

Regional distribution and ontogeny of the first exon variants of the rat growth hormone receptor mRNA in the brain and the pituitary gland

Haruo Nogami, Min chul Lee, Hideaki Soya and Setsuji Hisano

*Laboratry of Neuroendocrinology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan*

*Laboratory of Exercise Biochemistry, University of Tsukuba Graduate School of Comprehensive Human Sciences, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8574, Japan*

\*Corresponding author. Tel.:81-298-53-3342; fax:81-298-53-3100.

E-mail: [hnogami@md.tsukuba.ac.jp](mailto:hnogami@md.tsukuba.ac.jp) (H. Nogami)

## ABSTRACT

Expression of the first exon variants of the rat growth hormone receptor mRNA was studied in the brain and the pituitary gland. Four of the five different variant mRNA previously identified in the liver were detected in the cerebral cortex by a conventional reverse-transcription polymerase chain reaction, and, unlike the data reported previously, a quantitative analysis revealed that approximately 90% of the total growth hormone receptor mRNA in the cerebral cortex is V1 form. The present results suggest that the V1 was also a dominant transcript in other brain areas and the pituitary gland, not only in adult but also in fetal and postnatal period. The growth hormone receptor expression in the brain was lower during fetal period than in adults, while in the pituitary gland, the expression is markedly higher in fetuses, suggesting a yet unknown role of growth hormone in the development of this organ.

## Key words

Growth hormone receptor, rat, brain, pituitary gland, variant mRNA, fetus,

## **Introduction**

The distribution of the growth hormone receptor (GHR) mRNA, GHR protein and the binding sites for GH in the brain has been extensively studied and many of the brain regions studied have been shown to express GHR [1-5]. Although the involvement of stress, ovarian steroid or glucocorticoids in the regulation of hippocampal GHR expression have been reported [6,7], the factors that regulate GHR expression in the other brain regions and the molecular mechanisms underlying it are remained to be elucidated. Since a number of functions have been postulated for GH in the brain including proliferation and maturation of neurons during the development of brain [5, 8], the understanding of the mechanisms of GHR gene expression is required to reveal the mechanisms of regulation of neural function and development by GH.

The rat GHR gene transcript is known to give two different classes of mature mRNA through specific splicing, a long form and a short form, which encode membrane receptors for GH and soluble binding protein for GH, respectively [9,10]. In addition to these variants with different C-terminal structure, multiple forms of GHR mRNA have been also reported which differ in the sequences in their 5'-UTR [11]. Since translation initiation codon is in the second exon that is common to all of these variant mRNAs, they give the same translation products. However, the presence of the multiple first exons in the GHR gene suggests that each variant mRNA is transcribed under the regulation of each specific promoter [11], and It is conceivable that a detailed examination of the distribution of these first exon variants provides some new insights into the regulatory mechanisms responsible for the region specific of expression of GHR gene [1-5].

In this study, we aimed at identifying the species of the first exon variant of GHR expressed in the different brain regions in the adult and developing rats. The analysis was also carried out in the pituitary gland in which GHR expression has been demonstrated [12] but any detailed analysis has not been carried out.

## **2. Methods**

### *2.1 Animals*

Sprague-Dawley male and female rats at 6 weeks of age were purchased from CLEA Japan Inc. (Tokyo, Japan) and acclimatized for 2 weeks before experiment. The animals were housed in light (12h dark and 12h light) and temperature (22C) controlled room, and allowed free access to standard diet and tap water. The sexual cycles were determined by the vaginal smear test. The animals were killed by cardiac puncture under the light ether anesthesia and the cerebral cortex (CTX), hippocampus (HP), hypothalamus (HT), cerebellum (CB), brain stem (BS), spinal cord (SP), and anterior pituitary gland (PIT) were dissected out as described previously [13,14]. The timed pregnant

Sprague-Dawley rats were also obtained from CLEA Japan Inc. at day 12 of gestation and housed in a same condition as described above. The day on which spermatozoa were found in a vaginal smear was designated as day 0 of pregnancy. Fetuses were dissected out at embryonic day 16 (E16), E18 and E20 under light ether anesthesia, and tissues were removed under the surgical microscope. The brain tissues and the pituitary gland were obtained from rats at postnatal day 5 (P5) and P25. The tissues were stored frozen at -80 C until RNA extraction.

## 2.2 RNA extraction and RT-PCR

Total RNA was extracted by the method described previously [15], and RNA concentration was determined by spectrophotometry at 260 nm. The cDNA synthesis was carried out using murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) and random hexamer primers set (Takara Shuzo, Shiga, Japan) at 42 C for 15 min according to the manufacturer's protocol. The composition of the conventional PCR reaction consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.05 U/μl EX-Taq polymerase, and 0.2 mM each of dATP, dGTP, dCTP, and dTTP (all from Takara Shuzo Co., Ltd., Shiga, Japan). After incubation at 94 C for 1 min, a PCR was performed for 27 cycles (cyclophilin D) or 40 cycles (for others) at 95 C for 15 sec, 60 C for 30 sec, followed by an additional incubation at 72 C for 5 min. The sequences of the primers used in this study are shown in Table 1. The PCR products were separated in 2% agarose gel and stained with ethidium bromide.

## 2.3 RNase protection assay

The cDNA fragments of GHR-V1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified using a primer pair shown in Table 1, cloned into pCRII plasmid (Invitrogen, Groningen, The Netherlands), and the <sup>32</sup>P-labeled cRNA probe was prepared as described [13]. Probe length of V1 was 218 bp and the expected size of protected band was 147 for V1 and 119 bp for other GHR mRNA species. GAPDH probe was 189 bp and the expected protected size was 81 bp.

The total RNA (5μg of total RNA from cerebral cortex and 1μg from liver) was dissolved in 20 μl hybridization buffer [400 mM NaCl, 1 mM EDTA, 40 mM PIPES (pH 6.4), and 80% formamide] containing 2 x 10<sup>4</sup> cpm each of V1 cRNA and GAPDH cRNA. After the hybridization overnight at 55 C, 150 μl RNase digestion buffer (300 mM sodium acetate, 5 mM EDTA, and 10 mM Tris-HCl, pH 7.5) containing 10 μg/ml RNase A and 100 U/ml RNase T1 (both from Roche Molecular Biochemicals, Indianapolis, IN) was added and the reaction mixture was incubated for 30 min at 37 C. The mixture was incubated at 37 C for an additional 15 min after addition of 2.5 μl proteinase K

(Roche Molecular Biochemicals; 20 mg/ml) and 10 µl 10% SDS. The protected RNA fragment was precipitated and analyzed by electrophoresis on 8% polyacrylamide gel containing 8 M urea, and analyzed by BAS5000 image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

#### *2.4 Quantitative RT-PCR*

Real-time PCR was performed using a Fast start universal SYBR green master mix (Roche Applied Science, Mannheim, Germany) according to a protocol supplied by the manufacturer. A stock of PCR product of a GH-receptor variant or cyclophilin D cDNA generated by PCR using the reverse transcription of adult rat liver was purified and used to construct a standard curve in every assay. After 10 min incubation at 95°C, the PCR reaction was carried out for 40 cycles at 95°C for 15 sec and 60°C for 30 sec. The amounts of mRNA were normalized for cyclophilin D mRNA.

#### *2.5 Determination of the relative abundance of different variant mRNAs*

We tried to determine the relative abundance of variant mRNAs in CTX and liver using real-time PCR. In this study, various first exon variants were amplified using variant specific sense primers and an antisense primer that is located in the second exon common to all variants, and the resultant cDNA was used as standard cDNA for the real-time PCR. Therefore, standard cDNAs for determination of variant mRNAs contained the common 119 bp second exon sequence. By the real time PCR using a pair of primers (s1 and as1 shown in Table 1) both of which are in the second exon, the relative molar ratio of standard cDNAs was determined. Then the standard cDNAs were diluted to be equivalent with respect to the molar concentration. Similarly, the molar ratio of the standard cDNA for determination of long form (amplified by T/s1 and L/as1) and that for short form (amplified by T/s1 and S/as1) were determined by real-time PCR using a pairs of primers of T/s1 and T/as1, both are in the common sequences to long and short form (Table 1 and Fig. 1). The relative abundance of each mRNA was determined in the cerebral cortex and the liver using cDNAs prepared to be the same molar concentration as the standard.

#### *2.6 Statistical analyses*

The significance of differences was determined by one-way analysis of variance followed by Fisher's PLSD test, or the Student's t test.

### 3. Results

The expression of GHR variant mRNAs in the cerebral cortex and the liver was examined by a conventional RT-PCR (Fig. 1A). All variant mRNAs were detected in CTX as well as in liver, except for V5 mRNA which was not detectable at the present PCR condition. The RNA protection assay using V1 cRNA probe, detected only protected band for V1 mRNA in CTX, while both band of V1 and that of those other than V1 was detected in the liver (Fig. 1B). This suggests that V1 mRNA is a dominant transcript in CTX, while the other transcripts are also expressed in the liver.

Using real-time PCR, the relative abundance of each variant mRNA was determined in the CTX and the liver (Table 2). In agreement with the results of Fig. 1B, the major transcript in the CTX was V1 mRNA. The expression of V3 and V4 was detected, but it was much less than that of V1. The V2 mRNA expression was barely detected. In the liver, V1 mRNA was also largest in amount, and the substantial expression of V2 and V4 was detected. The amount of long form mRNA was larger than that of short form approximately 10-fold in the CTX.

The expression of total GHR, V1 and V4 mRNAs in the different region of the brain and the pituitary gland was examined and the results were shown in the Fig. 2. The expression level of the total GHR mRNA was found to be higher in CTX, HP, HT and PIT than in other regions of the brain. The relative expression levels of V1 mRNA in various brain regions were almost similar to that of total GHR mRNA, suggesting that the V1 mRNA is a dominant mRNA species also in other regions than CTX. The V4 mRNA expression was found to be relatively high in HT and PIT. The expression levels of total, V1 and V4 mRNA did not show sex-related difference, with only an exception of total GHR in spinal cord.

The expression of GHR mRNA was also examined in female CTX, HP, HT and PIT as a function of age (Fig. 3). The total GHR concentration in the CTX was significantly higher at postnatal day 5 (P5) than that of diestrus female at 8 weeks of age, but it was low during fetal period. The total GHR levels in HP and HT did not change during postnatal days. During gestation, a gradual increase in total GHR mRNA was seen in HT. The HP total GHR mRNA at embryonic day 16 (E16) was compatible to that of adult, but was significantly low in E18 and E20. Unexpectedly, pituitary total GHR mRNA was 4 to 5 times higher in fetal period than in adults, and it decreased during postnatal days. The levels of V1 mRNA in CTX, HP, HT and PIT also showed similar developmental change to that of total GHR, suggesting that the V1 is also preferentially expressed in these tissues during fetal and postnatal days.

#### 4. Discussion

Multiple first exon of the GHR mRNA has been identified in a variety of species including humans [17], mice [18-20], bovines [21], and rats [10, 11]. In the rat, five distinct first exons have been found so far, and they were shown to be expressed in a tissue specific manner [10, 11]. However, the 5'-UTR structure of the GHR mRNA in the different brain regions has not been studied. The present study revealed the expression of four of five variants in cerebral cortex in this study. The V5 mRNA could be detected neither in CTX nor in liver. The reason for this discrepancy is currently unknown. It is possible that this is the result of species difference of animals used. More importantly, we found that the V1 is preferentially expressed in the brain regions and in the pituitary gland, opposing to the results reported previously showing that only V4 was detected in the brain total RNA. In this study, V4 mRNA was detected in the brain, but its expression level was much smaller than that of V1.

The previous *in situ* hybridization and immunocytochemical studies revealed that the GHR mRNA is expressed in various regions of the brain including layers 2,3,5 and 6 of the cerebral cortex [22], arcuate and other nuclei of the hypothalamus [22-25], hippocampus [7,24], Purkinje cells of the cerebellum [22,26], a number of nuclei of brainstem including locus coeruleus and the area postrema [25], and the dorsal horn of the spinal cord [25]. The GH-binding site has been demonstrated in choroid plexus, hypothalamus, hippocampus, pituitary and spinal cord [2,3]. The present study demonstrated the GHR transcript in CTX, HP, HT, BS, CB, SP and PIT in agreement with those reported previously, and showed that not only the expression levels of the GHR mRNA, but also the developmental changes of the GHR mRNA expression differ between brain regions and the pituitary gland. These data suggest the presence of a tissue specific regulatory mechanism of transcription, however, it was revealed that this is not a results of the differential promoter usage, because, V1 mRNA is predominant in all the brain regions examined and pituitary gland. It is considered that the 5'-upstream region of the V1 contain the specific elements that enable the tissue specific regulation of the GHR gene. Since brain GHR mRNA appears to be mostly V1 mRNA, the effects of morphine [27] and estrogens [6] that decreased GHR expression in the brain may be mediated by V1 promoter. On the other hand, the involvement of not only V1 but also other promoters is possible for the up-regulation of brain GHR induced by hormone-treatment such as GH [28] and glucocorticoids [7]. The present results showing a dominant expression of V1 with a minor expression of V4 in the brain are contrast to the result reported previously that the V4 is only a variant form expressed in the brain [11]. The reason for this apparent inconsistency of these results is not presently unknown.

The GHR expression in the pituitary gland has been reported in rats [12], but little is known on the physiological role of GH in this tissue. Our data showed that the expression level of pituitary GHR is almost compatible to that of HT which is well known to be a site of GH feedback [29]. The

previous immunocytochemical study revealed the localization of GHR in somatotropes as well as in mammotropes and gonadotropes [30], or in all the anterior pituitary cell types including somatotropes [31] in rats. Therefore, it is possible that the pituitary GHR mediates feedback information to somatotropes, although a recent report showed that the changes in the GH-feedback status did not affect the expression levels of GHR in this tissue [32]. The present study revealed that the V1 mRNA is also expressed in the pituitary gland as well as in the brain, and its expression level is especially high in the fetal period. These results suggest the presence of yet unknown role of GH in the regulation of pituitary functions. Since congenital GH-deficiency always associates the marked decline in the population of pituitary mammotrophs [33-35], the high level of GHR in the fetal pituitary gland may be involved in the mammotroph development during gestation.

In this study, it is demonstrated that the GHR mRNA expressed in the rat brain and pituitary is mostly V1 mRNA, and V2, V3 and V4 were very small in amount. The expression of V1 GHR mRNA was developmentally regulated to increase with age in the brain, except for CTX where a transient increase at P5 and subsequent decline was observed. On the contrary, the GHR mRNA in the pituitary was high in the fetal period and decreased with age, suggesting a specific role of GH in gestation in the development of this tissue.

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## Legend for the figures

Fig. 1 Schematic diagram showing five different 5'-end variants (A) and two 3'-end variants, long and short forms (B) of GH-receptor mRNA. Arrows on each first exon indicate the position of sense primers used for the amplification of the variant mRNA. The s1 and as1 are primers within the second exon common to all 5'-variant mRNA which was used to estimate the relative abundance of the standard PCR fragments for the determination of each variant. \* translation initiation site.

The long and short forms of GH-receptor mRNA were amplified by the specific antisense primer for each and a T/s1 primer. The total GH-receptor mRNA was amplified by T/s1 and T/as1 primers.

Fig. 2 Expression of the rat GH-receptor mRNA variants in the brain and cerebral cortex as revealed by RT-PCR (A) and RNase protection assay (B),

Fig. 3. Distribution of total GH-receptor mRNA (A), V1 (B) and V4 (C) mRNA in the different regions of the brain and the pituitary gland of the rat.

Values are the means  $\pm$  SEM (n=6). \*p<0.05 vs female by the Student's t test.

Fig. 4. Developmental changes in the expression of total GH-receptor mRNA (A) and V1 (B) in cerebral cortex (CTX), hippocampus (HP), hypothalamus (HT) and pituitary gland (PIT). 8W, 8 weeks of age.

Values are the means  $\pm$  SEM (n=6).

\*p<0.05; \*\*p<0.01 vs 8W, by one-way analysis of variance followed by Fisher's PLSD test

Fig. 1

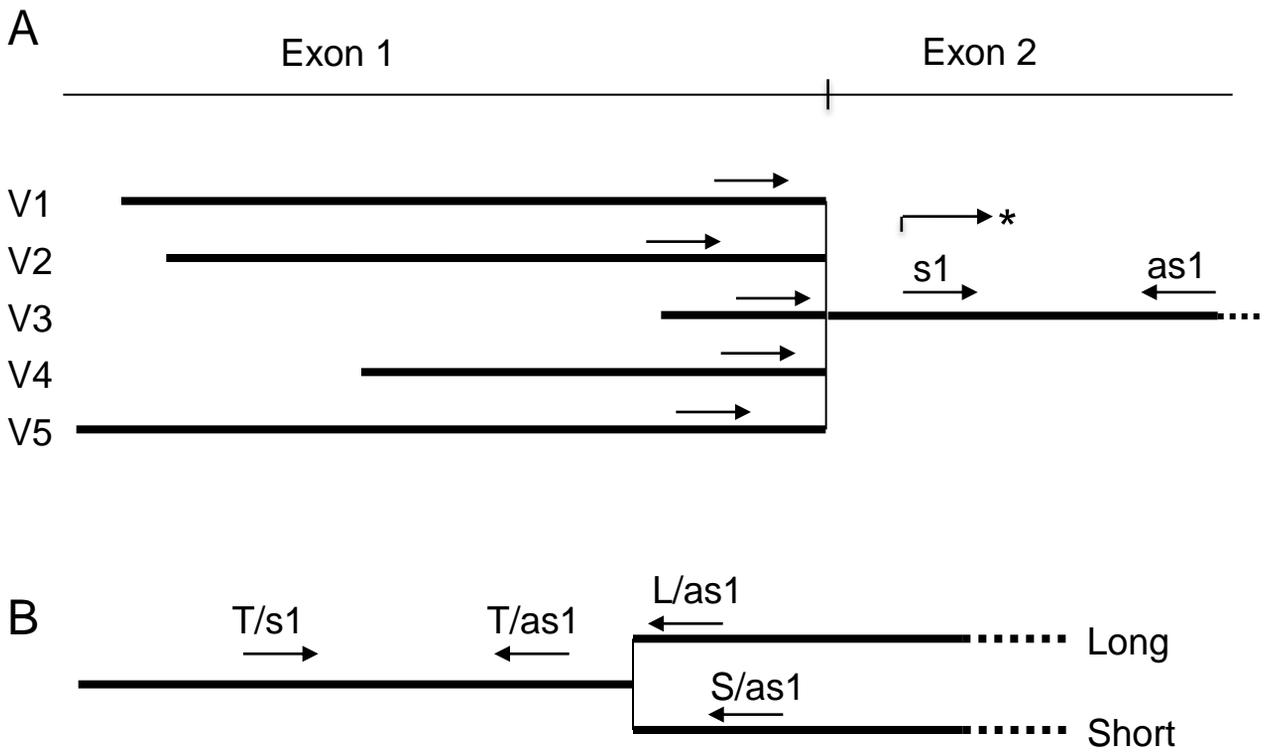


Fig. 2

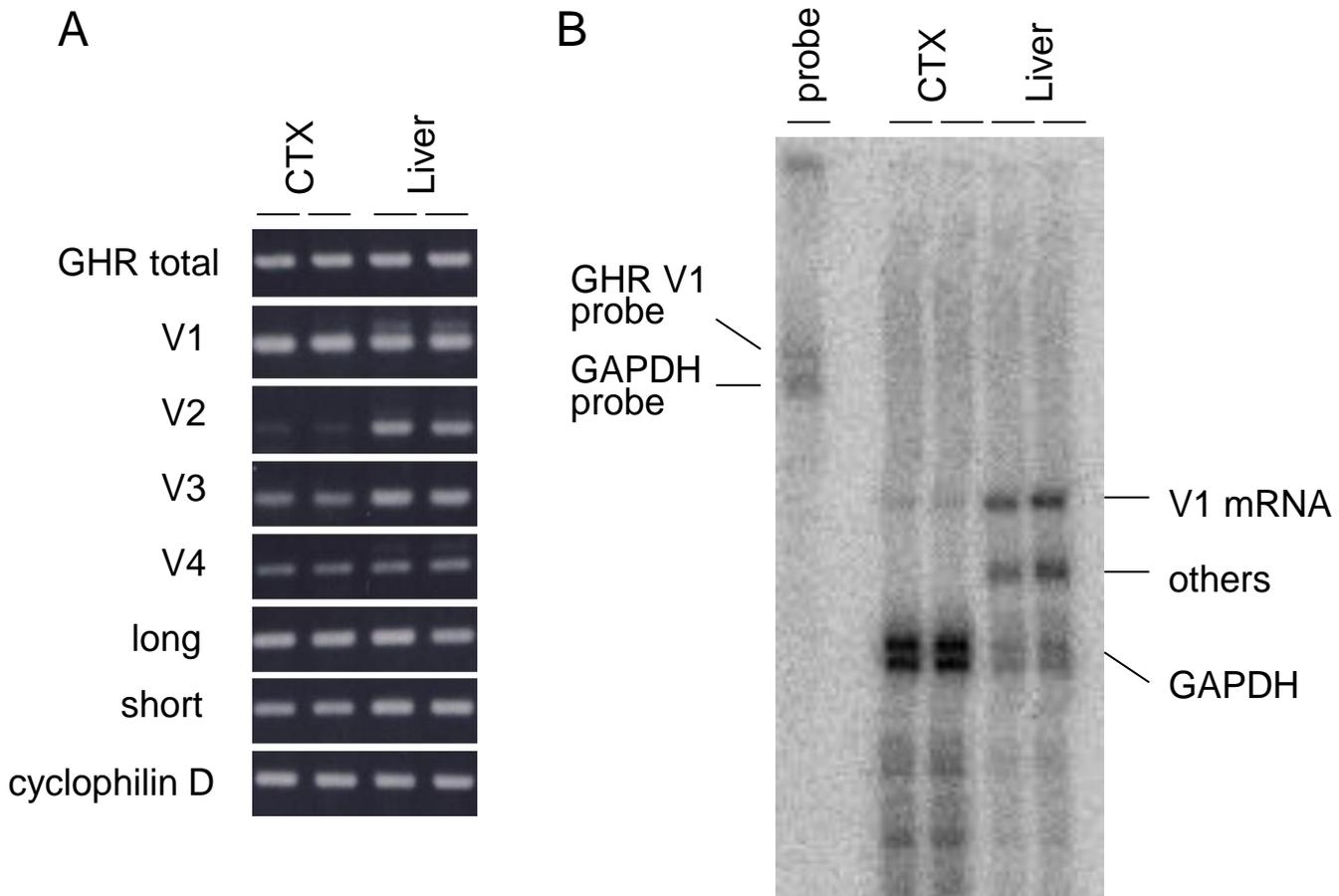


Fig. 3

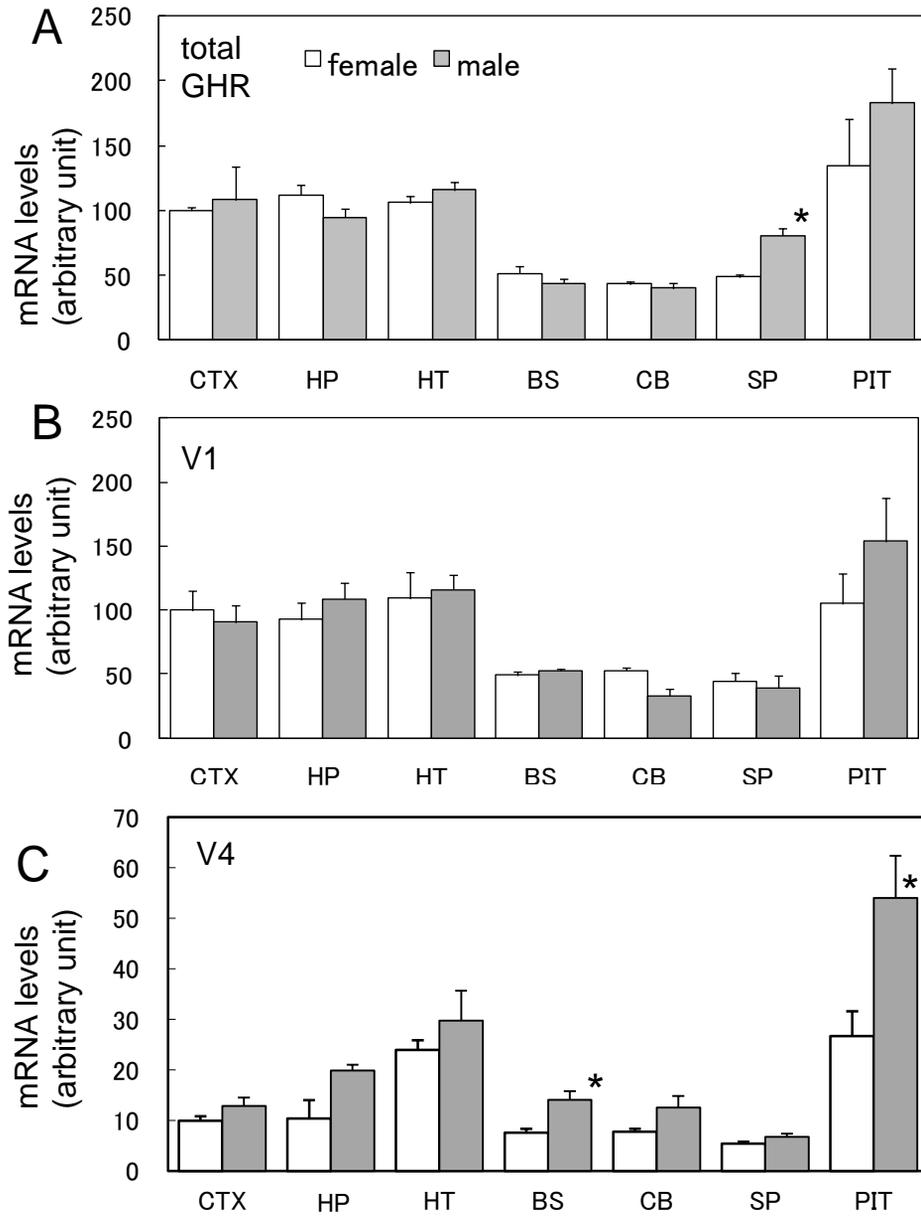


Fig. 4

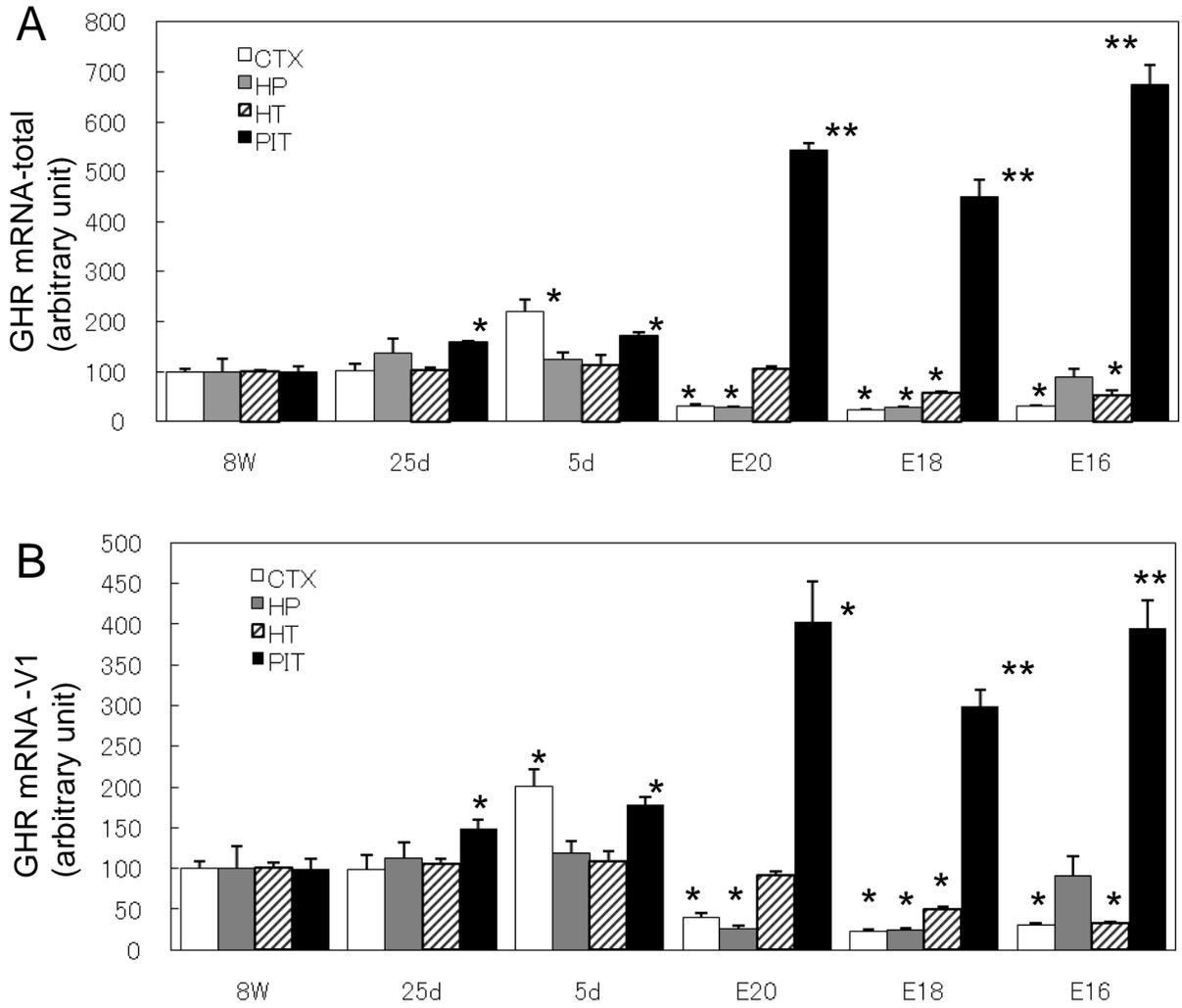


Table 1. Primer sequences used in this study

Primer name	Sequence	References or NCBI accession no.
s1	5'-atggatctttggcgggtgtt-3'	NM_017094
as1	5'-gccaagagtagctggtgtacg3'	NM_017094
V1	5'-gctctgtctcccaaggcgaa-3'	[11]
V2	5'-ccctagcttctctaccagat-3'	[11]
V3	5'-tgctgcagggaagtccgttt-3'	[11]
V4	5'-actgcagagtcttgagcgct-3'	[11]
T/s1	5'-agatccagacaacggagctt-3'	NM_017094
T/as1	5'-tttctcagatggacacact-3'	NM_017094
L/as1	5'-tccggttccatggttctta-3'	NM_017094
S/as1	5'-gcaccacatcaagagattg-3'	S49003
cyD/s	5'-actaggtgtggcaaggatgc-3'	NM_001004279
cyD/as	5'-tgactatccccagagccatc-3'	NM_001004279
GAPDHs	5'-atggtgaaggtcggtgtcaa-3'	[16]
GAPDHas	5'-gtccactttgtcacaagagaa-3'	[16]

Table 2. Relative abundance of the GH-receptor mRNA variants in the rat cerebral cortex and liver

	CTX	Liver
V1	100.00 $\pm$ 3.30	100.00 $\pm$ 12.06
V2	0.15 $\pm$ 0.06	65.28 $\pm$ 6.55
V3	1.75 $\pm$ 0.24	1.01 $\pm$ 0.09
V4	11.25 $\pm$ 2.21	23.19 $\pm$ 3.89
Long	100.00 $\pm$ 4.78	100.00 $\pm$ 10.62
Short	9.65 $\pm$ 2.26	87.44 $\pm$ 5.55

The abundance of each mRNA species is expressed as % of V1 or long form mRNA. Values are the mean  $\pm$  SEM (n=6). CTX, cerebral cortex