Prenatal Development of Gonadotropin Releasing Hormone (GnRH) Neurons in the Rat Brain

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The present experiment was carried out 1) to study the developmental topography of GnRH neuronal system and 2) to characterize the cellular localization of GnRH neurons in the prenatal brain development of the rat. At embryonic day (E) 14.5, immunoreactive cell bodies of GnRH were first seen in the nasal septum and in the ganglion terminale located in the ventral protion of the caudal olfactory bulb. Two days later (E 16.5), GnRH-containing neurons were observed at the level of olfactory tubercle and diagonal band of Broca, which is the first appearance in the intracerebral region. From E 18.5, the topographic pattern of immunoreactive GnRH perikarya was similar to that of adult rats. The present data suggest that GnRH neurons were originated from the nasal septum and gradually extended to the hypothalamic regions with increasing fetal age.

KEY WORDS: Gonadotropin releasing hormone (GnRH), Prenatal development, Immunohis-
tochemistry, Hypothalamus.

It is now firmly established that GnRH is a key brain regulator of the control of gonadotropin secretion in the mammalian reproduction. In the rat, numerous immunohistochemical studies showed that this neurohormone is produced in certain neurons which are predominantly located in the medial septum-diagonal band complex, ganglion terminale and preoptic area (Barry, 1979; Witkin and Silverman, 1983). From these neurons, an extensive network of GnRH-containing fibers projects to the median eminence and the organum vasculosum of lamina terminalis (OVLT) which are the main sites of GnRH release into the perivascular space of the fenestrated capillaries. In addition, smaller projections reach to a variety of brain areas including the nasal and

olfactory system, amygdala, periventricular hupothalamus, interpeduncular nucleus and central gray (King and Millar, 1980; Shivers et al., 1983; Barry et al., 1985).

While the neuroanatomy of GnRH neurons has been studied extensively in young and adult rats, little is known about the prenatal developmental topography of GnRH perikarya and nerve fibres during prenatal stage. In most studies, GnRH-synthesizing neurons were detected only postnatally, while the first immunoreactive cell processes appeared in the OVLT and median eminence at the embryonic day (E) 18.5 and 19.5, respectively (Daikoku et al., 1978; Kawano et al., 1980; Watanabe, 1980). These studies suggest that the initiation of gonadotropin expression in the pituitary gonadotrophs precedes the production and release of GnRH. Recently, it has been found that GnRH is synthesized in the certain neurons along with nervus terminalis as early as E 15, and GnRH

neurons migrate into the forebrain with age (Salis-
bury *et al.*, 1982; Schwanzel-Fukuda *et al.*, 1981,
1988; Schwanzel-Fukuda and Pfaff, 1989).
However, one of the important questions whether
GnRH peptide resided in the ganglion cells is
merely taken up from another sources such as the
placenta or is locally synthesized remains to be
resolved. Thus, the present study, using immuno-
histochemistry and *in situ* hybridization histoche-
mistry, aims to elucidate 1) the ontogenesis of
GnRH neurons in the rat brain and 2) whether
immunoreactive GnRH neruons in the nasal area
synthesize GnRH peptide by their own synthetic
machinery.

Materials and Methods

Animals

Adult Sprague-Dawley rats (Seoul National Uni-
versity Animal Breeding Center) were housed with
lights on 06:00-20:00 hr, food and water avaliable
ad libitum. The rats were mated overnight and
examined for the presence of semen in the vagina
in the next morning. This time was assigned to
embryonic day 0.5 (E 0.5). The pregnant rats were
caged individually after insemination, and thereaf-
ter embryos at various stages (E 10.5-E 19.5)
were taken for immunocytochemical and *in situ*
hybridization analyses.

Tissue Preparations

Pregnant female rats were anasesthetized with
ethyl ether in the morning (10:00-11:00 h). Sub-
sequently, fetal rats removed from the uterus were
decapitated, and their whole brains were im-
mediatly dissected and immersed overnight in 4%
paraformaldehyde in 0.1 M phosphate buffer (pH
7.4).

The brains were cut in the saggital or coronal
planes on a cryostat (American Optics) at 8 um
section thickness. The section were placed onto
gelatin-coated glass slides and stored at -80°C un-
til use. Since a prolonged storage of tissue sec-
tions seemed to decrease the intensity of GnRH
immunoreactivity and GnRH mRNA, tissue sec-
tions were used within one month. Solutions were
prepared with RNase-free reagents and disethyl-
pyrocarbonate (DEPC)-treated distilled water to

further eliminated the RNase contamination.

Immunohistochemistry

For a localization of immunoreactive GnRH,
avidin-biotin complex (ABC) method were used
(Hsu *et al.*, 1981). Tissue sections on slides were
air dried, dipped in 0.02 M phosphate buffered
saline (PBS, pH 7.4) twice for 5 min. Tissue sec-
tions were incubated with 40 μl of normal goat
serum diluted 1:20 for 30 min to exclude the
nonspecific binding before the primary antibody
application. Slides were then applied with 50 μl
of the primary antibody, rabbit-derived anti-
GnRH (Immuno. Nuclear Co.) with a final dilution
of 1:2000 for overnight at 4°C. Sections were
washed with PBS for 10 min twice, incubated with
0.05% periodic acid to exclude the endogenous
peroxidase and applied with 50 μl of biotinylated
goat anti-rabbit IgG (DAKO Co.) for 30 min at
room temperature. After washing an excess secon-
dary antiserum, avidin-biotin peroxidase complex
diluted 1:250 was treated for 30 min at room
temperature. After the last wash with PBS, the
sections were incubated with 0.5%
3,3′-diaminobenizidine (DAB)-Ni in 0.01% hyd-
rogen peroxide in PBS for 5 min, washed, dehy-
drated, mounted in synthetic mounting medium,
and observed under light microscope.

In situ Hybridization histochemistry

GnRH oligonucleotide probe (29-mer) com-
plementary to the rat mRNA encoding GnRH was
synthesized by automated DNA synthesizer (Gene-
tic Engineering Center, KIST, Seoul). This probe
was extensively used for GnRH mRNA determina-
tion (Park *et al.*, 1988; Kim *et al.*, 1989; Choi *et
al.*, et al., 1990; Lee *et al.*, 1990). This oligomer
was labeled by the 5′-end labeling method (Davis
et al., 1986).

In situ hybridization histochemistry were per-
formed as previously described (Shivers *et al.*,
1986; Choi *et al.*, 1990). The tissue sections were
air-dried at room temperature and soaked for 10
min in 0.25% acetic anhydride in 0.1 M triethano-
lamine hydrochloride-0.9% NaCl. Tissue sections
were then dehydrated through a series of ethanol
solution, and rehydrated to water in descending
series of ethanol. Fifty μl of prehybridization buf-
fer is the same as the hybridization buffer except

for labeled GnRH probe. It consists of 4 × SSC (1 × SSC = 0.15 M NaCl-0.015 M sodium citrate, pH 7.2), 50% deionized formamide, 10% dextran sulfate (Sigma), 250 μg/ml yeast tRNA, 500 μg/ml sonicated, heat denatured salmon sperm DNA, and 1 × Denhrdt's solution (0.02% ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA). The control sections were pretreated with RNase and unlabeled GnRH probe before hybridization step. Hybridization with ^{32}P-labeled probe proceeded for approximately 2 days at room temperature in a humid chamber. The sections were initially rinsed with 2 × SSC twice, and then washed with 0.5×SSC for overnight at room temperature. After drying, the tissues were exposed to NTB$_2$ (Eastman Kodak) emulsion for two weeks. Emulsions were developed, and tissues were counterstained with cresylviolet. GnRH mRNA signals were observed under both a dark-field and a light-field microscopes.

Results

E 14.5

Neither GnRH immunoreactivity nor GnRH mRNA hybridization signal was detected until E 14.5. Cells containing GnRH were first detected in the nasal septum at E 14.5 (Figs. 1 and 9). Several GnRH containing neurons were also found at the ventral portion of the caudal olfactory bulb close to the subarachnoid space within the anlage of ganglion terminale (Figs. 2, 9 and 10). In this region, non-immunoreactive neurons wer also seen with the GnRH-containing neurons. GnRH immunoreactive neurons were dispersed and did not form apparent group or clusters. The shape of GnRH perikarya was ovoid or elongated, while cell processes were not labeled. GnRH-contaiing neurons were not detected in any region of the cnetral nervous system (CNS) at this stage. In situ hybridization revealed that the distribution of GnRH mRNA was similar to that of immunoreactive GnRH. Particularly, in the region of nasal septum, GnRH mRNA-containing cells were intensely clustered (Fig. 3). No GnRH mRNA hybridization signal was found in the intracerebral region.

E 16.5

At this stage, the number of GnRH-containing neurons increased. The distribution of immunoreactive GnRH perikarya was more extensive. Most rostrally, GnRH-containing neurons were seen in the connective tissues next to the nasal septum. The perikarya of these neurons were elongated with short immunoreactive processes. The peripheral GnRH neurons appeared indistinguishable in their morphology, when compared to GnRH neurons in the CNS. Most neurons in the nasal septum were oriented parallel to the surface of adjacent nasal cavity and their processes did not reach to the epithelium.

In association with the olfactory bulb, a few GnRH-immunoreactive neurons were found at the ventromedial portion in the ganglion terminale. In the ganglion terminale, GnRH-immunoreactive neurons were intermingled with GnRH-negative neurons. In the nervus terminalis, GnRH-immunoreactive fibres were not seen at this stage of the brain development.

Further caudally, at the level of olfactory tubercle and diagonal band of Broca, GnRH-immunoreactive cells were detected. These GnRH neurons were the first ones that appeared in the intracerebral region and had a fusiform structure (Figs. 4 and 5). At this stage, GnRH cell processes were only in the close vicinity to the GnRH perikarya. No GnRH immunoreactivity was detected in the OVLT or in the median eminence at this stage.

E 18.5

At this stage, GnRH-containing neurons were present in all areas described at E 16.5. The number of GnRH-positive neurons increased in several areas, while the morphological appearance remained relatively unchanged. In addition to these regions, a large number of GnRH-positive neurons were located in the septum-diagonal complex with the majority of the cells residing close to the interhemisphere cleft (Figs. 6, 9 and 10). At the level of OVLT, most GnRH-immunoreactive neurons were found scattered throughout its externt and in the diagonal band of Broca (Fig. 7).
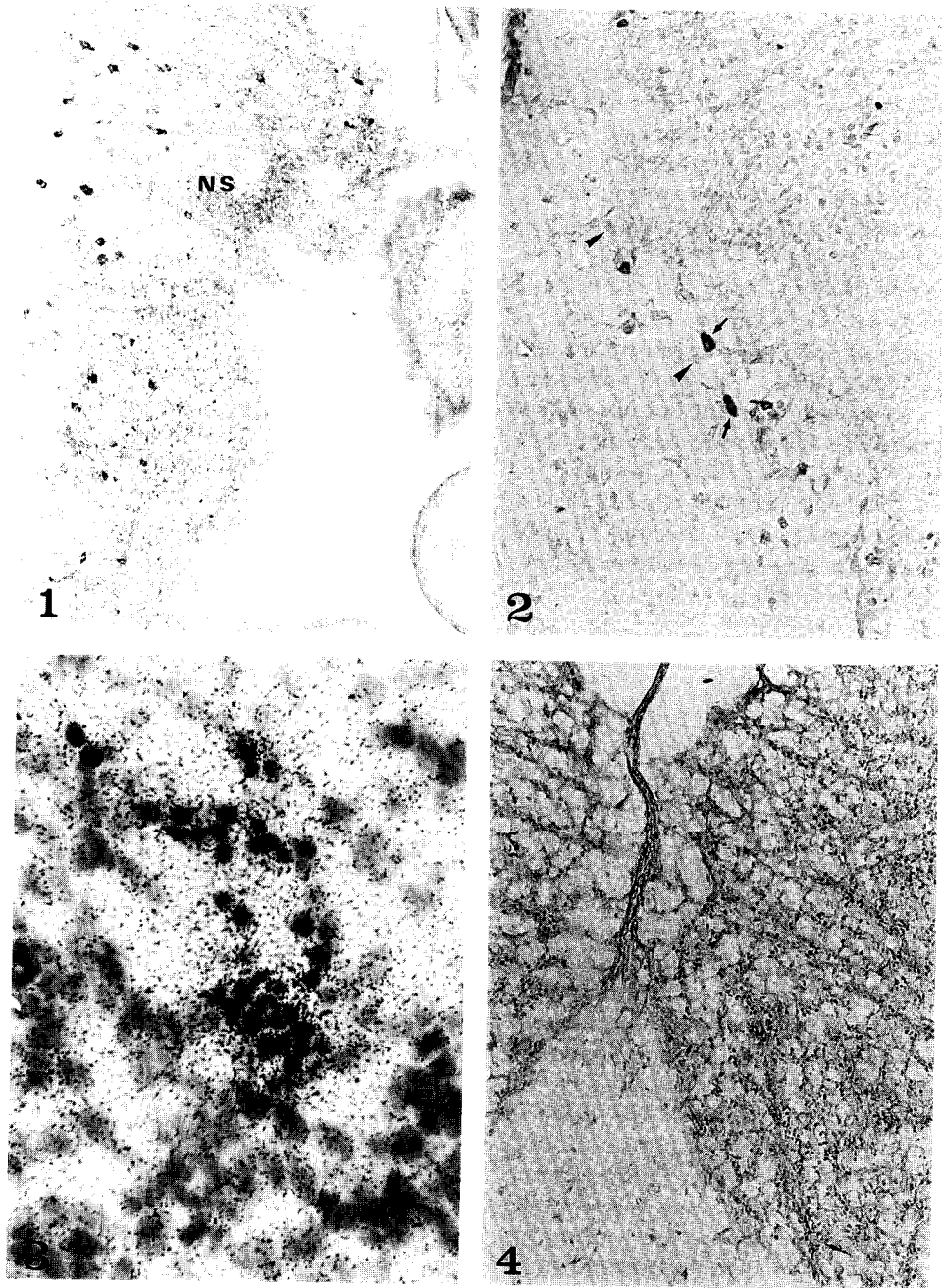
In the hypothalamus, clusters of GnRH-positive

Fig. 1. GnRH-immunoreactive neurons at the E 14.5. GnRH neurons are seen in the nasal septum (NS) and show ovoid type with their clear nucleus, × 200.

Fig. 2. Immunohistochemical demonstration of GnRH neurons at embryonic day 14.5 within the area of ganglion terminale (arrows). GnRH-immunoreactive neurons (arrowheads) are seen in the vicinity of GnRH immunoreactive neurons, × 200.

Fig. 3. Localization of GnRH mRNA at E 14.5 rat embryo by *in situ* hybridization histochemistyr. Hybrydization signals of GnRH mRNA are shown in the nasal septum, × 400.

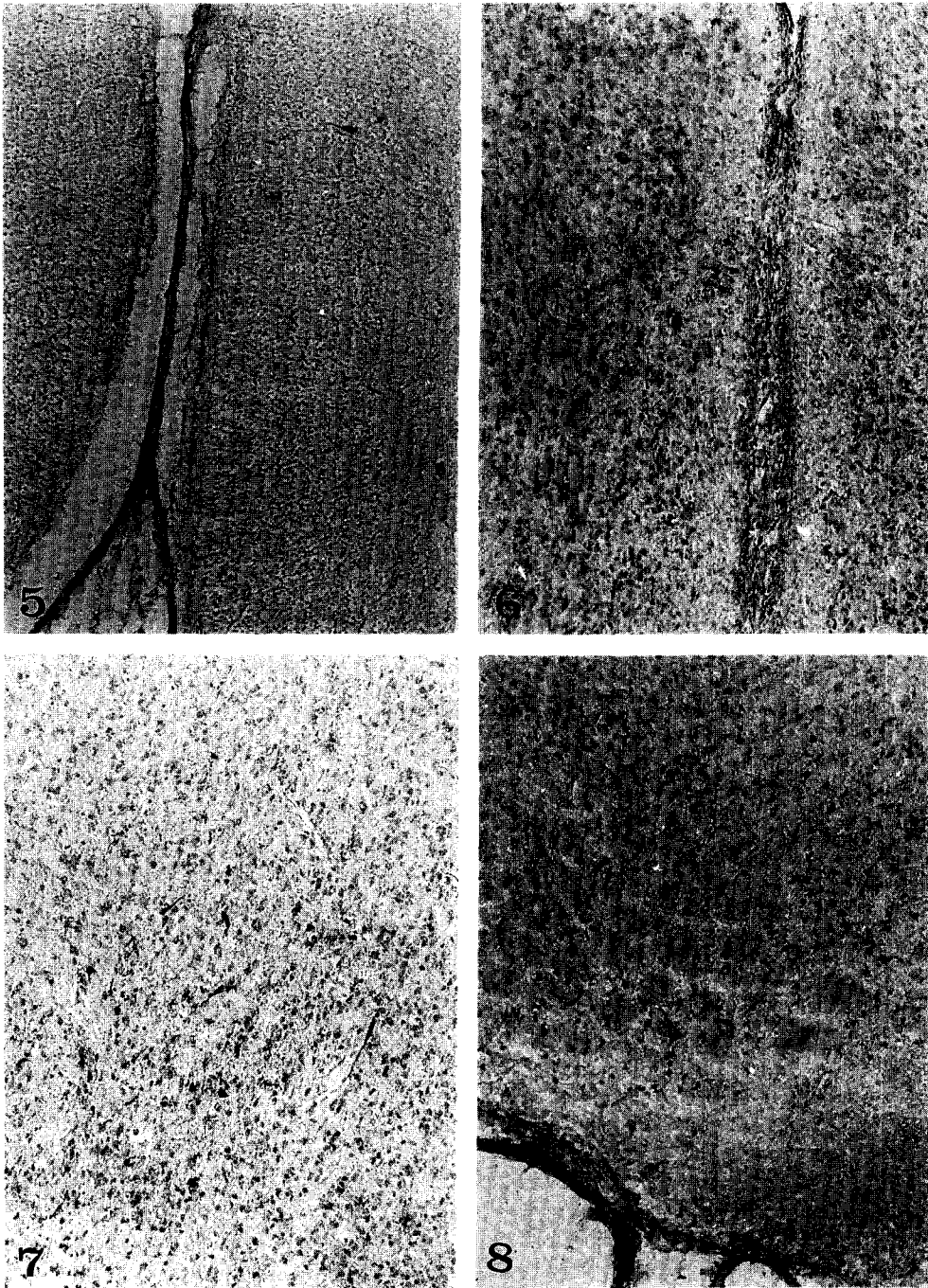**Fig. 4.** GnRH-immunoreactive neurons in the olfactory tubercle at E 16.5, × 200.

Fig. 5. Immunohistochemical demonstration of GnRH neurons in the diagonal band of Broca at E 16.5, × 200.
Fig. 6. GnRH-immunoreactive neurons at E 18.5. Note that GnRH neurons are seen close to the interhemisphere cleft at the level of diagonal band of Broca, × 200.
Fig. 7. GnRH-immunoreactive neurons at E 18.5. Several GnRH neurons are seen lateral to the OVLT, × 200.
Fig. 8. GnRH-immunoreactive features at E 18.5. Adult type-like GnRH neuron with a long processes were seen lateral to optic chiasma, × 200.

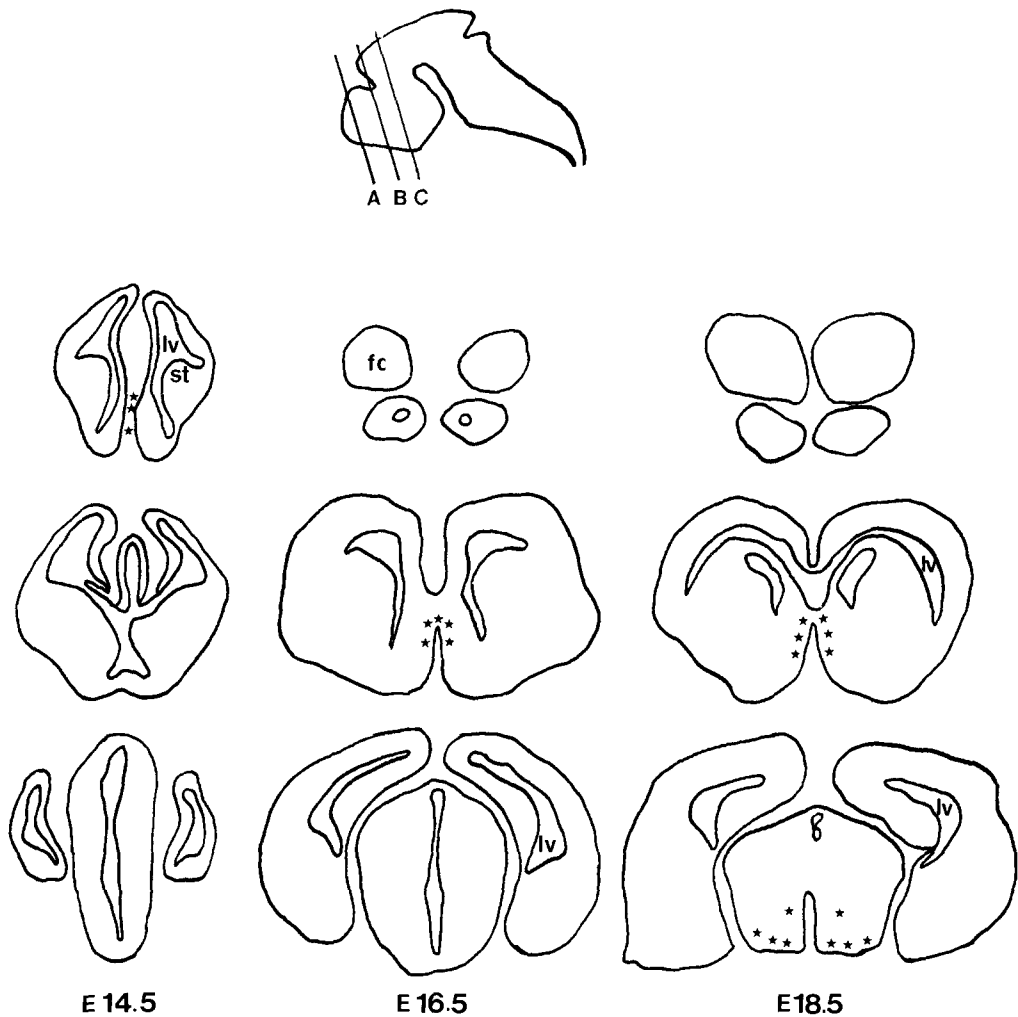E 14.5   E 16.5   E 18.5

Fig. 9. Schematic representation of GnRH-immunoreactive neurons in the fetal rat brain, coronal view. fc: frontal cortex, lv: lateral ventricle, st; striatum.

neurons were present lateral to the optic chiasm in an area next to the anterior hypothalamic nucleus, and in a more lateral area beneath the medial forebrain bundle. These neurons had well developed processes similar to the adult type (Fig. 8). At this state, the overall distribution of GnRH-containing fibres was similar to the one found in the mature rats. However, the total number of immunoreactive GnRH fibres was still much lower than that in the adult rats. The first immunoreactive GnRH cell processes were appeared in the OVLT at this stage.

Discussion

The present study showed that 1) GnRH-containing neurons are first detectable at E 14.5 in the nasal septum and ganglion cells in association with ganglion terminale near the ventral portion of the caudal olfactory bulb; 2) With in situ hybridization histochemistry, the hybridization signal of GnRH are also detected in the similar regions containing GnRH-immunoreactive peptide, which
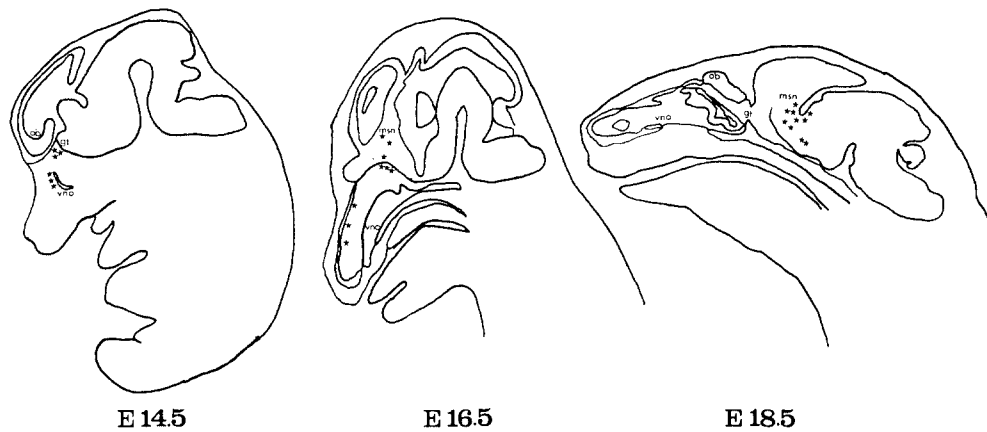
E 14.5   E 16.5   E 18.5

Fig. 10. Schematic reprsentation of GnRH-immunoreactive neurons in the fetal rat brain, sagittal view. gt: ganglion terminale, msn: medial septal nucleus, ob: olfactory bulb, vno: vomeronasal organ.

implies that the GnRH neuron itself made GnRH peptide; 3) GnRH-immunoreactive neurons which originated from in the nasal region appear to migrate into the brain, probably at the level of ganglion terminale on E 16.5; and 4) The migratory pathway within the brain seems to proceed in a rostral to caudal direction.

By applying the Golgi staining method, Larsell (1950) previously found that the bipolar type cells are distinguished among the olfactory nerve fibres below the cirbriform plate. These bipolar cells appear to be the same as GnRH neurons and may be a part of the nervus terminalis (Schwanzel--Fukuda and Silverman, 1980). In the human fetus, moreover, the nervus terminalis is known to originate from the developing vomeronasal organ and forms irregular masses along the medial side of olfactory nerve (Pearson, 1941). Considering the facts that cell bodies of the vomeronasal nerve reside in the nasal epithelium of the vomeronasal organ and project to the accessory olfactory bulbs (Bojsen-Moller, 1975), and nervus terminalis is formed in part by migration of neurons from the vomeronasal organ (Pearson, 1941), it appears that the ganglion cells resides along the nervus terminalis. Therefore, the presence of GnRH-immunoreactive neurons in the nasal septum and ganglion terminale shown in the present study indicates a very similar distribution to that of nervus terminalis. Moreover, hybridization signal of GnRH mRNA appears in the region of nasal sep-

tum, while neither GnRH immunoreactivity nor GnRH mRNA signal was detected in the intracerebral region at E 14.5. One to two days later, GnRH-immunoreactive neurons with fusiform structure were found in the intracerebral region, such as ventral olfactory bulb, olfactory tubercle and diagonal band of Broca. These results, taken together, clearly showed that GnRH-immunoreactive neurons are originated from the vomeronasal organ and migrated along the nervus terminalis.

The origin of hypothalamic GnRH neurons from the vomeronasal organ was first described in the guinea pig (Schwanzel-Fukuda et al., 1981). Therafter, similar findings have been reported in various animals (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989; Ronnekleiv and Resko, 1990). However, the factor(s) that regulates the migration of GnRH neurons into the intracerebral region is unknown. In the brain of rhesus moneky, GnRH appeared first in fibres that project into the brain from the nasal region and these fibres may play a role for the migation (Ronnekleiv and Resko, 1990).

The function of nervus terminalis is generally unknown. However, it appears to be the sensory neuron in the rat (Bojsen-Moller, 1975) and this is the primary chemosensory neuron for mediating response to pheromones in the goldfish (Goldfoot et al., 1978). In addition, hypogonadotropic hypogonadism with decreased secondary sex characteristics and deficiency in gonadotropins was shown

to be associated with the malformation of the olfactory apparatus. It is then of importance to note that in the Kallman's syndrome, anosmia is simultaneously observed with the gonadal failure (Tagatz et al., 1970). These studies appear to indicate the importance of nervus terminalis in the development of GnRH neurons.

The initial appearance of GnRH in ganglion cells of the ganglion terminale coincided with the time in which Leydig cells of the fetal testis synthesizes testosterone and reponds to luteinizing hormone (Feldman and Bloch., 1978). Anatomically, the ganglion terminale and pituitary are close, but the primary capillary plexus is not yet formed until the 5th postnatal day (Page, 1986). Thus, there is a demand for another route of GnRH delievery to anterior pituitary in this early stage, since luteinizing hormone is known to be detected in the anterior pituitary as early as E 17.5 (Salisbury et al., 1982; Schwanzel-Fukuda et al., 1988). The gonadotrophs are able to respond to intraperitoneal injection of synthetic GnRH by releasing luteinizing hormone into the circulatory system at this stage (Salisbury et al., 1982). Therefore, it is possible to presume that GnRH released into the blood vessels of nasal epithelium or cerebrospinal fluid from GnRH neurons of ganglion terminale, which is located in the subarachnoid space, can reach gonadotrophs of the anterior pituitary through either or both of these routes. It is, however, yet unknown whether GnRH are not produced in the intracerebral neuroepithelium in the rat.

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흰쥐 태아 뇌에서 GnRH 신경세포의 초기발생과정

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시상하부에서 합성, 분비되는 gonadotropin-releasing hormone(GnRH) 신경세포의 초기발생과정을 조사하기 위하여 본 실험이 행해졌다. 면역조직화학적 방법과 in situ hybridization histochemistry를 이용한 결과, GnRH 신경세포는 임신 14.5일이 되는 시기에 코 승각과 종말신경절에서 나타나기 시작하였으며, 뇌안의 어느 지역에서도 GnRH의 양성면역의 염색이나 GnRH mRNA hybridization signal이 검출되지 않았다. 임신 16.5일 되는 시기에 GnRH 신경세포는 뇌 안쪽지역인 후각결절과 diagonal band of Broca에서도 분포하고 있었으며, 임신 18.5일 되는 시기에는 이미 성숙한 흰쥐에서와 비슷한 분포양상을 보여주었다. 특히 이 시기에 organum vasculosum lamina terminalis 지역에서 GnRH 신경섬유가 존재하였다. 이와같은 결과는 GnRH 신경세포가 발생초기에 대뇌 바깥 쪽에서 발생되어 대뇌 안쪽으로 이동되는 것으로 사료된다.