show desensitization of long duration and that reactivation of the same neurons by a further application of NT after 20 min washing does not occur[2]. Desensitization of a long duration was also found in a study involving oocytes injected with poly(A)$^+$ mRNA from rat brain[9]. In contrast to these findings, our study showed that the desensitization did not continue for a long time, because re-application of NT, 4 min after transient elevation of $[Ca^{2+}]_i$, failed to cause an increase in $[Ca^{2+}]_i$ in some cells, but caused such elevation in cells which had been washed for 15 min. The discrepancy between our and other studies is attributed to the different ages of the rats used: we used neonatal cerebral cortex and the others used adult cortex. Accordingly, it may be concluded that the nature of the NT receptor which appears transiently in the cerebral cortex is different from that in adults.

We further showed that NN also increased $[Ca^{2+}]_i$ in primary cultured cortical cells from neonatal rats. With regard to elevation of $[Ca^{2+}]_i$, the property of NN is quite similar to that of NT. The coexistence of NT and NN is easily supposed, because they originate from the same precursor[6,11]. In addition, Checler et al.[5] have reported the possibility that NN binds to NT receptors. Our study provided evidence supporting this and further clarified the character of the interaction between NT and NN at the postsynaptic level, as described below. As shown in Fig. 4A, cells once activated by NT or NN were

re-activated by NN or NT, respectively. There were no cells in which the $[Ca^{2+}]_i$ change was first evoked by NT or NN but not reactivated on re-application of NN or NT after washing for 15 min, respectively. These findings suggest that NT and NN, which are released from the same terminals, acted on the same target cells, particularly in the neonatal rat cerebral cortex. In addition, as shown in Fig. 4B, re-application of NN or NT immediately after transient elevation of $[Ca^{2+}]_i$ evoked by NT or NN, respectively, failed to cause an increase in $[Ca^{2+}]_i$ in the same cells, but after washing for 10 min they both did. These findings revealed the occurrence of an interaction between NT and NN at the postsynaptic level. Possible interpretations are as follows. (1) NT and NN bind to the same receptor and subsequent application fails to mobilize Ca^{2+} because of desensitization of receptors of a short duration. (2) NT and NN bind to different receptors, but there is an interaction between the receptors, for example, the NT–NT receptor complex might inhibit NN binding to the NN receptor. (3) NT and NN bind to different receptors sharing the same intracellular mechanisms, and reapplication of NN or NT fails to elevate the $[Ca^{2+}]_i$ because almost all the Ca^{2+} ions have already been mobilized from the intracellular Ca^{2+} store by the first application of NT or NN, respectively; or (4) because the substances which mediate the signal transduction from the receptor to the intracellular Ca^{2+} store are exhausted transiently. As no conspicuous changes of $[Ca^{2+}]_i$ were observed from when we reapplied NN or NT until the cells had responded to the final application of NN or NT, the intracellular Ca^{2+} had already been sequestered before the reapplication of NN or NT. Therefore it is difficult to accept case (3). We cannot completely rule out the possibility of case (2), but NT and NN gave the same results when we exchanged them for each other, and they always act on the same cells and they are structurally related substances, so case (1) is the most probable. Namely, our findings suggested that NT and NN act on the same receptor, though we cannot rule out the possibility of case (4).

Little is known about the functions or origins of NT and NN in neonatal rats. The existence of a ventral tegmental area–prefrontal cortex pathway has been demonstrated[21,24]. However, the number of NT positive cells in the ventral tegmental area increases during the postnatal period and reaches a plateau level which is maintained in the adult rat[8]. As the NT-binding sites appear transiently in the cortex[13], other NT-positive cells which appear transiently during development, for example, NT cells in the retrosplenial (posterior cingulate) cortex[8,18], are more suitable candidates as the cells of origin. Further studies are needed to address this problem.

Acknowledgements. We wish to thank Drs. A. Kaneko and U. Tanaka of the First Department of Medicine, Osaka University Medical School, for calibrating the fura-2 signals and some technical advice. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 Amar, S., Kitabgi, P. and Vincent, J.-P., Activation of phosphatidylinositol turnover by neurotensin receptors in the human colonic adenocarcinoma cell line HT29, *FEBS Lett.*, 201 (1986) 31–36.
2 Audinat, E., Hermel, J.-M. and Crepel, F., Neurotensin-induced excitation of neurons of the rat's frontal cortex studied intracellularly in vitro, *Exp. Brain Res.*, 78 (1989) 358–368.
3 Boyer, J.L., Hepler, J.R. and Harden, T.K., Hormone and growth factor receptor-mediated regulation of phospholipase C activity, *Trends Pharmacol. Sci.*, 10 (1989) 360–364.
4 Carraway, R. and Leeman, S.E., The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami, *J. Biol. Chem.*, 248 (1973) 6854–6861.
5 Checler, F., Vincent, J.-P. and Kitabgi, P., Neuromedin N: high affinity interaction with brain neurotensin receptors and rapid inactivation by brain peptidases, *Eur. J. Pharmacol.*, 126 (1986) 239–244.
6 Dobner, P.R., Barber, D.L., Villa-Kimaroff, L. and McKieren, C., Cloning and sequence analysis of cDNA for the canine neurotensin/neuromedin N precursor, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 3516–3520.
7 Grynkiewicz, G., Poenie, M. and Tsien, R.Y., A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, *J. Biol. Chem.*, 260 (1985) 3440–3450.
8 Hara, Y., Shiosaka, S., Senba, E., Sakanaka, M., Inagaki, S., Takagi, H., Kawai, Y., Takatsuki, K., Matsuzaki, T. and Tohyama, M., Ontogeny of the neurotensin-containing neuron system of the rat: immunohistochemical analysis. I. Forebrain and diencephalon, *J. Comp. Neurol.*, 208 (1982) 177–195.
9 Hirono, C., Ito, I. and Sugiyama, H., Neurotensin and acetylcholine evoke common responses in frog oocytes injected with rat brain messenger ribonucleic acid, *J. Physiol.*, 382 (1987) 523–535.
10 Kanba, K.S., Kanba, S., Okazaki, H. and Richelson, E., Binding of [^{3}H]neurotensin in human brain: properties and distribution, *J. Neurochem.*, 46 (1986) 946–952.
11 Kislauskis, E., Bullock, B., McNeil, S. and Dobner, P.R., The rat gene encoding neurotensin and neuromedin N, *J. Biol. Chem.*, 263 (1988) 4963–4968.
12 Kitabgi, P., Carraway, R., Van Rietschoten, J., Granier, C., Morgat, J.L., Menez, A., Leeman, S. and Freychet, P., Neurotensin: specific binding to synaptic membranes from rat brain, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 1846–1850.
13 Kiyama, H., Inagaki, S., Kito, S. and Tohyama, M., Ontogeny of [^{3}H]neurotensin binding sites in the rat cerebral cortex: autoradiographic study, *Dev. Brain Res.*, 31 (1987) 303–306.
14 Mazella, J., Poustis, C., Labbe, C., Checler, F., Kitabgi, P., Granier, C., Van Rietschoten, J. and Vincent, J.-P., Monoiodo-[Trp^{11}]neurotensin, a highly radioactive ligand of neurotensin receptors, *J. Biol. Chem.*, 258 (1983) 3476–3481.
15 Mills, A., Demoliou-Mason, C.D. and Barnard, E.A., Characterization of neurotensin binding sites in intact and solubilized bovine brain membranes, *J. Neurochem.*, 50 (1988) 904–911.
16 Minamino, N., Kangawa, K. and Matsuo, H., Neuromedin N: a novel neurotensin-like peptide identified in porcine spinal cord, *Biochem. Biophys. Res. Commun.*, 122 (1984) 542–549.

17 Palacios, J.M., Pazos, A., Dietl, M.M., Schlumpf, M. and Lichtensteiger, W., The ontogeny of brain neurotensin receptors studied by autoradiography, *Neuroscience*, 25 (1988) 307–317.
18 Sato, M., Lee, Y., Zhang, J.-H., Shiosaka, S., Noguchi, K., Morita, Y. and Tohyama, M., Different ontogenetic profiles of cells expressing prepro-neurotensin/neuromedin N mRNA in the rat posterior cingulate cortex and the hippocampal formation, *Dev. Brain Res.*, 54 (1990) 249–255.
19 Schotte, A., Leysen, J.E. and Laduron, P.M., Evidence for a displaceable non-specific [^{3}H]neurotensin binding site in rat brain, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 333 (1986) 400–405.
20 Schotte, A. and Laduron, P.M., Different postnatal ontogeny of two [^{3}H]neurotensin binding sites in rat brain, *Brain Res.*, 408 (1987) 326–328.
21 Seroogy, K.B., Mehta, A. and Fallon, J.H., Neurotensin and cholecystokinin coexistence within neurons of the ventral mesencephalon: projections to forebrain, *Exp. Brain Res.*, 68 (1987) 277–289.
22 Sternweis, P.C. and Robishaw, J.D., Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain, *J. Biol. Chem.*, 259 (1984) 13806–13813.
23 Stockbroekx, R.A., Luyckx, M.G., Willems, J.M., Janssen, M.A.C., Bracke, J.O.M.M., Joosen, B.L.P. and Van Wauwe, J.P., Levocabastine (R 50 547), the prototype of a chemical series of compounds with specific H_1-antihistamine activity, *Drug Dev. Res.*, 8 (1986) 87–93.
24 Studler, J.M., Kitabgi, P., Tramu, G., Herve, D., Glowinski, J. and Tassin, J.P., Extensive co-localization of neurotensin with dopamine in rat meso-cortico-frontal dopaminergic neurons, *Neuropeptides*, 11 (1988) 95–100.
25 Tani, E., Shiosaka, S., Sato, M., Ishikawa, T. and Tohyama, M., Histamine acts directly on calcitonin gene-related peptide- and substance P-containing trigeminal ganglion neurons as assessed by calcium influx and immunocytochemistry, *Neurosci. Lett.*, 115 (1990) 171–176.
26 Tsien, R.Y., Fluorescent probes of cell signaling, *Annu. Rev. Neurosci.*, 12 (1989) 227–253.
27 Ui, M., Islet-activating protein, pertussis toxin: a probe for function of the inhibitory guanine nucleotide regulatory component of adenylate cyclase, *Trends Pharmacol. Sci.*, 5 (1984) 277–279.