Semiquantitative Analysis of the Expression of GABA-A Receptor Subunits in the Developing Embryonic Chick Brain Stem

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Abstract: The expression levels of seven types of gamma-aminobutyric acid-A (GABA-A) receptor subunits ($\alpha 1$, $\beta 2$, $\beta 3$, $\beta 4$, $\gamma 1$, $\gamma 2$, and $\gamma 4$) were quantified in the embryonic chick brain stem at 2 to 20 d of incubation (E2 to E20) and just after hatching. The expression level of mRNA was measured by using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). When property regions were amplified, two products were observed for each of the $\beta 2$, $\beta 4$, and $\gamma 2$ subunits because of alternative splicing. These were named $\beta 2S$ and $\beta 2L$, $\beta 4S$ and $\beta 4L$, and $\gamma 2S$ and $\gamma 2L$ for shorter and longer

fragments, respectively. Transcripts of $\alpha 1$, $\beta 2L$, $\beta 2S$, $\beta 3$, $\beta 4L$, $\beta 4S$, $\gamma 1$, and $\gamma 2S$ subunits were first detected from E2 to E5 brain stems. The expression level of each subunit increased gradually with development and reached a plateau at E9 to E12. In contrast, a delay occurred in the appearance of both the $\gamma 4$ and $\gamma 2L$ subunits, which were not detected until E8 to E10. The absence of $\gamma 4$ and/or $\gamma 2L$ subunits may explain differences in the pharmacological characteristics of GABA-A receptors at the early stages of development. [Japanese Journal of Physiology, 51, 53–61, 2001]

Key words: GABA-A receptor subunit, alternative splicing, RT-PCR, developmental expression, embryonic brain stem.

GABA receptors are classified into two major groups: Cl^- -channel ionotropic receptors (GABA-A and GABA-C receptors) [1, 2], and G-protein-coupled metabotropic receptors (GABA-B receptor) [3, 4]. In the central nervous system, ionotropic and metabotropic GABA receptors cause fast and slow postsynaptic potential changes, respectively [3, 4]. GABA-A and GABA-C receptors can be pharmacologically distinguished by their differential sensitivity to benzodiazepines and barbiturates [1, 2, 5].

The GABA-A receptor is a pentamer composed of five subunits. Each subunit consisted from over 15 types, and different subunit compositions may produce significant differences in receptor properties [6–9]. In fact, in the 7-d-old chick brain stem, a pharmacological different GABA-A receptor has been reported [10]. However, qualitative and/or quantitative changes in GABA-A receptor subunits during the

early stages of development have not been well documented. In the present study, we investigated the developmental changes in the expression levels of mRNA coding GABA-A receptor subunits in embryos at 2 to 20 d of incubation (E2 to E20) and just after hatching, using a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Preparation and dissections. Fertilized chicken eggs (Barred Plymouth Rock) were incubated in a forced draft incubator (Type Baby (B), Showa Incubator Lab.) at 38°C and 60% humidity and were turned once an hour. The embryos were isolated at E2 to E18, and the brain stems were dissected under a binocular microscope. The brain stem is defined as the caudal part of the brain from the boundary between

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the prosencephalon (diencephalon) and the mesencephalon. At the early stages of development, rhombencephalon and mesencephalon were dissected. After the cerebellum became identifiable, the cerebellum and possibly a large part of the cerebellar peduncle were removed.

Extraction of total RNA and RT-PCR. Immediately after the dissection, total RNA was extracted and purified from brain stems by using an RNA isolation kit (QIAGEN). To improve the quality of RNA sample, extracted RNA was retreated with same kit. To test the quality of the extracted RNA, agarose-gel electrophoresis was carried out for all samples, and the integrity of 18S and 28S ribosomal RNAs was confirmed. The contamination of genome DNA in the RNA sample was tested with PCR amplification of β -actin fragment without a reverse transcription step. Since no amplification was detected, it was confirmed that the contamination had practically no effect on the following experiments. A master mixture of cDNA was synthesized from total RNA, using MuLV reverse transcriptase (Perkin Elmer) and a random hexamer. The experimental process basically followed the manufacturer's protocol, except that the reaction mixture was incubated for 4 to 12 h at 37°C. The quality of cDNA was tested by PCR, using β -actin primers (Table 1) [11]. Since DNA fragments of the expected length for amplified β -actin were detected in all samples (data not shown), it was concluded that the master mixture was adequately prepared.

The qualitative PCR was conducted by using a portion of the master mixture as a template and a primer pair specific to each subunit. Taq Gold DNA polymerase (Perkin Elmer) and premix Taq (TaKaRa) or Ready-To-Go PCR beads (Amersham) were used. The PCR consisted of 35 cycles of 30 s at 94°C, 30 s at a fixed temperature (54 to 62°C) and 45 s at 72°C. Primer pairs specific for each subunit were designed based on the nucleotide sequences cited in the following references: $\alpha 1$ [12], $\beta 2$ [13, 14], $\beta 3$ [15], $\beta 4$ [16, 17], $\gamma 1$ [18], $\gamma 2$ [19], and $\gamma 4$ [20] (Table 1). In this paper, an underlined subunit name, e.g., $\beta 2$, represents the primer pair for the subunit. In an adult chicken, alternative splicing occurs in the mRNA of $\beta 2$, $\beta 4$, and $\gamma 2$ subunits. We have thus designated the primer pairs that include the splicing sites within the amplified area of the mRNAs as $\beta 2SL$, $\beta 4SL$, or $\gamma 2SL$, respectively. Two different sets of primer pairs were designed for the $\beta 2$ and $\beta 4$ subunits, one that included the splicing sites, designated $\beta 2SL$ and $\beta 4SL$, and one that excluded these sites, designated $\beta 2$ or $\beta 4$.

DNA sequencing. The mRNA from embryos at E8 to E9 was used as a template. When a single fragment was amplified by RT-PCR, cDNA was purified from the reaction mixture by using a Microcon 100 (Amicon) and directly sequenced. To determine the sequence of fragments involving splicing sites ($\beta 2S$, $\beta 2L$, $\beta 4S$, $\beta 4L$, and $\gamma 2L$ subunits), amplified DNA fragments were separated by using agarose gel electrophoresis and purified with SUPREC-01 (TaKaRa). These fragments were cloned into a plasmid vector, pCRII-TOPO, by a TOPO-TA cloning kit (Invitrogen). Plasmid DNA was prepared by using a Miniprep kit (Promega) and sequenced by using a dideoxy terminator cycle sequencing kit (Applied Biosystems and Amersham) and a DNA sequencer Model 377 (Applied Biosystems).

Semi-quantitative RT-PCR. To quantify the amount of mRNA, amplified fragments of GABA-A receptor cDNA were compared with internal β -actin cDNA. β -Actin is a housekeeping protein, and the level of β -actin mRNA should be consistent among cells throughout the tested developmental stages. Thus the ratio of GABA-A subunit PCR products to β -actin PCR products was proportional to the concentration of GABA-A receptor subunit mRNA [11, 21]. To obtain a linear relationship between the amount of mRNA and PCR product, a suitable PCR cycle number and annealing temperature (i.e., 30 s at 94°C, 30 s at 54°C, and 45 s at 72°C for 22 cycles) were selected for semiquantitative experiments. After electrophoresis, agarose or polyacrylamide gels were stained with SYBR gold (Molecular Probes) or ethidium bromide and photographed by a CCD camera with UV transillumination. The γ -compensation of the camera was set at 1 to ensure a linear relationship between the band intensity on the photo and the amount of PCR product, and care was taken to avoid the saturation of any bands on the image. The band intensity on the photocopy was then digitized with a density scanner, and the ratio of the band intensity of each GABA-A subunit to β -actin was calculated. The ratios for each subunit were then normalized to the highest observed ratio for that subunit. Experiments were repeated 2 to 11 times, and the mean $\pm$ SE are shown.

Computer programs for DNA sequence analysis. Primers were designed by consulting PC-rare [22]. Sequence analysis and alignments were conducted with seqaid (Rhoads, D. D. and Roufa, D. J. (1991); the freeware program was obtained at <http://mama.indstate.edu/pfaffle/software/>) and macaw [23]. The densities of photocopies of gel patterns were measured by using BAG2000 (Inohara, N. (1996); the shareware program was obtained at fbio.Nifty-serve).

Anthept [24] was used to analyze protein structures and to search phosphorylation sites in deduced amino acid sequences.

RESULTS

Nucleotide sequence of RT-PCR products

Figure 1A shows typical electrophoresis patterns of the PCR products using specific primer pairs for GABA-A receptor $\alpha 1$, $\beta 3$, $\gamma 1$, and $\gamma 4$ subunits. Each DNA fragment exhibited a single band in all the tested developmental stages. A single band was also obtained with primer pairs $\beta 2$ and $\beta 4$ (data not shown). Alternative splicing has been reported in GABA-A receptor $\beta 2$, $\beta 4$, and $\gamma 2$ subunits in adult chicken [3, 4, 7, 9, 16, 25]. The $\beta 2SL$, $\beta 4SL$, and $\gamma 2SL$ primer pairs were thus designed to contain these splicing sites (see MATERIALS AND METHODS), and double bands appeared with such primer pairs as expected. Double bands (short and long sequences, respectively) were thus observed with $\beta 2SL$ (designated $\beta 2S$ and $\beta 2L$), $\beta 4SL$ (designated $\beta 4S$ and $\beta 4L$), and $\gamma 2SL$ (designated $\gamma 2S$ and $\gamma 2L$; Figs. 2–4). The length of the fragments agreed with those predicted from published values, including the lost base pairs as a result of the alternative splicing (Table 1).

To confirm the accuracy of the PCR, the nucleotide sequences of the PCR products were determined and compared with published sequences. A few differ-

ences were found in the fragments from the $\alpha 1$, $\beta 2$, $\beta 4$, and $\gamma 1$ subunits, but the sequences of the $\beta 3$, $\gamma 2$, and $\gamma 4$ fragments were identical to the published reports (Table 2). Furthermore, the splicing sites of $\beta 2$, $\beta 4$, and $\gamma 2$ exactly matched previously reported sequences [3–7, 9, 17]. From these results, we conclude that the PCR amplified the correct segments of the GABA-A receptor subunits.

Expression of $\alpha 1$, $\beta 3$, $\gamma 1$, and $\gamma 4$ subunits during development

The electrophoresis patterns of the RT-PCR products of $\alpha 1$, $\beta 3$, $\gamma 1$, and $\gamma 4$ subunits are illustrated in Fig. 1A, lanes a to d. In $\alpha 1$, $\beta 3$, and $\gamma 1$, a single band was detected at E2 or E3 (Fig. 1A, lanes a to c). There was a distinct delay in the time of appearance of the $\gamma 4$ subunit: only slight traces could be detected from embryos younger than E7, and the expression clearly increased after that stage (Fig. 1A, lane d).

The early onset of $\alpha 1$, $\beta 3$, and $\gamma 1$ fragments and the delayed appearance of $\gamma 4$ were confirmed by semiquantitative RT-PCR (Fig. 1B). An expression of the $\alpha 1$, $\beta 3$, or $\gamma 1$ subunits also started at E2 to E6 (Fig. 1B), but $\gamma 4$ appeared first at E9.

The results of the semiquantitative analysis of these subunits at each developmental stage are summarized in Fig. 1C. The expression of $\alpha 1$, $\beta 3$, and $\gamma 1$ subunits started from E2 to E4. The expression level increased gradually as development proceeded and reached a

Table 1. Primer pairs and length of PCR products.

Primer	Target	Sequence	Location	Amplified sequence (bp)
<u>Actin</u>	β -Actin	CGGAACCGCTCATTGCC ACCCACACTGTGCCCATCTA		289
<u>$\alpha 1$</u>	$\alpha 1$	GACTTATCTGCCATGCATCATG TAAATAGGTGGCCCAATAGAC	961–1519	559
<u>$\beta 2$</u>	$\beta 2$	GATTGAGAGCTATGGCTATACGAC CCTTATTGACATCCATTCGCAACT	722–1268	547
<u>$\beta 2SL$</u>	$\beta 2$	CAACAATTAACACTCATCTTCGAG GTAAGCCAGCTTCCGGTATTGGA	1046–1467	422, 371
<u>$\beta 3$</u>	$\beta 3$	GAGAGGTGGAGATAATGCGG TAAGGCCAAGAAGACGAATA	670–1054	385
<u>$\beta 4$</u>	$\beta 4$	CTGACTGGGGGGGGTCGGTT GTTGAGAGGAAGGTCGTTAT	93–529	437
<u>$\beta 4SL$</u>	$\beta 4$	CAGAAGAAACAGAGTGAACG AGGACATGACAGAGTTTCGA	1228–1416	189, 177
<u>$\gamma 1$</u>	$\gamma 1$	AGCTTCATAACTTTCCTATGG TAGATAAAGATAGCCAATCC	560–1394*	835
<u>$\gamma 2SL$</u>	$\gamma 2$	TGACCACCCTAAGTACGATT CATGCTCCTGTTCGACAATC	1611–1977	367, 343
<u>$\gamma 4$</u>	$\gamma 4$	TATCGATGTGGATATCTACG GACCTCAATGGAATAGCGTC	310–723	414

Primer pairs are shown with underlines. * Recalculated from sequence data in Greka *et al.* [5].

Fig. 1. Expression pattern of the $\alpha 1$, $\beta 3$, $\gamma 1$, and $\gamma 4$ GABA-A receptor subunit mRNAs during development in the embryonic chick brain stem analyzed by RT-PCR.

The GABA-A receptor subunits that did not exhibit alternative splicing are summarized. M is a DNA marker and numerals indicate the embryonic days.

A: Typical agarose gel electrophoresis patterns of GABA-A receptor subunits at E2 to E10 or E14. $\alpha 1$, $\beta 3$, $\gamma 1$, and $\gamma 4$ subunits are shown in lanes a–d, respectively. X in lane c shows the PCR products obtained with both $\gamma 1$ and β -actin (Actin). **B:** Electrophoresis patterns of RT-PCR products using the $\alpha 1$, $\beta 3$, $\gamma 1$, and $\gamma 4$ primer pairs in the presence of β -actin. $\alpha 1$, $\beta 3$, $\gamma 1$, and $\gamma 4$ subunits are shown in lanes a–d, respectively. **C:** Time course of the expression level of $\alpha 1$ (filled circle), $\beta 3$ (open inverted triangle), $\gamma 1$ (filled square), and $\gamma 4$ (diamond) GABA-A receptor subunit mRNA. Data were obtained from Fig. 1B and other similar experiments. In this and the subsequent figures, the ratio of the band intensity of each subunit to that of β -actin was calculated in each assay, and the ratios for each subunit were normalized to the highest observed ratio for that subunit.

plateau at E9 to E12. In contrast, the $\gamma 4$ subunit exhibited a late onset in expression. It was first detected at E8, and the expression level reached a plateau at E14.

Expression of $\beta 2$ and $\beta 4$ subunits during development

RT-PCR with the $\beta 2$ SL primer pair amplified double fragments ($\beta 2$ S and $\beta 2$ L for short and long sequences, respectively) because of alternative splicing (Table 2). Both fragments were already present at E3 (Fig. 2A). Since the densities of both bands increased together, it seems that the expression of $\beta 2$ S and $\beta 2$ L subunits started simultaneously and increased at simi-

lar rates. Figure 2B shows a typical electrophoresis pattern of fragments obtained by using semiquantitative RT-PCR with $\beta 2$ SL and β -actin primer pairs. Again, the $\beta 2$ S and $\beta 2$ L subunits increased with a similar time course. The developmental expression of $\beta 2$ S and $\beta 2$ L is summarized in Fig. 2C. PCR with the $\beta 2$ primer amplified a segment of the $\beta 2$ subunit mRNA lacking the splicing site and yielded a single band. The temporal expression pattern of this fragment was identical to those observed with the $\beta 2$ SL primer.

A similar tendency was observed in the experiments using $\beta 4$ SL and $\beta 4$. Double bands ($\beta 4$ S and $\beta 4$ L for short and long fragments, respectively) were

Table 2. Comparison of observed nucleotide sequence and reference in GABA-A receptor subunits in the chick.

Subunit	Analyzed region	Position	Present data	Reference data	GenBank accession number
$\alpha 1$	1084–1422	1089	ACT(T)	AGT(S)	X54244
		1284	ATA(I)	AAA(K)	
		1308	GTT(T)	GCT(A)	
		1371	ATG(M)	ACG(T)	
		1392	ATG(M)	AAG(K)	
		1399	GAT(D)	GAA(E)	
		1407	CTT(L)	CCT(P)	
		1413	GTA(V)	GAA(E)	
$\beta 2$	764–1244	no differences			X72806
$\beta 2L$	1050–1480	1154	GAG(E)	AAG(K)	
$\beta 2S$	1050–1480	1154	GAG(E)	AAG(K)	
	1267–1317		GGACAGAAGAATAATTGGCACATATCATT GTCCAGAAATGTATTCAACAAA (DRRIIGTYHCPEMYSTK) [deleted]		
$\beta 3$	726–994	no differences			X54243
$\beta 4$	117–440	280	GTT(V)	GTC(V)	X56647
		385	GGT(G)	GGA(G)	
		386	AGT(S)	GGT(G)	
		389	AAA(K)	AAC(N)	
$\beta 4L$	1260–1436	no differences			
$\beta 4S$	1260–1436	1294–1305	GGTGAGAGAGCA (VREQ) [deleted]		
$\gamma 1$	651–1252	663	GTG(V)	GTT(V)	Z25466, Z25467
		1177	CAT(H)	AAT(N)	
$\gamma 2L$	1632–1957	no differences			
$\gamma 2S$	1648–1957	1796–1819	CTTCTTCGGATGTTTTCTTCAAG (LLRMMFSFK) [deleted]		X54944 X73533
$\gamma 4$	339–703	no differences			

Letters in parentheses indicate corresponding amino acid sequence.

Fig. 2. Expression pattern of the $\beta 2$ GABA-A receptor subunit mRNA during development in the embryonic chick brain stem analyzed by RT-PCR. **A:** Typical agarose gel electrophoresis patterns of fragments of $\beta 2$ subunits amplified using the $\beta 2SL$ primer pair. RT-PCR with $\beta 2SL$ produced double bands, since 51 base pairs were spliced between the primer pair. An analysis of the nucleotide sequences confirmed that these represented fragments of the $\beta 2$ subunit with ($\beta 2S$) and without ($\beta 2L$) splicing. **B:** Three

fragments ($\beta 2L$, $\beta 2S$, and β -actin (Actin)) were observed with RT-PCR using primer pairs of both $\beta 2SL$ and β -actin. H indicates the developmental stage just after hatching. **C:** Developmental expression of mRNA coding GABA-A receptor $\beta 2L$ (filled circle), $\beta 2S$ (open circle), and total $\beta 2$ mRNA from a region in which alternative splicing was not present (filled inverted triangle). Data were obtained from Fig. 2B and similar experiments.

Fig. 3. Expression pattern of the $\beta 4$ GABA-A receptor subunit mRNA during development in the embryonic chick brain stem analyzed by RT-PCR. **A:** Typical polyacrylamide gel electrophoresis patterns of DNA fragments of the $\beta 4$ subunit amplified using the $\beta 4SL$ primer pair. An alternatively spliced region 12 base pairs long was present between the primer pair. An analysis of the nucleotide sequence confirmed that these were $\beta 4$ GABA-A receptor

subunits with ($\beta 4S$) and without ($\beta 4L$) splicing. **B:** Three fragments ($\beta 4L$, $\beta 4S$, and β -actin (Actin)) were observed with RT-PCR using both $\beta 4SL$ and β -actin. **C:** Developmental expression of $\beta 4L$ (filled circle) and $\beta 4S$ (open circle) subunits and a region in which alternative splicing was not present (filled inverted triangle). Data were obtained from Fig. 3B and similar experiments.

Fig. 4. Expression pattern of the $\gamma 2$ GABA-A receptor subunit mRNA during development in the embryonic chick brain stem analyzed by RT-PCR. **A:** Typical agarose gel electrophoresis patterns of PCR products of the $\gamma 2$ subunit using the $\gamma 2SL$ primer pair. An alternatively spliced region, which deleted 24 base pairs, was present between the primer pair. An analysis of the nucleotide sequence con-

firmed that these were the $\gamma 2$ GABA-A receptor subunits with ($\gamma 2S$) and without ($\gamma 2L$) splicing. **B:** Three fragments ($\gamma 2S$, $\gamma 2L$, and β -actin (Actin)) were observed with RT-PCR using both $\gamma 2SL$ and β -actin. **C:** Developmental expression of GABA-A receptor $\gamma 2L$ (filled circle) and $\gamma 2S$ (open circle) subunits. Data were obtained from Fig. 4B and similar experiments.

observed with $\beta 4SL$. Both bands were first detected at E2 in the qualitative experiments (Fig. 3A) and at E5 in the semiquantitative one (Fig. 3B). The time course of expression of the $\beta 4$ fragment (amplified with $\beta 4$) was the same as the $\beta 4S$ and $\beta 4L$ fragments (electrophoresis data not shown). Figure 3C summarizes the time courses of developmental expression of the $\beta 4$ subunit in three different fragments. The temporal expression patterns of these three fragments were also identical. From Figs. 1C, 2C, and 3C, it can be con-

cluded that the time course of expression of the $\alpha 1$, $\beta 3$, $\beta 2$, $\beta 4$, and $\gamma 1$ fragments were similar, but $\beta 4$ differed.

Expression of the $\gamma 2$ subunit during development

Figure 4A shows the results of qualitative PCR with $\gamma 2SL$. In brain stems at E4 to E7, only a single band was detected. However, after E8, another fragment with a higher molecular weight appeared. Similar re-

sults were obtained by using semiquantitative RT-PCR (Fig. 4B). The shorter fragment (γ 2S) appeared much earlier than the longer one (γ 2L). Figure 4C summarizes the time courses of the developmental expression of γ 2S and γ 2L subunits. γ 2S was first detected at E4; the expression level increased gradually and reached a plateau at E9. On the other hand, γ 2L first appeared at E9 and reached a plateau at E15. Thus the temporal expression pattern of γ 2S resembled the early appearance group which included the α 1, β 3, β 2, β 4, and γ 1 genes. In contrast, the time course of expression of γ 2L was similar to that of γ 4, and these belonged to the late expression group. It should be noted that the subunits belonging to the late expression group were absent in embryonic brain stem younger than E8.

DISCUSSION

The GABA-A receptor/ Cl^- channel complex is a transmembrane protein consisting of five subunits selected from a pool of more than 15 [6–9]. GABA-A receptors in the central nervous system may have different characteristics because of their different subunit combinations. There is a correlation between the spatio-temporal expression patterns of subunit mRNAs and subunit proteins [6]. Differences in neuron-specific gene expression of GABA-A receptor subunits can explain the diversity of GABA-A receptor properties, such as the difference in benzodiazepine potentiation of the response to GABA [8].

To evaluate the developmental time course of gene expression of GABA-A receptor subunits, we used a semiquantitative RT-PCR technique to measure the amount of mRNA at different stages of development. We did not examine the quantity of GABA-A receptor proteins. Miranda *et al.* [26] reported a good correlation between the level of GABA-A receptor α 1 subunit mRNA and α 1 polypeptide in the embryonic chick cerebral cortex. It is very likely that the amount of mRNA coding other GABA-A receptor subunits is also proportional to that of the synthesized proteins.

Our results indicate that the expression of the α 1, β 2S, β 2L, β 3, β 4S, β 4L, γ 1, and γ 2S subunits started in the early stage of embryogenesis. The mRNA of these subunits could be detected as early as E2 to E5 (Figs. 1–4). A time lag occurred in the appearance of the γ 2L and γ 4 subunits. They appeared first at E8 to E10 (Figs. 1 and 4). Thus in the embryonic chick brain stem younger than E8, both γ 2L and γ 4 subunits are lacking, but the α 1, β 2S, β 2L, β 3, β 4S, β 4L, γ 1, and γ 2S subunits are present. The absence of γ 4 and/or γ 2L subunits from GABA-A receptors might explain the existence of pharmacologically different

GABA receptors in the early stage of development [10].

In bovine, human, and rat brains, two subtypes of γ 2 subunits are known because of the alternative splicing of mRNA [19, 25] (Table 2). In γ 2S, eight amino acids are missing relative to γ 2L (Table 2), including an “SFK” sequence that confers a phosphorylation site for protein kinase C (PKC) [25]. The γ 2L subunit can be phosphorylated at this site, and PKC activators can inactivate the GABA-A receptor [27]. Similar alternative splicing occurs in the β 2 subunit, which also includes a phosphorylation site within the splicing site [3, 4]. This site [3, 16] is outside the alternatively spliced region in the β 4 subunit [6, 17]. The alternative splicing of mRNA further increases the potential diversity of GABA-A receptor [27].

Tissue-specific quantitative differences in the expression of γ 2S and γ 2L have been reported. γ 2S was more prominent than γ 2L in the bovine cortex, whole human brain, and rat hippocampus, whereas γ 2L was dominant in the rat cortex, rat striatum, and rat cerebellum [17]. Whether tissue-specific expression patterns of spliced mRNA derivatives are in the brain stem remains to be elucidated. According to Glen-corse *et al.* [28], in the nuclei of visual and auditory pathways in the chick brain at 1 d after hatching, γ 1 subunit transcripts are absent but γ 2 transcripts are present. Moreover, both α 1 and γ 2S+ γ 2L transcripts are present in many brain regions in the same preparation [28]. Thus it is probable that mRNAs of various subunits localize to distinct brain regions and that there are varying degrees of overlap in their distribution patterns.

Using a multiple-site optical recording technique employing a fast voltage-sensitive dye, Momose-Sato *et al.* [10] found a novel type of GABA response in the embryonic brain stem slice preparation at E7–E8 stage. This GABA receptor was blocked by either GABA-A antagonists, including picrotoxin, dieldrin, bicuculine, and SR95531, or GABA-B antagonists, 2-hydroxysaclofen, CGP35348, and phacofen. Furthermore, it is stimulated by either GABA-A or GABA-B agonists, musimol, and baclofen. On the other hand, several investigators compared subtype specific single-channel properties, using gene expression in *Xenopus* oocyte [29], fibroblast cell line L929 [30, 31], Chinese hamster ovary cells [32], or targeted disruption of the γ 2 subunit [33]. Sensitivity to GABA, single-channel conductance, and the number of open and closed states differ in combinations α 1 β 3, α 1 β 3 δ , and α 1 β 3 γ 2 subunits [30–32], or in wild and mutant mice lacking the γ 2 subunit [33]. They concluded that in GABA-A receptors, the γ 2 subunit enhances the ef-

ficacy of GABA by prolonging the open channel lifetime [30–33]. Thus it is highly possible that the lack of a γ subunit, such as $\gamma 4$ and $\gamma 2L$, causes different characteristics in the GABA-A receptor, as occurred in the early stage of development (Figs. 1 and 4). In fact, in the GABA-A receptor consisted of $\alpha 1\beta 1$ isoform, open duration increases with pentobarbital, and both open duration and opening frequency slightly reduce by the application of picrotoxin [32]. However, the pharmacological characteristics of GABA response without γ subunit remain to be elucidated.

In the present study, we used chick brain stems at different developmental stages and showed that there were differences in the onset and alternative splicing of GABA-A receptor subunits. The expression of the $\alpha 1$, $\beta 2L$, $\beta 2S$, $\beta 3$, $\beta 4L$, $\beta 4S$, $\gamma 1$, and $\gamma 2S$ subunits started early at E2 to E5, and the expression of the $\gamma 4$ and $\gamma 2L$ subunits was delayed; they first appeared at E8 to E10. The absence of $\gamma 4$ and/or $\gamma 2L$ subunits until E8 to E10 may explain the pharmacological differences in GABA response at this stage, which was observed by Momose-Sato *et al.* [10].

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