FEBS Letters 417 (1997) 208–212 FEBS 19443

Expression of B-type endothelin receptor gene during neural development

Meei-Ling Tsaur[a,*], Yi-Chiou Wan[a], Fang-Pin Lai[a], Hwei-Fang Cheng[b]

[a]*Institute of Neuroscience, National Yang-Ming University, Taipei 112, Taiwan, ROC*
[b]*National Laboratories of Foods and Drugs, Department of Health, Executive Yuan, Taipei 115, Taiwan, ROC*

Received 16 September 1997

Abstract Mutations of the B-type endothelin receptor (ETRB) gene have been found to cause defects in the development of enteric neurons, which resulted in aganglionic megacolon in rodents and humans. To determine the distribution of ETRB mRNA during neural development, mainly in the CNS, in situ hybridization was applied at various developmental stages of rat. ETRB gene was abundantly expressed prenatally in the ventricular and subventricular zones, as well as postnatally in the ependymal and subependymal cells. ETRB mRNA was also strongly detected prenatally in the dorsal root ganglia, as well as postnatally in the cerebellar Bergmann glial cells and epithelial cells of choroid plexus. Our data suggest that ETRB acts as a regulator in the differentiation, proliferation, or migration of neural cells during development.

Key words: B-type endothelin receptor; mRNA distribution; Nervous system; In situ hybridization; Neural development

1. Introduction

Endothelins (ETs), including ET1, ET2, and ET3, were originally characterized as potent vasoactive peptides secreted by vasoactive endothelial cells [1,2]. Two types of ET receptor cDNAs have been cloned and both belong to the superfamily of G protein-coupled heptahelical receptors [3–5]. The B-type endothelin receptor (ETRB) exhibited equal affinity for the three endothelin isopeptides [4]. ETRB mRNA was widely distributed, with a prominent expression in the adult central nervous system (CNS) [4,6,7].

Recent evidence indicated that the function of ETRB was important for neural development. Mutations in the ETRB gene produced congenital aganglionic megacolon associated with pigment abnormalities in rodents and humans, which was due to defects in the development of two neural crest-derived cell lineages to enteric neurons and epidermal melanocytes [8–12]. In addition, it was found that selective induction of ETRB mRNA was associated with the differentiation of an embryonic cell line to neural lineage [13].

To elucidate the physiological role of ETRB during neural development, one of the useful steps is to determine its distribution in the nervous system at various developmental stages. In this report, using in situ hybridization, we found that the ETRB gene was most abundantly expressed in the developing nervous system, such as the ventricular and subventricular zones, further supporting that the function of ETRB is important for neural development.

*Corresponding author. Fax: (886) (2) 820-0259.
E-mail: mltsaur@ym.edu.tw

2. Materials and methods

2.1. Animal preparation

Sprague-Dawley rats were used, including day 10–18 embryos (E10–E18) from pregnant females, postnatal day 2–15 pups (P2–P15), and adult males (>2 months). Animals were anesthetized with halothane and decapitated. Whole embryos from uterus, as well as tissues from pup and adult rats, were quickly submerged in dry ice powder and frozen at −80°C before use.

2.2. Synthesis of riboprobes

A 0.6 kbp *Eco*RI fragment of rat ETRB cDNA [7], containing the seventh transmembrane domain to the polyA tail of the 3′ non-coding region, was subcloned into SK^+ vector in the sense orientation. In the presence of [α-^{35}S]UTP, antisense and sense cRNA probes were made from plasmids linearized with *Bam*HI and *Hin*dIII, followed by in vitro transcription with T7 and T3 RNA polymerases respectively.

2.3. In situ hybridization (ISH)

Large sizes of frozen samples were sectioned directly by cryostat, while small sizes of frozen samples were embedded in OCT compound before sectioning. Sections of 10 μm were mounted onto pre-treated slides, air-dried, and fixed with 3% formaldehyde in DEPC-treated phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate [pH 7.4]) for 15 min. The following steps were exactly the same as described previously [14]. Neuroanatomy was identified according to Paxinos et al. [15,16].

3. Results

3.1. General distribution of ETRB mRNA in rat nervous system

The distribution of ETRB mRNA in the rat nervous system, including embryonic (E12–E18), early postnatal (P2–P15) and adult stages, was examined by ISH using the antisense riboprobe. The sense riboprobe giving background signal was used as negative control. In the developing CNS, the ETRB gene was abundantly expressed in cells lining the central canal of spinal cord and ventricles, including the aqueduct of Sylvius, the 3rd and the 4th ventricles, but not the lateral ventricle (Fig. 1). In the peripheral nervous system (PNS), ETRB signal was concentrated in the developing dorsal root ganglia (Fig. 1), which will form clusters of sensory neurons. The ETRB gene was also expressed in other regions of the PNS; however, this is not described here.

In adult rat brain, ETRB mRNA was greatly distributed not only in cells lining the ventricles, including the lateral, the 3rd and the 4th ventricles, but also within the ventricles (Fig. 2A,C). In addition, ETRB mRNA was strongly detected in the cerebellum (Fig. 2C). When compared with negative control of ISH in the hippocampus, the ETRB signal was weakly but significantly detected in the stratum lacunosum moleculare (Fig. 2B); however, it was at background level in regions with high cell density, such as the dentate granule cell layer and CA1 pyramidal cell layer (Fig. 2C,D). Other brain areas showed weak but significant expression, including midbrain, brain stem, and olfactory bulb (Fig. 2C,D). Re-

PII S0014-5793(97)01295-7

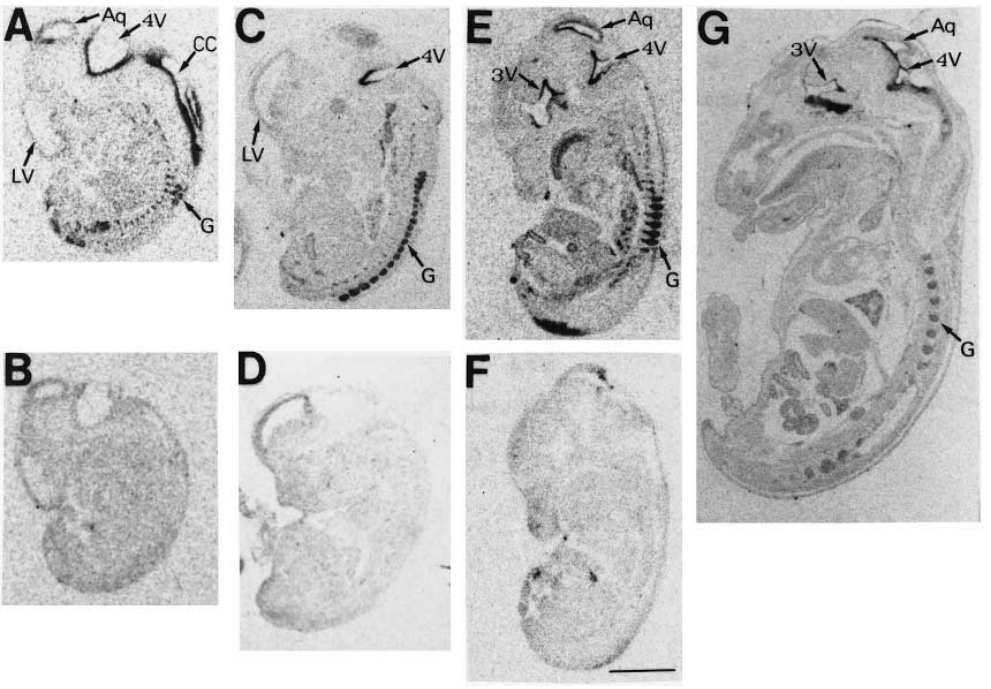

Fig. 1. Distribution of ETRB mRNA in rat embryo. X-ray film autoradiograms of sagittal sections after ISH with antisense (A, C, E, G) and sense (B, D, F) cRNA probes. A and B: at E13. C and D: at E14. E and F: at E15. G: at E18. Aq, aqueduct of Sylvius; CC, the central canal of the spinal cord; G, a dorsal root ganglion; 3V, 3rd ventricle; 4V, 4th ventricle; LV, lateral ventricle. Scale bar: A and B, 2.0 mm; C–F, 2.3 mm; G, 2.9 mm.

gions showing a strong ETRB signal in adult brain or the nervous system of embryo will be further demonstrated as follows.

3.2. Expression of ETRB gene in cells lining the ventricles

During the differentiation of the walls of the neural tube, ETRB mRNA was most strongly detected in the ventricular and subventricular zones (Fig. 3A,E). The ventricular zone contains cells in a single layer immediately lining the ventricles, whereas the subventricular zone is in multiple cell layers surrounding the ventricular zone. Around E13, the subventricular zone was extremely thick and compacted with cells, in which ETRB mRNA was distributed in the inner layers but was little detected in the outer layers (Fig. 3A,E). As the embryo grew, the ETRB signal remained in the subventricular zone, which was thinner at E16 than it was at E13 (Fig. 3B,F). Just before birth, ETRB mRNA was localized in the ependymal cells (Fig. 3C,G), which belong to a type of glia and are derived from the ventricular cells [17]. In addition to the ependyma, ETRB mRNA was concentrated in the subependyma of certain regions surrounding the adult lateral ventricle (Fig. 3D,H).

3.3. Expression of ETRB gene in the cerebellum

ETRB signal was scattered over the primitive cerebellum during development, with only a little higher density in the

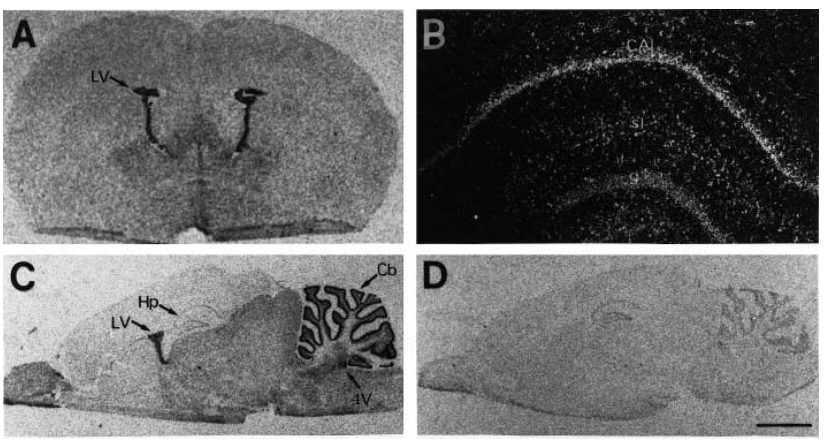

Fig. 2. Distribution of ETRB mRNA in adult rat brain. A: X-ray film autoradiogram of coronal section with antisense probe. B: Dark-field microscopic photograph of emulsion autoradiograms in coronal section with antisense probe. C and D: X-ray film autoradiograms of sagittal sections with antisense and sense probes respectively. CA1, CA1 pyramidal cells; Cb, cerebellum; g, dentate granule cells; Hp, hippocampus; LV, lateral ventricle; sl, stratum lacunosum moleculare; 4V, 4th ventricle. Scale bar: A, 1.9 mm; B, 375 μm; C and D, 3.2 mm.

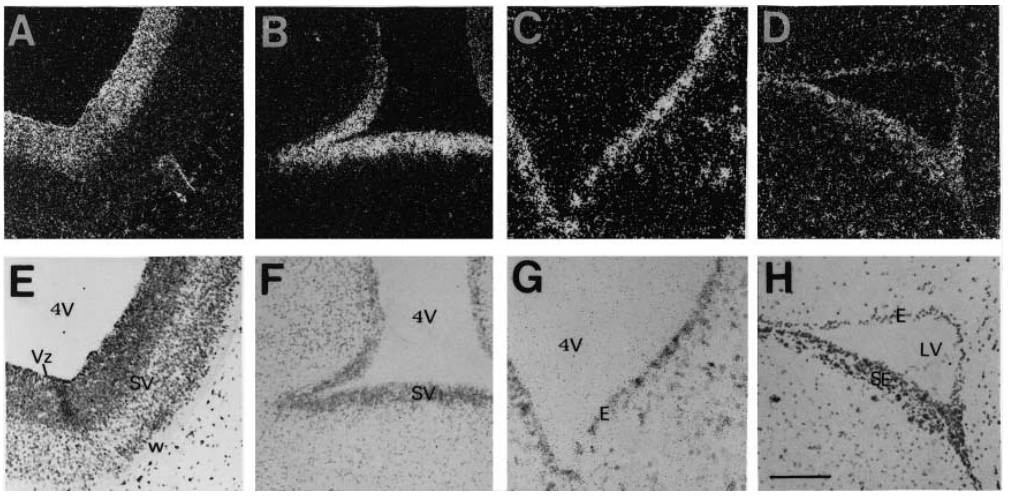

Fig. 3. Localization of ETRB mRNA in cells lining the ventricles during development. Dark-field (A–D) and bright-field (E–H) microscopic autoradiograms of sagittal sections are shown. A/E: at E13. B/F: at E16. C/G: at E18. D/H: in adulthood. E, ependyma; 4V, 4th ventricle; LV, lateral ventricle; SE, subependyma; SV, subventricular zone; Vz, ventricular zone; w, white matter. Scale bar: 150 μm, except C/G, 75 μm.

multiple Purkinje cell layers (Fig. 4A,E). As granule neuron precursors proliferated in the external germinal layer, the ETRB signal was found mainly in the Purkinje cell layer (Fig. 4B,F). Gradually, when most granule neuron precursors had migrated to the granular layer and the molecular layer had also become broader, ETRB mRNA was concentrated in the zigzag Purkinje cell layer (Fig. 4C,G). During the proliferation and migration of granule neuron precursors, little ETRB mRNA was detected in the external germinal layer, the molecular layer, or the granular layer (Fig. 4A–C,E–G).

After completion of neuron migration, the ETRB gene was strongly expressed in the single Purkinje cell layer and localized just beside the cell bodies of Purkinje neurons (Fig. 4D,H). Nevertheless, a weak but significant signal was also present in the granular layer. Since the localizations of ETRB mRNA and the Bergmann glia overlap in the Purkinje cell layer [18], the cell type expressing ETRB is very likely to be Bergmann glia.

3.4. Expression of ETRB gene in the dorsal root ganglia

During the development of PNS, the ETRB gene was strongly expressed in the dorsal root ganglia (around E13–E15, Fig. 1A,C,E), especially in their axon bundles toward visceral organs (Fig. 5A,B,D). In the developing axon bundles near the dorsal root ganglion at E13, ETRB mRNA was localized in the spindle-shaped cells, but little in the polygonal or rounded cells (Fig. 5D). Similar to the enteric neurons and epidermal melanocytes as described above, cells in the dorsal root ganglia are also derived from neural crest cells [18]. However, the ETRB signal in the dorsal root ganglia and their axon bundles gradually decreased afterwards (Fig. 1G), and could not be detected postnatally (Fig. 5C,F).

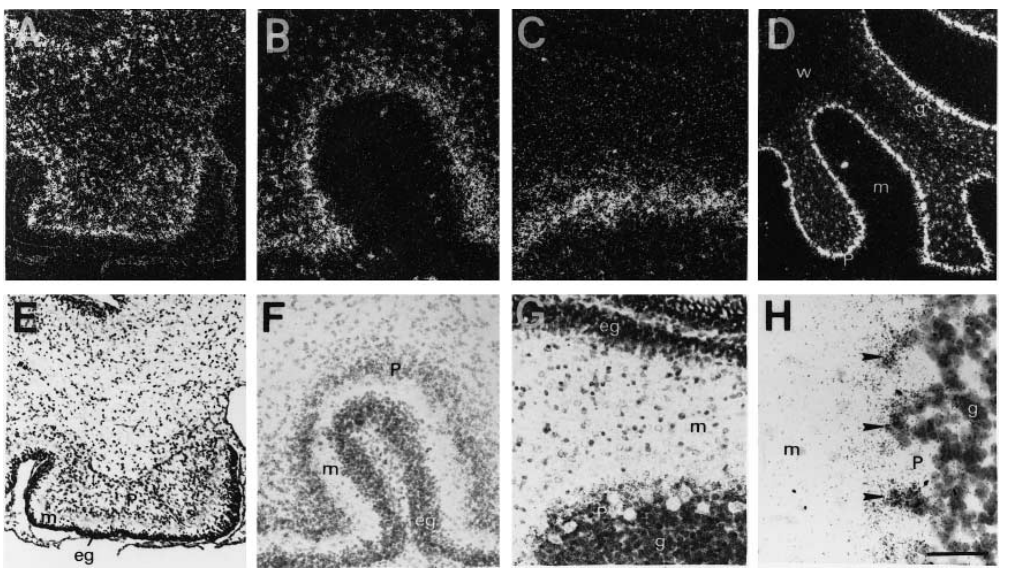

Fig. 4. Localization of ETRB mRNA in the cerebellum during development. Dark-field (A–D) and bright-field (E–H) microscopic autoradiograms are shown. A/E: at P2 (coronal section). B/F: at P4 (coronal section). C/G: at P15 (coronal section). D and H: in adulthood (sagittal section). Arrowheads indicate that signal was located between the cell bodies of Purkinje neurons. eg, external germinal layer; g, granular layer; m, molecular layer; P, Purkinje cell layer; w, white matter. Scale bar: A/E and B/F, 150 μm; C/G, 75 μm; D, 375 μm; H, 38 μm.

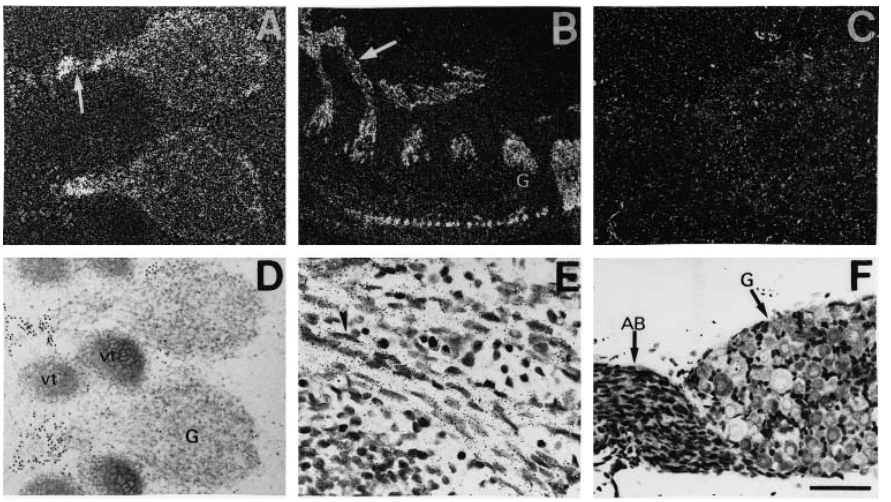

Fig. 5. Expression of ETRB gene in the dorsal root ganglia. Dark-field (A–C) and bright-field (D–F) microscopic autoradiograms of sagittal sections are shown. A/D: two intact dorsal root ganglia and their axon bundles (arrow) at E15. B: axon bundles (arrow) and several incomplete dorsal root ganglia at E14. E: signal in the spindle-shaped cells (arrowhead) of developing axon bundles at E13. C/F: a dorsal root ganglion and its axon bundles at P6. AB, axon bundles; G, a dorsal root ganglion; vt, vertebra. Scale bar: A/D, 150 μm; B, 375 μm; C/F, 75 μm; E, 38 μm.

3.5. *Expression of ETRB gene in the choroid plexus*

The choroid plexus, a capillary network extending into the ventricle cavity, constitutes the blood-brain barrier [18]. Choroid plexus primordia were formed no later than E12, however, little ETRB signal could be detected prenatally (Fig. 6A,B,E,F). The ETRB gene was strongly expressed postnatally in the choroid plexus within the lateral, the 3rd and the 4th ventricles (Figs. 2 and 6C,D,G,H). At the cellular level, ETRB mRNA was localized in the epithelial cells of the choroid plexus (Fig. 6C,D,G,H), which are modified ependymal cells [17,18]. Consistent with previous observations [6], the ETRB gene was highly expressed in the glial cells of adult CNS, including the ependymal cells lining the ventricles, the epithelial cells of the choroid plexus within the ventricles, and the Bergmann glial cells in the cerebellar Purkinje cell layer.

In summary, we have shown the spatiotemporal expression of the ETRB gene in rat nervous system. In the CNS, the ETRB gene was abundantly expressed in cells lining the ventricles throughout life (except in the lateral ventricle, where it was expressed postnatally only), as well as postnatally in the cerebellar Bergmann glia and epithelial cells of the choroid plexus. In the PNS, ETRB mRNA was strongly detected in the dorsal root ganglia and their axon bundles at the embryonic stage.

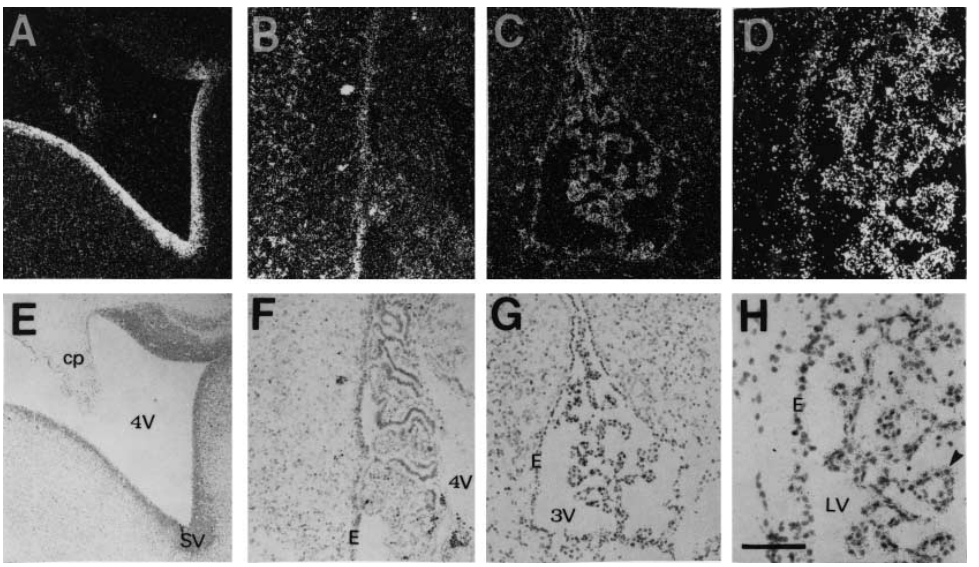

Fig. 6. Expression of the ETRB gene in the choroid plexus. Dark-field (A–D) and bright-field (E–H) microscopic autoradiograms of sagittal (A/E, B/F) and coronal (C/G, D/H) sections are shown. A/E: a choroid plexus primordium (cp) at E14. B/F: a longitudinal choroid plexus at E18. C/G: a complete choroid plexus in adulthood. D/H: part of the choroid plexus in adulthood. The arrowhead indicates the epithelial cell of the choroid plexus. E, ependyma; LV, lateral ventricle; 3V, 3rd ventricle; 4V, 4th ventricle. Scale bar: A/E, 375 μm; B/F and C/G, 150 μm; D/H, 75 μm.

4. Discussion

We are the first to demonstrate that the ETRB gene is highly expressed prenatally in the ventricular and subventricular zones, as well as the subependyma of adult lateral ventricle. Both findings are highly significant because these areas contain neural stem cells, which are crucial for the formation of the mammalian nervous system during embryogenesis [19], and for neuroregeneration in adulthood [20]. These neural stem cells are of great value for patients with neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases. Consistent with our results, a recent report indicated that activation of ETRB had a mitogenic effect in cultured astrocytes [21]. In the CNS, since ETRB mRNA was mainly localized in glial cells and neural stem cells ([6], this report), it is likely that expression of the ETRB gene is important for the differentiation and proliferation of glial cells from neural stem cells, as well as the initiation of gliosis in adulthood after brain damage [22].

It has been found that activation of ETRB could increase the effectiveness of EGF to stimulate wound closure by promoting cell migration [23]. Interestingly, activation of ETRB could also induce cytoskeletal actin reorganization in cultured astrocytes [24]. It is possible that ETRB plays a role in the activity of glia in directing neuronal migration, in addition to differentiation and proliferation. For example, ETRB mRNA was localized in the Bergmann glia. During cerebellar development, Bergmann glias can direct granule neuron precursors to migrate along their long processes from the external germinal layer to the granular layer. After that, Bergmann glias retract their long processes and become satellite cells to protect Purkinje neurons [18].

Taken together, it is likely that activation of ETRB can evoke different functions in different types of cells at different developmental stages, such as proliferation, differentiation and migration. Although only aganglionic megacolon and white coat color phenotypes have been found to be associated with ETRB mutations, the significance of abundant expression of the ETRB gene in neural stem cells reported here remains to be determined.

Acknowledgements: We are grateful to Larry Hung and Dr. Sue Lin-Chao for useful suggestions. This work was supported by Grant NSC 87-2314-B-010-108 (the National Scientific Council, ROC).

References

[1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 332, 411–415.
[2] Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989) Proc. Natl. Acad. Sci. USA 86, 2863–2867.
[3] Arai, H., Hori, S., Aramori, I., Ohkubo, H. and Nakanishi, S. (1990) Nature 348, 730–732.
[4] Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) Nature 348, 732–735.
[5] Huggins, J.P., Pelton, J.T. and Miller, R.C. (1993) Pharmacol. Ther. 59, 55–123.
[6] Hori, S., Komatsu, Y., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1992) Endocrinology 130, 1885–1895.
[7] Cheng, H.-F., Su, Y.-M., Yeh, J.-R. and Chang, K.-J. (1993) Mol. Pharmacol. 44, 533–538.
[8] Puffenberger, E.G., Hosoda, K., Washington, S.S., Nakao, K., deWit, D., Yanagisawa, M. and Chakravarti, A. (1994) Cell 79, 1257–1266.
[9] Hosoda, K., Hammer, R.E., Richardson, J.A., Baynash, A.G., Cheung, J.C., Giaid, A. and Yanagisawa, M. (1994) Cell 79, 1267–1276.
[10] Baynash, A.G., Hosoda, K., Giaid, A., Richardson, J.A., Emoto, N., Hammer, R.E. and Yanagisawa, M. (1994) Cell 79, 1277–1285.
[11] Kapur, R.P., Sweetser, D.A., Doggett, B., Siebert, J.R. and Palmiter, R.D. (1995) Development 121, 3787–3795.
[12] Gariepy, C.E., Cass, D.T. and Yanagisawa, M. (1996) Proc. Natl. Acad. Sci. USA 93, 867–872.
[13] Monge, J.C., Stewart, D.J. and Cernacek, P. (1995) J. Biol. Chem. 270, 15385–15390.
[14] Tsaur, M.-L., Sheng, M., Lowenstein, D.H., Jan, Y.N. and Jan, L.Y. (1992) Neuron 8, 1055–1067.
[15] Paxinos, G. and Watson, C. (1986) The Rat Brain in Stereotaxic Coordinates, Academic Press, San Diego, CA.
[16] Paxinos, G., Ashwell, K.W.S. and Törk, I. (1994) Atlas of the Developing Rat Nervous System, Academic Press, San Diego, CA.
[17] Kettenmann, H. and Ransom, B.R. (1995) Neuroglia, Oxford University Press, New York.
[18] Parent, A. (1996) Carpenter's Human Neuroanatomy, Williams and Wilkins, Baltimore, MD.
[19] Reynolds, B.A. and Weiss, S. (1996) Dev. Biol. 175, 1–13.
[20] Gritti, A., Parati, E.A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morassutti, D.J., Roisen, F., Nickel, D.D. and Vescovi, A. (1996) J. Neurosci. 16, 1091–1100.
[21] Lazarini, F., Strosberg, A.D., Couraud, P.O. and Cazaubon, S.M. (1996) J. Neurochem. 66, 459–465.
[22] Hama, H., Kasuya, Y., Sakurai, T., Yamada, G., Suzuki, N., Masaki, T. and Goto, K. (1997) J. Neurosci. Res. 47, 590–602.
[23] Tao, W., Liou, G.I., Wu, X. and Reinach, P.S. (1995) Invest. Ophthal. Vis. Sci. 36, 2614–2622.
[24] Koyama, Y. and Baba, A. (1996) Glia 16, 342–350.